JOURNAL OF BACTERIOLOGY, Mar. 1995, p. 1505–1510 Vol. 177, No. 6
0021-9193/95/$04.00+0

The Genes Encoding the Delta Subunits of Dinitrogenases 2 and 3 Are Required for Mo-Independent Diazotrophic Growth by *Azotobacter vinelandii*

SHEELA I. WAUGH,† DENISE M. PAULSEN, PHOTINI V. MYLONA, ROBERT H. MAYNARD, R. PREMAKUMAR, AND PAUL E. BISHOP*

USDA Agricultural Research Service and Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615

Received 4 November 1994/Accepted 17 January 1995

***vnfG* and *anfG* encode the δ subunits of alternative nitrogenases 2 and 3 in *Azotobacter vinelandii*, respectively. As a first step towards elucidating the role of these subunits, diazotrophic growth and acetylene reduction studies were conducted on mutants containing alterations in the genes encoding these subunits. Mutants containing a stop codon (C36stop) or an in-frame deletion in *anfG* were unable to grow in N-free, Mo-deficient medium (Anf$^-$). Mutants in which cysteine 36 of AnfG (a residue conserved between VnfG and AnfG) was changed to Ala or Ser were Anf$^+$. Thus, this conserved cysteine is not essential for the function of AnfG in dinitrogenase 3. A mutant with a stop codon in *vnfG* (C17stop) grew after a lag of 25 h in N-free, Mo-deficient medium containing V_2O_5. However, a Nif$^-$ Anf$^-$ strain with this mutation was unable to grow under these conditions. This shows that the *vnfG* gene product is required for nitrogenase 2-dependent growth. Strains with mutations in *vnfG* and *anfG* reduced acetylene to different degrees. This indicates that the δ subunits are not required for acetylene reduction by nitrogenases 2 and 3.**

Azotobacter vinelandii is able to reduce atmospheric N_2 to NH_4^+ by any one of three genetically distinct nitrogenases depending on the metal content of the growth medium (3, 10). The molybdenum (Mo)-containing nitrogenase (nitrogenase 1) is expressed in medium containing Mo and has been extensively characterized (for reviews, see references 30 and 42). Nitrogenase 2 contains vanadium (V) and is synthesized in Mo-deficient medium containing V, while nitrogenase 3 is expressed in Mo- and V-deficient medium.

Nitrogenase 1 is composed of two components: dinitrogenase reductase 1 and dinitrogenase 1. Dinitrogenase reductase 1 is a dimer of two identical subunits with an M_r of approximately 60,000, and these subunits are encoded by *nifH*. A single (4Fe-4S) cluster is bridged between the two subunits (14, 17). Dinitrogenase 1, with an M_r of about 220,000, is a tetramer that is made up of two pairs of nonidentical subunits ($\alpha_2\beta_2$) encoded by *nifDK*. Dinitrogenase 1 contains two types of metal centers that are involved in the redox reactions of N_2 reduction: two P clusters each containing two 4Fe-4S clusters bridged by cysteine thiol ligands (21) and two identical FeMo cofactors which are the sites for binding and reduction of N_2 (37, 38).

Nitrogenase 2 is composed of dinitrogenase reductase 2 and dinitrogenase 2. Dinitrogenase reductase 2 has an M_r of about 62,000 and is a dimer of two identical subunits that are encoded by *vnfH* (15, 19, 33). Dinitrogenase reductase 2 contains four Fe atoms and four acid-labile sulfide groups per dimer (12, 15). Dinitrogenase 2 has been purified from both *A. vinelandii* and *Azotobacter chroococcum* (13, 16). Dinitrogenase 2 from *A. chroococcum* has an M_r of 240,000 and is composed of three subunits ($\alpha_2\beta_2\delta_2$) (13). This enzyme contains two V atoms, 23 Fe atoms, and 20 acid-labile sulfide groups per molecule (13). A cofactor (FeV) analogous to the FeMo cofactor has been extracted from dinitrogenase 2 with *N*-methylformamide (39). The α, β, and δ subunits have approximate M_rs of 54,000, 53,000, and 13,000 and are encoded by *vnfD*, *vnfK*, and *vnfG*, respectively. These genes form a contiguous operon, *vnfDGK*, located 2.5 kb downstream from an operon encoding dinitrogenase reductase 2 (*vnfH*) and a ferredoxin-like gene (33, 34). The organization of the structural genes for *A. vinelandii* nitrogenase 2 is the same as that for *A. chroococcum* except that for *A. vinelandii* only 1 kb separates the ferredoxin-like gene from the *vnfDGK* operon (19).

Nitrogenase 3 is made up of two components: dinitrogenase reductase 3 and dinitrogenase 3 (7). Dinitrogenase reductase 3 (encoded by *anfH*) is a dimer with a subunit M_r of 32,500. Dinitrogenase 3, like dinitrogenase 2, is composed of α, β, and δ subunits (7, 31). The α (encoded by *anfD*), β (encoded by *anfK*), and δ (encoded by *anfG*) subunits have molecular weights of 58,000, 50,000, and 15,000, respectively (7, 31). Dinitrogenase 3 contains approximately 24 Fe atoms and 18 acid-labile sulfide groups per molecule; however, it lacks significant amounts of Mo and V. The genes encoding the structural subunits of nitrogenase 3 lie in a contiguous operon, *anfHDGK* (18).

The presence of *vnfG* and *anfG* is a genetic feature that distinguishes alternative nitrogenase structural genes from the genes (*nifHDK*) that encode the conventional Mo-containing nitrogenase (18, 34, 35, 41, 43). Interestingly, in *Anabaena variabilis*, *vnfDG* are fused (41). The functional roles of the *vnfG* and *anfG* products in dinitrogenases 2 and 3 are unknown; however, Kim and Rees (22) have suggested that the *vnfG* product might be involved in the stabilization of the quaternary structure of dinitrogenase 2.

The results described in this article indicate that the *vnfG* and *anfG* products are essential for nitrogenase 2- and nitrogenase 3-dependent diazotrophic growth of *A. vinelandii*.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains, plasmids, and phages used in this study are given in Table 1. The *A. vinelandii* strains were grown in

* Corresponding author.
† Present address: Nexagen, Inc., Boulder CO 80301.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
CJ236	*dut-1 ung-1 thi-1 relA1*, pCJ105 (Cm^r)	Bio-Rad
K12 71-18	Δ(*lac-proAB*) F′ *lacI*^{q} *lacZ*ΔM15 *pro*$^+$ *supE*	28
MV1190	Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*) *306*::Tn*10* (Tet^r) (F′:*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15)	Bio-Rad
A. vinelandii		
CA	Wild type	6
CA1	Δ*vnfDGK1*::*spc*	This study
CA11	Δ*nifHDK*	4
CA11.1	Δ*nifHDK* Δ*vnfDGK1*::*spc*	This study
CA11.1.71	Δ*nifHDK* Δ*vnfDGK1*::*spc* Δ*anfDGK71*::*kan*	This study
CA11.1.76	Δ*nifHDK* Δ*vnfDGK1*::*spc* AnfG (C36stop)	This study
CA11.1.77	Δ*nifHDK* Δ*vnfDGK1*::*spc* AnfG (C36A)	This study
CA11.1.78	Δ*nifHDK* Δ*vnfDGK1*::*spc* AnfG (C36S)	This study
CA11.1.79	Δ*nifHDK* Δ*vnfDGK1*::*spc* Δ*anfG*	This study
CA11.71	Δ*nifHDK* Δ*anfDGK71*::*kan*	18
CA11.71.119	Δ*nifHDK* Δ*anfDGK71*::*kan* VnfG (C17stop)	This study
CA76	AnfG (C36stop)	This study
CA77	AnfG (C36A)	This study
CA78	AnfG (C36S)	This study
CA79	Δ*anfG*	This study
CA119	VnfG (C17stop)	This study
Plasmids		
pJSM1	Amp^r, 1.4-kbp *Bgl*II fragment containing *vnfDGK* replaced with a 2-kbp Spc^r cartridge in pUC9	19
pJWD3	Amp^r, 3.1-kbp *Eco*RI fragment containing *anfDGK* in pUC9	18
pDB303	Amp^r, 1.7-kbp *Eco*RI fragment containing *rpoB* in pUC8	Dennis Dean

modified Burk medium (40) at 30°C. Precautions were taken to minimize contamination with metals as previously described (2). Contaminating Mo was removed from the medium by extraction with 8-hydroxyquinoline as previously described (1). When required, V_2O_5 was added to a final concentration of 1 μM to N-free, Mo-deficient medium. Fixed N was added as ammonium acetate (final concentration, 28 mM). *Escherichia coli* K-12 71-18 was maintained on M9 minimal medium and was grown in tryptone-yeast extract or Luria-Bertani medium for transformation or phage infection (25). Strains CJ236 and MV1190, purchased from Bio-Rad Laboratories, Richmond, Calif., were maintained and grown according to the supplier's instructions. When required, antibiotics were added to the following final concentrations (in micrograms per milliliter): ampicillin, 50; chloramphenicol, 30; kanamycin, 10; rifampin, 10; spectinomycin, 20; tetracycline, 20.

DNA manipulations. All manipulations involving DNA were done as suggested by Maniatis et al. (25) or as directed by the manufacturer.

Site-directed mutagenesis. The sense strand of the 3.1-kbp *Eco*RI fragment containing *anfDGK* and the noncoding strand (mRNA strand) of the 2.9-kbp *Xho*I-*Kpn*I fragment containing *vnfDGK* were cloned into M13mp18 and M13mp19, respectively. Site-directed mutagenesis was carried out with these M13 templates by the method of Kunkel et al. (23) according to the instruction manual for the Muta-gene M13 in vitro mutagenesis kit purchased from Bio-Rad Laboratories, Richmond, Calif. The following primers were employed for site-directed mutagenesis (the codon change is indicated in parentheses, and the site of mutation is boldfaced): C36stop in AnfG (TGC→TGA), TGAAAAACT G**A**CTCTGGCAG; C36S in AnfG (TGC→AGC), TGAAAAAC**A**GCCTCTG GCAG; C36A in AnfG (TGC→GCG), ACATCATGAAAAAC**GCG**CTCTG GCAGTTC; and C17stop in VnfG (TGC→TGA), TCCTCGCGACTG**A**CAC CGTCA.

Construction of mutant strains. Fragments (3.1 kbp each) containing mutations in *anfG* were isolated after digestion of M13 replicative-form DNA with *Eco*RI. A 2.9-kbp fragment carrying the *vnfG* mutation was obtained by digestion of replicative-form DNA with *Hin*dIII and *Bam*HI. The fragments were used in congression crosses (4) with *A. vinelandii* CA1 (Δ*vnfDGK1*::*spc*) or CA71 (Δ*anfDGK71*::*kan*). The selection marker used in the congression cross was an *rpoB* mutation conferring rifampin resistance (Rif^r) contained on a 1.7-kbp *Eco*RI fragment isolated from pDB303 (obtained from Dennis Dean, Virginia Polytechnic Institute, Blacksburg). Rif^r transformants were selected on ammonium-supplemented Burk medium containing rifampin (10 μg/ml). To test for the replacement of the interposons by DNA carrying *vnfG* or *anfG* mutations, individual colonies of Rif^r transformants were tested for sensitivity to kanamycin (for *anfG* mutations) or spectinomycin (for *vnfG* mutations). Antibiotic-sensitive transformants were presumed to contain the desired mutation. An in-frame deletion (258 bp) in *anfG* was obtained by introducing *Nsi*I sites at positions 2743 and 3003 of the *anfHDGKorf1orf2* sequence (nucleotide sequence positions are those cited in reference 18). This deletion was transferred to the *A. vinelandii* chromosome as described above for mutations involving Cys codon changes in *anfG* and *vnfG*. The presence of this deletion in the chromosome was confirmed by Southern blot hybridization analysis. By using CA11.71 (Δ*nifHDK* Δ*anfDGK 71*::*kan*) as the parent strain, the *anfG* mutations were placed in a background containing deletions in the genes encoding nitrogenases 1 and 2. Since CA11.71 is Rif^r, the spectinomycin-resistant (Spc^r) interposon from pJSM1 was used as a congression marker. This interposon is located in a *vnfDGK* deletion carried by pJSM1. Competent CA11.71 cells were transformed with crude lysate DNA of CA76, CA77, CA78, or CA79 plus *Bam*HI-digested pJSM1 (19). Spc^r transformants were selected and then screened for kanamycin sensitivity. The double-deletion strain with a stop codon in *vnfG* of nitrogenase 2 (CA11.71.119) was made by transforming CA11.1 with the 2.9-kbp *Bam*HI-*Hin*dIII fragment from M13 replicative-form DNA (*vnfG* mutation) and genomic DNA from strain CA11.71 as a congression marker. Kan^r transformants were tested for sensitivity to spectinomycin. A spectinomycin-sensitive transformant which had lost its spectinomycin resistance gene because of replacement by DNA containing the stop codon in *vnfG* was selected.

Growth studies. *A. vinelandii* mutant and wild-type strains were cultured for 2 days at 30°C on Mo-deficient agar medium supplemented with ammonium acetate. Cells from these plate cultures were used to inoculate liquid Mo-deficient medium containing ammonium acetate (30 ml in a 300-ml sidearm flask). These cultures were incubated at 30°C with vigorous shaking (250 to 300 rpm) overnight. Fresh medium (30 ml) of the same composition was inoculated with cells from the overnight cultures and grown to a cell density of 100 Klett units. Three-hundred microliters from each culture was used to inoculate N-free Mo-deficient medium (30 ml in a 300-ml sidearm flask), and the inoculated cultures were incubated with vigorous shaking at 30°C. For nitrogenase 2-dependent growth, V_2O_5 was added to a final concentration of 1 μM. Growth was monitored with a Klett-Summerson colorimeter (no. 64 red filter) for at least 60 h.

Two-dimensional gel electrophoresis. *A. vinelandii* CA119 was derepressed for nitrogenase 2 in Mo-deficient medium containing 1 μM V_2O_5 for 3.5 h. Strains CA76 and CA79 were derepressed for nitrogenase 3 by incubation in Mo-deficient medium for 16 h. Two-dimensional gel electrophoresis of cell protein extracts from mutants was conducted by the method of O'Farrell (29) with modifications as described by Bishop et al. (2).

Whole-cell acetylene reduction assays. The acetylene reduction assay was conducted as described by Bishop et al. (2). Mutant strains of *A. vinelandii* were derepressed for nitrogenase 2 or 3 as described above for two-dimensional gel electrophoresis. Ethylene was measured with a Carle (model 311H) gas chromatograph equipped with a column of Porapak N (0.32 by 182 cm) and a flame ionization detector. The amount of protein in whole cells was determined by the bicinchoninic acid method as described by the supplier (Pierce, Rockford, Ill.), after the cells were solubilized by heating with NaOH (0.5 N) in a boiling water bath for 15 min. Bovine serum albumin was used as the standard.

RESULTS AND DISCUSSION

Strains containing mutations in *anfG* and *vnfG*. As a first step towards elucidating the functions of *anfG* and *vnfG* gene products, it is necessary to determine if these subunits are essential when Mo-independent nitrogenases are required for diazotrophic growth of *A. vinelandii*. Therefore, we changed a Cys codon which is conserved between AnfG and VnfG (Cys-36 in AnfG and Cys-17 in VnfG) to a translational stop codon (TGC to TGA) in both *anfG* and *vnfG*. To test for possible polar effects by the stop codons on the translation of downstream genes, protein extracts from strains CA76 and CA119 (containing stop codon mutations in *anfG* and *vnfG*, respectively) were analyzed by two-dimensional protein gel electrophoresis. The stop codon in *vnfG* did not seem to have a polar effect on the translation of *vnfK*, since the products of *vnfH*, *vnfD*, and *vnfK* were present in approximately equal amounts in both strains, CA and CA119 (Fig. 1A and B). This

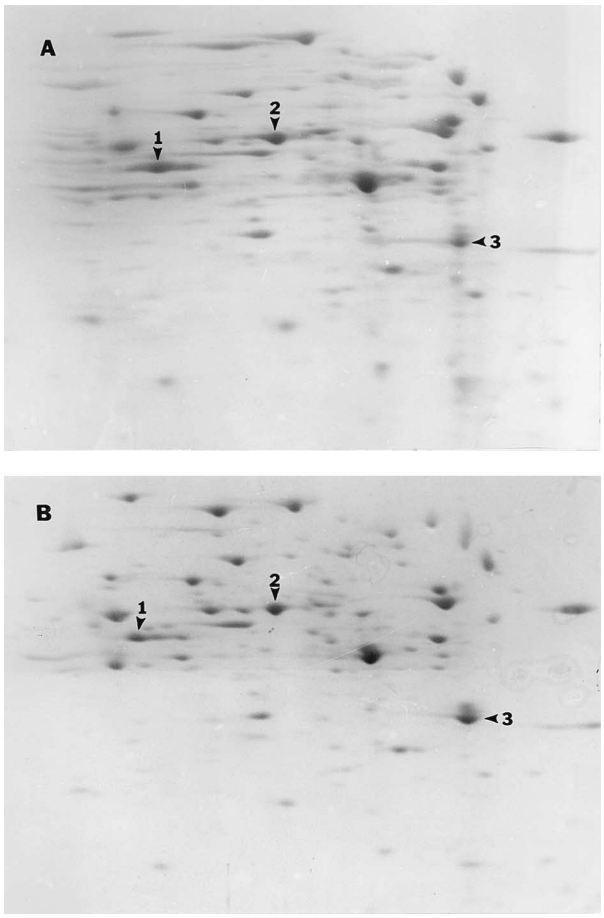

FIG. 1. Two-dimensional gels of protein extracts from CA (A) and CA119 (B) derepressed in N-free medium containing 1 μM V_2O_5. 1, α subunit of dinitrogenase 2; 2, β subunit of dinitrogenase 2; 3, dinitrogenase reductase 2.

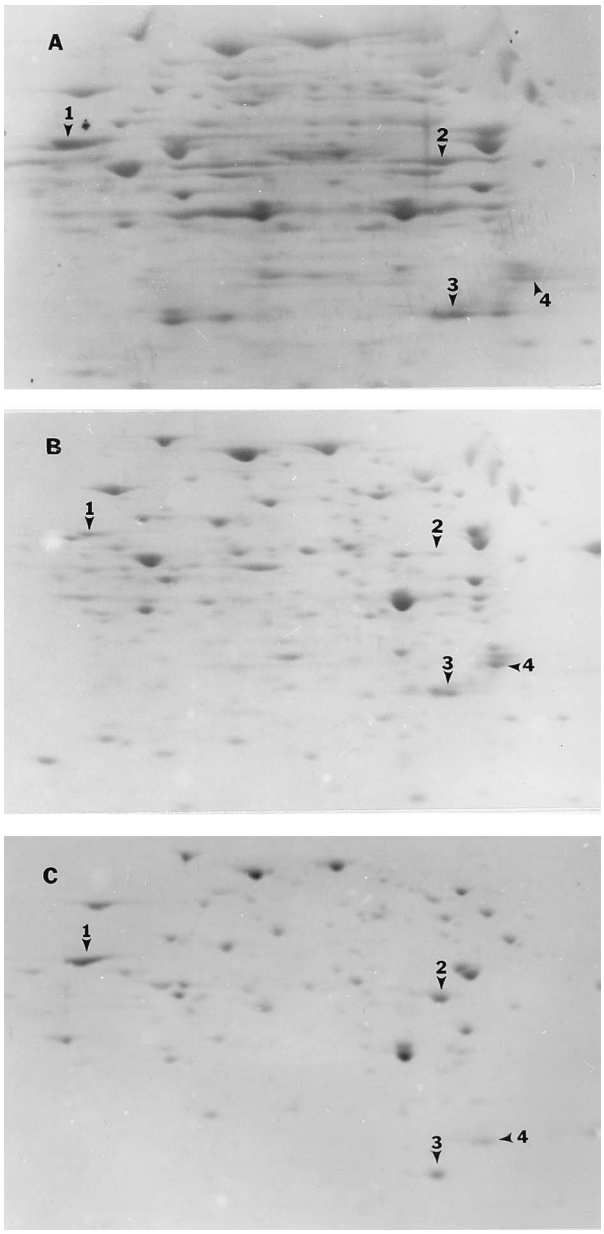

FIG. 2. Two-dimensional gels of protein extracts from *A. vinelandii* strains CA (A), CA76 (B), and CA79 (C) derepressed in N-free Mo-deficient medium. 1, α subunit of dinitrogenase 3; 2, β subunit of dinitrogenase 3; 3, dinitrogenase reductase 3; 4, dinitrogenase reductase 2.

result is not surprising, since 41 bases lie between the natural translational stop of *vnfG* and the ribosome binding site of *vnfK* (19). The stop codon mutation in *anfG* may have a polar effect on the translation of *anfK* since the amount of the β subunit appeared to be reduced compared with those of the other nitrogenase 3 subunits in strain CA76 (Fig. 2B). This could be due to translational coupling of *anfG* and *anfK* since there is an overlap between the potential ribosomal binding site for *anfK* and natural translational stop for *anfG* (18). Because the stop codon mutation in *anfG* appeared to reduce the amount of the β subunit on two-dimensional protein gels, an in-frame deletion of 258 bp was made in this gene (CA79). In contrast to CA76, cell extracts of CA79 contained normal amounts of the dinitrogenase 3 β subunit (Fig. 2C).

Cysteine residues are known to serve as ligands for Fe-S clusters in proteins such as nitrogenase. Additionally, cysteine residues may serve as nucleophiles in catalysis, as redox centers in electron transfer, or as structural cross-links in the form of disulfides (17). The α and β subunits of all known nitrogenases have five conserved cysteine residues in the α subunit and three in the β subunit. These conserved cysteine residues have been the targets of site-directed mutagenesis in *A. vinelandii* (5, 8) and *Klebsiella pneumoniae* (20). In most cases the substitution of alanine or serine for the conserved cysteine residues resulted in either complete loss or reduced ability to fix nitrogen. Thus, to ascertain the functional requirement of the conserved cysteine (Cys-36) in AnfG, the cysteine was replaced by alanine (CA77) or serine (CA78).

Diazotrophic growth of strains with mutations in *anfG* and *vnfG*. Diazotrophic growth of strains carrying mutations in *anfG* was monitored in Mo-deficient medium. Strain CA (wild type) grew with a generation time of 5.6 h, while CA77 (C36A) and CA78 (C36S) had generation times of 5.9 and 5.8 h, respectively (Fig. 3A). Strains CA76 (C36stop) and CA79 (Δ*anfG*) did not grow under these conditions (Fig. 3A). An interpretation of the effects of mutations in *anfG* could be complicated because of the possible contribution by other functional nitrogenases coupled with the potential for cofactor swapping (11, 31). Therefore, each of these mutations was introduced into double-deletion strains lacking nitrogenases 1

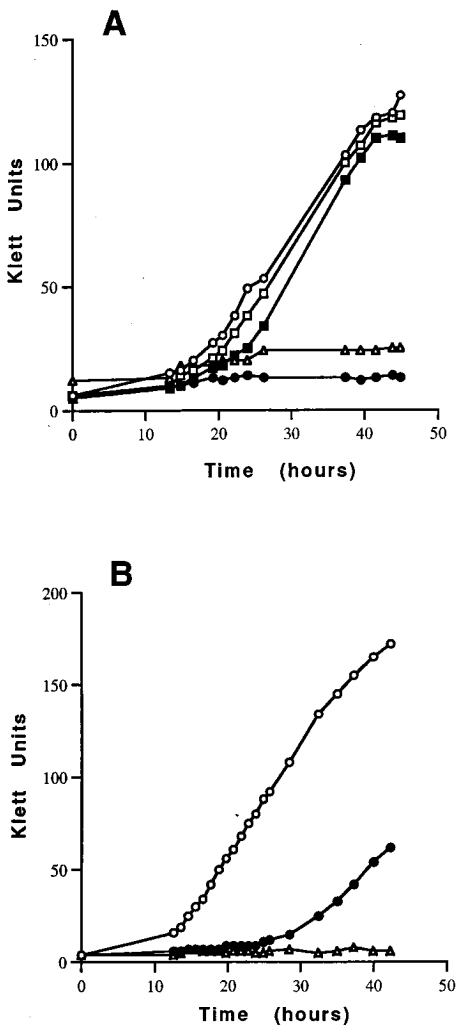

FIG. 3. (A) Diazotrophic growth of strains CA (○), CA76 (●), CA77 (□), CA78 (■), and CA79 (△) in Mo-deficient medium. (B) Diazotrophic growth of strains CA (○), CA119 (●), and CA11.71.119 (△) in medium containing 1 μM V_2O_5.

and 2. Growth experiments with these strains (CA11.1.76, CA11.1.77, and CA11.1.78) gave essentially the same results as those for strains containing nitrogenases 1 and 2 (data not shown). These results indicate that the *anfG* gene product is essential for nitrogenase 3-dependent growth. Furthermore, it can be concluded that the C36A and C36S substitutions have only a minor effect on nitrogenase 3-dependent growth. Thus, Cys-36 is probably not acting as an essential ligand. It is possible, however, that one of the other three cysteine residues (positions 63, 76, and 83) in AnfG can substitute for Cys-36. Martin et al. (26) showed that replacement of Cys-20 by Ala in *A. vinelandii* ferredoxin I leads to the formation of a new 4Fe-4S cluster in which the ligating function of Cys-20 is replaced by Cys-24. Although AnfG does not appear to be a ferredoxin, this example brings up the possibility that cysteine residues in AnfG might be interchangeable. In summary, the growth experiments suggest that Cys-36 of AnfG is not essential for nitrogenase 3 function.

Strain CA119 (C17stop in VnfG) was unable to grow diazotrophically in Mo-deficient medium containing V until after approximately 25 h of incubation (Fig. 3B). On the other hand,

TABLE 2. Acetylene reduction activities of AnfG and VnfG mutant strains of *A. vinelandii*

Incubation and strain	Phenotype	Acetylene reduction activity[a] (nmol of $C_2H_4 \cdot 30\ min^{-1} \cdot mg\ of\ protein^{-1}$)
16 h in Mo-deficient medium		
CA11.1	$Nif^- Vnf^-$	6.3
CA11.71	$Nif^- Anf^-$	0.9
CA11.1.71	$Nif^- Vnf^- Anf^-$	0.3
CA11.1.76	$Nif^- Vnf^-$ AnfG (C36stop)	1.2
CA11.1.77	$Nif^- Vnf^-$ AnfG (C36A)	3.3
CA11.1.78	$Nif^- Vnf^-$ AnfG (C36S)	5.4
CA11.1.79	$Nif^- Vnf^- AnfG^-$	4.4
3.5 h in Mo-deficient medium containing 1 μM V_2O_5		
CA11.71	$Nif^- Anf^-$	41.4
CA11.1	$Nif^- Vnf^-$	1.1
CA11.1.71	$Nif^- Vnf^- Anf^-$	0.8
CA11.71.119	$Nif^- Anf^-$ VnfG (C17stop)	14.4

[a] Determined as described in Materials and Methods.

a strain containing this mutation and lacking nitrogenases 1 and 3 (CA11.71.119) did not grow under these conditions even after prolonged incubation (Fig. 3B). The *vnfG* product, therefore, appears to be necessary for nitrogenase 2-dependent growth. Growth by CA119 after 25 h of incubation was probably due to nitrogenase 3, even though 1 μM V_2O_5 normally represses this nitrogenase. Pau et al. (32) observed a similar growth pattern in the presence of V by a strain of *A. vinelandii* carrying deletions in the structural genes for nitrogenases 1 and 2. Thus, with strains lacking nitrogenase 2, it appears that nitrogenase 3 can be expressed even in the presence of normally repressive amounts of V.

Acetylene reduction assays. Since $NifV^-$ mutants of *K. pneumoniae* (27) and *A. vinelandii* (24) are able to reduce acetylene even though they are unable to grow diazotrophically, it was of interest to test the AnfG and VnfG mutant strains for their abilities to reduce acetylene. The results of a typical experiment are given in Table 2. It should be mentioned that there was a rather large variation between acetylene reduction values from experiment to experiment, but within a given experiment the values observed for the mutants relative to the control were consistent. Strains CA11.1 and CA11.71 were used as positive controls for nitrogenase 3 and nitrogenase 2 activities, respectively.

AnfG mutant strains CA11.1.76 (C36stop), CA11.1.77 (C36A), CA11.1.78 (C36S), and CA11.1.79 (Δ*anfG*) reduced acetylene at 19, 52, 86, and 70%, respectively, of the rate observed with strain CA11.1 derepressed in Mo-deficient medium (Table 2). The difference between the acetylene reduction rates exhibited by CA11.1.76 and CA11.1.79 could be due to a polar effect of C36stop on the accumulation of AnfK (Fig. 2B). The ability of CA11.1.79 to reduce acetylene coupled with its inability to grow diazotrophically in Mo-deficient media suggests that the *anfG* product is required for the reduction of N_2 but not for acetylene reduction. There is evidence that the reduction of N_2 to NH_3 requires more specific cofactor-polypeptide interactions than the reduction of other substrates (9, 36, 39). Thus, the *anfG* product may be involved with cofactor-polypeptide interactions in dinitrogenase 3 (31). It should be pointed out that CA11.1.71, which carries deletions

in the structural genes of all three nitrogenases (Table 1), still has about 5% of the acetylene reduction activity of the control. The reason for this low level of activity in a strain lacking all three nitrogenases is not known. However, it may be dependent on the presence of cofactors since acetylene reduction is undetectable with a NifB$^-$ mutant.

After derepression in Mo-deficient medium containing V, the VnfG mutant, strain CA11.71.119, reduced acetylene at 35% of the rate for the control strain CA11.71 (Table 2).

The wild-type strain CA gave acetylene reduction activities of 621 and 63 nmol of $C_2H_4 \cdot 30\ min^{-1} \cdot mg$ of $protein^{-1}$ for nitrogenase 2 and nitrogenase 3, respectively. The reason that these activities are approximately 10 times greater than the activities for the double-deletion strain controls (CA11.71 for nitrogenase 2 and CA11.1 for nitrogenase 3) is unclear.

In summary, our results show that VnfG and AnfG are required for nitrogenase 2- and nitrogenase 3-dependent diazotrophic growth, respectively. However, these subunits do not seem to be required for acetylene reduction by nitrogenases 2 and 3.

ACKNOWLEDGMENTS

This work was done in a cooperative study between the Agricultural Research Service, U.S. Department of Agriculture, and the North Carolina Agricultural Research Service.

This work was supported by U.S. Department of Agriculture competitive grant 92-37305-7722.

REFERENCES

1. **Bishop, P. E., M. E. Hawkins, and R. R. Eady.** 1986. Nitrogen fixation in molybdenum-deficient continuous culture by a strain of *Azotobacter vinelandii* carrying a deletion of the structural genes for nitrogenase (*nifHDK*). Biochem. J. **238:**437–442.
2. **Bishop, P. E., D. M. L. Jarlenski, and D. R. Hetherington.** 1980. Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. Proc. Natl. Acad. Sci. USA **77:**7342–7346.
3. **Bishop, P. E., and R. Premakumar.** 1992. Alternative nitrogen fixation systems, p. 736–762. *In* G. Stacey, R. H. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman & Hall, New York.
4. **Bishop, P. E., R. Premakumar, D. R. Dean, M. R. Jacobson, J. R. Chisnell, T. M. Rizzo, and J. Kopczynski.** 1986. Nitrogen fixation by *Azotobacter vinelandii* strains having deletions in structural genes for nitrogenase. Science **232:**92–94.
5. **Brigle, K. E., R. A. Setterquist, D. R. Dean, J. S. Cantwell, M. C. Weiss, and W. E. Newton.** 1987. Site-directed mutagenesis of the nitrogenase molybdenum-iron protein of *Azotobacter vinelandii*. Proc. Natl. Acad. Sci. USA **84:**7066–7069.
6. **Bush, J. A., and P. W. Wilson.** 1959. A non-gummy chromogenic strain of *Azotobacter vinelandii*. Nature (London) **184:**381.
7. **Chisnell, J. R., R. Premakumar, and P. E. Bishop.** 1988. Purification of a second alternative nitrogenase from a *nifHDK* deletion strain of *Azotobacter vinelandii*. J. Bacteriol. **170:**27–33.
8. **Dean, D. R., K. E. Brigle, H. D. May, and W. E. Newton.** 1988. Site-directed mutagenesis of the Mo-Fe protein, p. 107–113. *In* H. Bothe, F. J. deBruijn, and W. E. Newton (ed.), Nitrogen fixation: hundred years after. Gustav Fischer Verlag, Stuttgart, Germany.
9. **Dilworth, M. J., M. E. Eldridge, and R. R. Eady.** 1993. The molybdenum and vanadium nitrogenases of *Azotobacter chroococcum*: effect of elevated temperature on N_2 reduction. Biochem. J. **289:**395–400.
10. **Eady, R. R.** 1991. The Mo-, V- and Fe-based nitrogenase systems of *Azotobacter*. Adv. Inorg. Chem. **36:**77–102.
11. **Eady, R. R., R. N. Pau, D. J. Pau, and F. J. Luque.** 1990. Vanadium nitrogenase of *Azotobacter*, p. 125–133. *In* P. M. Gresshoff, L. E. Roth, G. Stacey, and W. E. Newton (ed.), Nitrogen fixation: achievements and objectives. Chapman & Hall, New York.
12. **Eady, R. R., T. H. Richardson, R. W. Miller, M. Hawkins, and D. J. Lowe.** 1988. The vanadium nitrogenase of *Azotobacter chroococcum*. Purification and properties of the Fe protein. Biochem. J. **256:**189–196.
13. **Eady, R. R., R. L. Robson, T. H. Richardson, R. W. Miller, and M. Hawkins.** 1987. The vanadium nitrogenase of *Azotobacter chroococcum*. Purification and properties of the V-Fe protein. Biochem. J. **244:**197–207.
14. **Gillum, W. O., L. E. Mortenson, J. S. Chen, and R. H. Holm.** 1977. Quantitative extrusions of the Fe_4S_4* cores of the active sites of ferredoxins and the hydrogenase of *Clostridium pasteurianum*. J. Am. Chem. Soc. **99:**586–595.
15. **Hales, B. J., D. J. Langosch, and E. E. Case.** 1986. Isolation and characterization of a second nitrogenase Fe-protein from *Azotobacter vinelandii*. J. Biol. Chem. **261:**15301–15306.
16. **Hales, B. J., E. E. Morningstar, M. F. Dzeda, and L. Mauterer.** 1986. Isolation of a new vanadium containing nitrogenase from *Azotobacter vinelandii*. Biochemistry **25:**7251–7255.
17. **Hausinger, R. P., and J. B. Howard.** 1983. Thiol reactivity of the nitrogenase Fe-protein from *Azotobacter vinelandii*. J. Biol. Chem. **258:**13486–13492.
18. **Joerger, R. D., M. R. Jacobson, R. Premakumar, E. D. Wolfinger, and P. E. Bishop.** 1989. Nucleotide sequence and mutational analysis of the structural genes (*anfHDGK*) for the second alternative nitrogenase from *Azotobacter vinelandii*. J. Bacteriol. **171:**1075–1086.
19. **Joerger, R. D., T. M. Loveless, R. N. Pau, L. A. Mitchenall, B. H. Simon, and P. E. Bishop.** 1990. Nucleotide sequences and mutational analysis of the structural genes for nitrogenase 2 of *Azotobacter vinelandii*. J. Bacteriol. **172:**3400–3408.
20. **Kent, H. M., I. Ioannidis, C. Gormal, B. E. Smith, and M. Buck.** 1989. Site-directed mutagenesis of the *Klebsiella pneumoniae* nitrogenase. Effects of modifying conserved cysteine residues in the α- and β- subunits. Biochem. J. **264:**257–264.
21. **Kim, J. S., and D. C. Rees.** 1992. Crystallographic structure and functional implications of the nitrogenase molybdenum-iron protein from *Azotobacter vinelandii*. Nature (London) **360:**553–560.
22. **Kim, J. S., and D. C. Rees.** 1994. Nitrogenase and biological nitrogen fixation. Biochemistry **33:**389–397.
23. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
24. **Madden, M. S., T. D. Paustian, P. W. Ludden, and V. K. Shah.** 1991. Effects of homocitrate, homocitrate lactone, and fluorohomocitrate on nitrogenase in NifV$^-$ mutants of *Azotobacter vinelandii*. J. Bacteriol. **173:**5403–5405.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Martin, A. E., B. K. Burgess, C. D. Stout, V. L. Cash, D. R. Dean, G. M. Jensen, and P. J. Stephens.** 1990. Site-directed mutagenesis of *Azotobacter vinelandii* ferredoxin I: [Fe-S] cluster-driven protein rearrangement. Proc. Natl. Acad. Sci. USA **87:**598–602.
27. **McLean, P. A., and R. A. Dixon.** 1981. Requirement of *nifV* gene for production of wild-type nitrogenase enzyme in *Klebsiella pneumoniae*. Nature (London) **292:**655–656.
28. **Messing, J., B. Gronenborn, B. Müller-Hill, and P. H. Hofschneider.** 1977. Filamentous coliphage M13 as a cloning vehicle: insertion of a *Hin*dII fragment of the *lac* regulatory region in M13 replicative form *in vivo*. Proc. Natl. Acad. Sci. USA **74:**3642–3646.
29. **O'Farrell, P. H.** 1975. High resolution two-dimensional gel electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
30. **Orme-Johnson, W. H.** 1985. Molecular basis of biological nitrogen fixation. Annu. Rev. Biophys. Biophys. Chem. **14:**419–459.
31. **Pau, R. N., M. E. Eldridge, D. J. Lowe, L. A. Mitchenall, and R. R. Eady.** 1993. Molybdenum-independent nitrogenases of *Azotobacter vinelandii*: a functional species of alternative nitrogenase 3 isolated from a molybdenum-tolerant strain contains an iron-molybdenum cofactor. Biochem. J. **293:**101–107.
32. **Pau, R. N., L. A. Mitchenall, and R. L. Robson.** 1989. Genetic evidence for an *Azotobacter vinelandii* nitrogenase lacking molybdenum and vanadium. J. Bacteriol. **171:**124–129.
33. **Robson, R. L., P. R. Woodley, R. N. Pau, and R. R. Eady.** 1988. Cloning and analysis of the structural genes (*vnfD*, *vnfG* and *vnfK*) for the V-protein of the V-containing nitrogenase from *Azotobacter chroococcum*, p. 107–113. *In* H. Bothe, F. J. deBruijn, and W. E. Newton (ed.), Nitrogen-fixation: hundred years after. Gustav Fischer Verlag, Stuttgart, Germany.
34. **Robson, R. L., P. R. Woodley, R. N. Pau, and R. R. Eady.** 1989. Structural genes for the vanadium nitrogenase from Azotobacter chroococcum. EMBO J. **8:**1217–1224.
35. **Schüddekopf, K., S. Hennecke, U. Liese, M. Kutsche, and W. Klipp.** 1993. Characterization of *anf* genes specific for the alternative nitrogenase and identification of *nif* genes required for both nitrogenases in *Rhodobacter capsulatus*. Mol. Microbiol. **8:**673–684.
36. **Scott, D. J., H. D. May, W. E. Newton, K. E. Brigle, and D. R. Dean.** 1990. Role for the nitrogenase Mo-Fe protein α-subunit in FeMo-cofactor binding and catalysis. Nature (London) **343:**188–190.
37. **Shah, V. K., and W. J. Brill.** 1977. Isolation of an iron molybdenum cofactor from nitrogenase. Proc. Natl. Acad. Sci. USA **74:**3249–3253.
38. **Smith, B. E., P. E. Bishop, R. A. Dixon, R. R. Eady, W. A. Filler, D. J. Lowe, A. J. M. Richards, A. J. Thomson, R. N. F. Thorneley, and J. R. Postgate.** 1985. The iron-molybdenum cofactor of nitrogenase, p. 597–603. *In* H. J. Evans, P. J. Bottomley, and W. E. Newton (ed.), Nitrogen fixation research.

Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
39. **Smith, B. E., R. R. Eady, D. J. Lowe, and C. Gormal.** 1988. The vanadium-iron protein of vanadium nitrogenase from *Azotobacter chroococcum* contains an iron-vanadium cofactor. Biochem. J. **250:**299–302.
40. **Strandberg, G. W., and P. W. Wilson.** 1968. Formation of the nitrogen-fixing enzyme system in *Azotobacter vinelandii*. Can. J. Microbiol. **14:**25–31.
41. **Thiel, T.** 1993. Characterization of genes for an alternative nitrogenase in the cyanobacterium *Anabaena variabilis*. J. Bacteriol. **175:**6276–6286.
42. **Yates, M. G.** 1992. The enzymology of molybdenum dependent nitrogen fixation, p. 685–735. *In* G. Stacey, H. J. Evans, and R. H. Burns (ed.), Biological nitrogen fixation. Chapman & Hall, New York.
43. **Zinoni, F., R. M. Robson, and R. L. Robson.** 1993. Organization of potential alternative nitrogenase genes from *Clostridium pasteurianum*. Biochim. Biophys. Acta **1174:**83–86.