JOURNAL OF BACTERIOLOGY, Jan. 1996, p. 204–208 Vol. 178, No. 1
0021-9193/96/$04.00+0

Characteristics of *orf1* and *orf2* in the *anfHDGK* Genomic Region Encoding Nitrogenase 3 of *Azotobacter vinelandii*

PHOTINI V. MYLONA,[1] R. PREMAKUMAR,[1] RICHARD N. PAU,[2] AND PAUL E. BISHOP[1]*

Agricultural Research Service, U.S. Department of Agriculture, and Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615,[1] and Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, United Kingdom[2]

Received 17 August 1995/Accepted 31 October 1995

In *Azotobacter vinelandii*, the *anfHDGK* operon encodes the subunits for the third nitrogenase complex. Two open reading frames (*orf1* and *orf2*) located immediately downstream of *anfK* were shown to be required for diazotrophic growth under Mo- and V-deficient conditions. We have designated *orf1* and *orf2 anfO* and *anfR*, respectively. Strains (CA115 and CA116) carrying in-frame deletions in *anfO* and *anfR* accumulate the subunits for nitrogenase 3 under Mo-deficient diazotrophic conditions. AnfO and AnfR are required for nitrogenase 3-dependent diazotrophic growth and $^{15}N_2$ incorporation but not for acetylene reduction. AnfO contains a putative heme-binding domain that exhibits similarity to presumed heme-binding domains of P-450 cytochromes. Amino acid substitutions of Cys-158 show that this residue is required for fully functional AnfO as measured by diazotrophic growth under Mo- and V-deficient conditions. The nucleotide sequence of the region located immediately downstream of *anfR* has been determined. A putative ρ-independent transcription termination site has been identified 250 bp from the 3′ end of *anfR*. A third open reading frame (*orf3*), located downstream of *anfR*, does not appear to be required for diazotrophic growth under Mo- and V-deficient conditions.

Nitrogen fixation, the reduction of dinitrogen to ammonia, is an enzymatic process that occurs in a wide range of bacterial species, including the soil bacterium *Azotobacter vinelandii*. This process is catalyzed by the enzyme complex nitrogenase. Nitrogenase is a metalloenzyme composed of two protein components called dinitrogenase reductase and dinitrogenase. *A. vinelandii* harbors three genetically distinct nitrogenases (3, 4). The first nitrogenase is the molybdenum (Mo)-containing enzyme (nitrogenase 1) that is made in the absence of a fixed nitrogen source when Mo is available (9). The second enzyme, a vanadium (V)-containing nitrogenase (nitrogenase 2), is synthesized under Mo-deficient conditions in the presence of V (4, 9, 12). The third nitrogenase (nitrogenase 3) is expressed only in the absence of Mo and V (3, 6, 17, 30).

The protein subunits for the Mo nitrogenase are encoded by the *nifHDK* operon (10). The structural genes for V nitrogenase comprise two operons, *vnfHorfFd* and *vnfDGK* (12). The subunits for the third nitrogenase are encoded by the *anfHDGK* operon (11).

Recently, three other diazotrophs, *Clostridium pasteurianum* (41), *Rhodobacter capsulatus* (16, 33), and *Rhodospirillum rubrum* (4), were found to have alternative nitrogenases that are similar to nitrogenase 3 of *A. vinelandii*. These nitrogenases are expressed only in the absence of Mo. Nitrogenase 3 is composed of dinitrogenase 3 and dinitrogenase reductase 3. Dinitrogenase reductase 3 is a homodimer (γ_2) encoded by the *anfH* gene, while dinitrogenase 3 is a hexamer ($\alpha_2\beta_2\delta_2$). The α- and β-subunits are encoded by the *anfD* and *anfK* genes, respectively. The δ-subunit, a small protein, characteristic of the alternative nitrogenases (33, 39, 41), is encoded by the *anfG* gene.

In addition to the structural genes for nitrogenase 3, the *anfHDGK* operon contains two additional open reading frames (ORFs) (*anfO* and *anfR*) located immediately downstream of the *anfK* gene (4, 11). Recent studies show that *anfO* and *anfR* are cotranscribed with the *anfHDGK* genes in one polycistronic message that apparently undergoes posttranscriptional processing (31). *anfO* codes for a putative polypeptide with a molecular weight of 26,943 and a pI of 5.99 (4, 11). A region (amino acids 37 to 136) of the *anfO* translation product exhibits some degree of identity with the N-terminal domain of dinitrogenase reductases (11). Another region of this putative peptide (amino acids 150 to 168) exhibits 57.9% identity to a putative heme-binding domain of cytochrome P-450 (11, 15, 25).

The putative product of *anfR* has an estimated molecular mass of 20,241 Da and a pI of 5.38. This product does not show any significant similarity to sequences in the database. To date, the possible functional role(s) of these two ORFs has not been identified. Here we present evidence for the involvement of *anfO* and *anfR* in nitrogenase 3-dependent diazotrophic growth. We also report on the characterization of a third ORF downstream of *anfHDGK*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *Escherichia coli* and *A. vinelandii* used in this study are listed in Table 1. *E. coli* K-12 71-18 and CJ236 were cultured in Luria-Bertani medium. *A. vinelandii* strains were grown in modified Burk medium (36) as previously described (3). When required, antibiotics were added to the following concentrations: ampicillin (50 μg/ml), kanamycin (10 μg/ml), spectinomycin (20 μg/ml), and rifampin (5 μg/ml). When required, Na_2MoO_4 and V_2O_5 were each added to a final concentration of 1 μM. Fixed nitrogen was added in the form of ammonium acetate to a final concentration of 28 mM.

Growth of *A. vinelandii* strains. *A. vinelandii* strains were transferred, at least three times, on solid Mo- and V-deficient medium containing fixed nitrogen (2, 3). The strains were then transferred to Mo-deficient, N-containing Burk medium and grown to mid-log phase for use as inocula. Thirty milliliters of N-free, Mo- and V-deficient medium in a 300-ml side-arm flask was inoculated to a density of approximately 10 Klett units (1 Klett unit equals 5×10^6 CFU/ml). Growth at 30°C was monitored with a Klett-Summerson colorimeter equipped with a no. 66 red filter.

$^{15}N_2$ incorporation. Cells were grown in 25 ml of Mo- and V-deficient medium in sealed conical flasks at 30°C for 40 h. Air in the space (43 ml) above the culture represented 10% $^{15}N_2$ of the gas phase at 1 atm (ca. 100 kPa). Control cultures contained air. The atom percent excess of $^{15}N_2$ from the cells after oxidation with

* Corresponding author.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) and/or genotype	Reference or source
E. coli strains		
K-12 71-18	*supE thi* Δ(*lac-proAB*) F′ (*proAB*$^+$) *lacI*^{q}*Z* ΔM15	19
CJ236	*dut ung thi relA* pCJ105 (Cm^r)	14
Plasmids		
M13mp19	M13 cloning vector	23, 40
pUC18	Amp^r	38
pDB303	Amp^r, 1.7-kbp *Eco*RI fragment containing *rpoB* in pUC18	10
pJSM1	Amp^r Spec^r, 1.3-kbp *Bgl*II fragment from pLAM3 replaced by Spec^r gene	R. Pau
pLAM1031	Amp^r Kan^r, 1.8-kbp *Bcl*I fragment of pDB86 replaced by Kan^r gene	R. Pau
pJFL33	Amp^r, 2.2-kbp *Sma*I fragment containing *anfO-anfR* of the *anf* cluster in pTZ18R	R. Pau
pPAU147.8	Amp^r, 3.2-kbp *Eco*RI fragment containing *anfO*, *anfR*, and part of *orf3* in pTZ18R	R. Pau
pPM110	Amp^r, 0.9-kbp *Sma*I-*Eco*RI fragment containing part of *orf3* (derived from pPAU147.8) in pUC18	This study
pPM114	Amp^r Kan^r, same as pPAU147.8, carries a Kan^r cartridge in *anfO* in pUC18	This study
pPM115	Amp^r, 3.4-kbp *Bam*HI-*Kpn*I fragment containing the 3′ end of *orf3* and the region downstream in pUC18	This study
pPM1	Amp^r, 2.0-kbp *Eco*RI-*Sma*I fragment containing the 3′ end of *anfK* and all of *anfO* and *anfR* in pUC18	This study
pPM12	Amp^r, 1.2-kbp *Eco*RI-*Sma*I fragment containing the 3′ end of *anfK* and all of *anfR* in pUC18	This study
pPM13	Amp^r, 1.7-kbp *Eco*RI fragment containing the 3′ end of *anfK* and all of *anfO* and the 5′ end of *orf3* in pUC18	This study
pPM52	Amp^r Kan^r, same as pPM12 except the *Bam*HI fragment containing *anfO* is replaced by the 1.6-kbp Kan^r cartridge from pKIXX	This study
pPM53	Amp^r Kan^r, 2.6-kbp *Sma*I-*Eco*RI fragment containing *orf3*, insertion of Kan^r cartridge in *Bam*HI site	This study
pPM54	Amp^r, same as pPM1, C158A in AnfO	This study
pPM55	Amp^r, same as pPM1, C158S in AnfO	This study
pKISS	Amp^r Kan^r, pUC4 with Kan^r cartridge derived from Tn*903*	Pharmacia
pKIXX	Amp^r Kan^r, pUC4 with Kan^r cartridge derived from Tn*5*	Pharmacia
A. vinelandii strains		
CA	Wild type	11
CA11.71	Δ*nifHDK* Δ*vnfDGK*::*kan*	11
RP1.11	Δ*nifHDK* Δ*vnfDGK*	17
CA114	*anfO*::*kan*	This study
CA115	Δ*anfO*	This study
CA116	Δ*anfR*	This study
CA120	Δ*nifHDK*::*kan* Δ*vnfDGK*::*spec* Δ*anfO*	This study
CA121	Δ*nifHDK*::*kan* Δ*vnfDGK*::*spec* Δ*anfR*	This study
CA122	*anfO*, C158A	This study
CA123	*anfO*, C158S	This study
CA124	*orf3*::*kan*	This study
CA130	Δ*anfO*::*kan*	This study

copper oxide for 10 min at 600°C was measured with a VG Siara mass spectrometer.

Construction of *A. vinelandii* mutant strains. *A. vinelandii* strains were made competent and transformed with plasmid DNA as described by Page and von Tigerstrom (28). Plasmid pPAU 147.8 was partially digested with *Pst*I and ligated to a kanamycin resistance cartridge (*kan*) contained on a 1.3-kbp *Pst*I fragment from pKISS (Pharmacia, Piscataway, N.J.). *E. coli* K-12 71-18 cells were transformed with the ligation mixture, and Kan^r Amp^r transformants were selected. The location of the *kan* interposon in the plasmid construct was confirmed by restriction endonuclease analysis. One of the recombinant plasmids, pPM114, was used to transform *A. vinelandii* CA to Kan^r. Kan^r Amp^s transformants (indicative of a double-crossover event) were transferred at least four times on medium containing kanamycin (10 μg/ml) to ensure segregation of the *kan* insertion prior to tests for the Anf (ability to grow diazotrophically under Mo- and V-deficient conditions) phenotype of the transformants. An isolate, designated strain CA114, was unable to grow diazotrophically in the absence of Mo and V (Anf$^-$); however, in the presence of Mo or V growth was normal. The presence of the *kan* interposon in CA114 was verified by Southern hybridization analysis.

In-frame deletions were placed in *anfO* and *anfR* to determine if one or both ORFs were required for diazotrophic growth. The sense strand of a 2.0-kbp *Eco*RI-*Sma*I insert (containing *anfO*, *anfR*, and part of *anfK*) derived from plasmid pFJL-33 (31) was cloned into M13mp19. With this insert as a template, site-directed mutagenesis was carried out by the method of Kunkel et al. (14) according to the instruction manual for the Muta-gene M13 in vitro mutagenesis kit purchased from Bio-Rad Laboratories (Richmond, Calif.). The nucleotide bases that were altered to produced new restriction sites are located at positions 4620 and 5394 (according to the numbering in reference 11). Each mutant derivative was subcloned into pUC18 (*Eco*RI-*Sma*I sites) and then was subjected to transformation into *E. coli* K-12 71-18.

Plasmid pPM1 with *Bam*HI sites at the 5′ and 3′ ends of *anfO* was digested with *Bam*HI, and the resulting 4.2-kbp fragment was ligated to itself. *E. coli* K-12 71-18 cells were transformed with the ligation mixture, and Amp^r transformants were selected. The in-frame deletion of *anfO* (spanning nucleotides 4620 to 5341) was confirmed by restriction endonuclease analysis, and one of the Amp^r recombinants, pPM12, was transformed into *A. vinelandii* CA114 by congression with rifampin resistance as the selection marker as described previously (10). Rif^r Kan^s Amp^s transformants were transferred at least four times on Burk medium to ensure segregation of the *anfO* deletion. One of the transformants was designated CA115. A similar procedure was followed for plasmid pPM13, which contains *Sma*I sites at the 5′ and 3′ ends of *anfR*. The in-frame deletion of *anfR* spanned nucleotides 5341 to 6108 according to the numbering in reference 15. The resulting strain was designated CA116. Southern hybridization analysis was used to verify the deletions of *anfO* and *anfR* in strains CA115 and CA116.

Construction of an *anfO* deletion plus insertion mutant was as follows: pPM12 DNA was digested with *Bam*HI, and the resulting 4.2-kbp fragment was ligated to a Kan^r cartridge isolated from pKIXX (Pharmacia) following cleavage with *Bam*HI. Competent *E. coli* K-12 71-18 cells were transformed with the ligation mixture, and Kan^r Amp^r transformants were selected. Restriction endonuclease analysis confirmed the replacement of *anfO* by the Kan^r cartridge. One of the plasmids (pPM52) was used to transform *A. vinelandii* CA. Kan^r Amp^s transformants were selected and transferred at least four times on medium containing kanamycin (10 μg/ml). The resulting strain was designated CA130. Site-directed mutagenesis was used to make the following amino acid substitutions: Cys-158 to Ala (C158A) (TGC→**GC**C) and C158S (TGC→**A**GC). (The position of the amino acid is according to the numbering in reference, 15 and the altered bases are shown in boldface type.) pPM54 carries the mutation for the C158A substitution and pPM55 carries the mutation for the C158S substitution. These mutations were transferred to *A. vinelandii* with CA130 in congression crosses as

described above. Two transformants, CA122 (C158A) and CA123 (C158S), were isolated.

Plasmid pPM110 carries a 0.9-kbp *Sma*I-*Eco*RI insert harboring the genomic region located downstream of *anfR*. This plasmid was digested with *Bam*HI, and the linearized plasmid was ligated to a 1.6-kbp *kan*-containing *Bam*HI fragment from pKIXX. *E. coli* K-12 71-18 cells were transformed with the ligation mixture and screened for Kan^r and Amp^r. A plasmid isolated from one of the Kan^r Amp^r transformants, pPM53, was used to transform *A. vinelandii* CA. The resulting strain was designated CA124. Southern hybridization analysis was used to verify the presence of the *kan* interposon in *orf3*.

Two-dimensional gel electrophoresis. *A. vinelandii* CA115, CA116, and CA were derepressed for nitrogenase 3 in N-free, Mo-deficient Burk medium for 12 h. Cell protein extracts were obtained as previously described (3), and the isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis steps were conducted as described by O'Farrell (27) with modifications as described by Bishop et al. (3).

Acetylene reduction assays. Samples (5 ml) of culture were prepared as previously described (3). The C_2H_2 reduction assay was initiated by injecting 0.5 ml of C_2H_2 into each vial. After 15 min of incubation at 30°C with vigorous shaking, samples of 0.5 ml were withdrawn to determine C_2H_4 with a Carle model 311H gas chromatograph equipped with a flame ionization detector and a column (182 by 0.32 cm) of Porapak N.

DNA manipulations and sequencing. Plasmid DNA was isolated as described by Maniatis et al. (19). Transformation of competent *E. coli* cells by electroporation was performed as previously described (19). A 0.9-kbp *Sma*I-*Eco*RI fragment isolated from pPAU147.8, containing the region downstream of *anfR*, was cloned into pUC18. In addition to this, a 3.4-kbp *Bam*HI-*Kpn*I fragment was also isolated from *A. vinelandii* genomic chromosomal DNA. This fragment contains the 3′ end of the 0.9-kbp *Sma*I-*Eco*RI and the region downstream. This 3.4-kbp *Bam*HI-*Kpn*I fragment was cloned into pUC18 and the plasmid was designated pPM115. pPM115 along with plasmid pPM110 was used to determine the nucleotide sequence of the region located downstream of *anfR*. Sequencing was carried out by the method of Sanger et al. (32) with ^{35}S-dCTP (Dupont, NEN Research Products, Boston, Mass.). All nucleotides were purchased from United Scientific Biochemical, Cleveland, Ohio. Site-specific primers were designed to extend the known sequence by a "primer walking" strategy (37). Site-specific oligonucleotides were purchased from Integrated DNA Technologies, Coralville, Iowa. The DNA sequence was analyzed for base and codon preference with the Genetics Computer Group computer programs (7, 13). Amino acid sequences were compared with other sequences by using the Swissprot and GenBank databases (1).

Nucleotide sequence accession number. The nucleotide sequence of the 1.366-kbp region of the *A. vinelandii* genome has been submitted to GenBank under accession no. U37517.

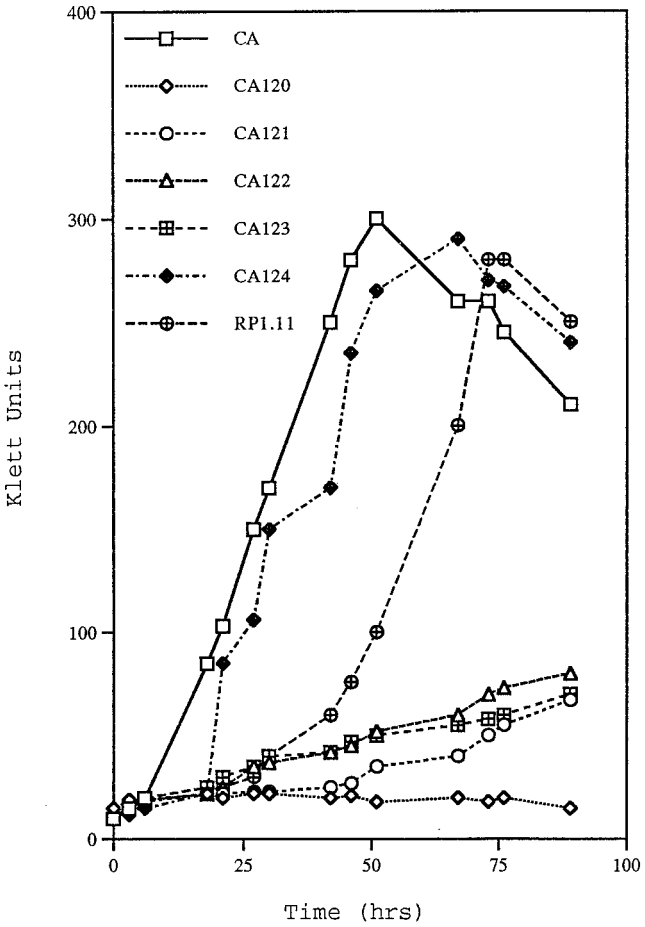

FIG. 1. Diazotrophic growth of *A. vinelandii* mutant strains in Mo- and V-deficient medium.

RESULTS

Nucleotide sequence analysis. In order to define the complete *anfHDGKorf1orf2* operon, the nucleotide sequence of a 1.366-kbp region of the *A. vinelandii* genome (containing the region located immediately downstream of *anfR*) has been determined. The nucleotide sequence for this region (data not shown) was analyzed for base and codon preferences. One potential ORF (designated *orf3*) was found. Within this nucleotide sequence two putative ρ-independent transcription terminator sites (5, 20) were identified. The first ρ-independent terminator site is located 250 nucleotides downstream of *anfR*. The presence of this site suggests the end of the *anfHDGK orf1orf2* transcriptional unit.

Growth of mutants carrying deletion or insertion mutations. To determine if *orf3* is involved in nitrogenase 3-dependent diazotrophy, a *kan* interposon was inserted in this ORF. This insertion mutant (CA124) grew well under diazotrophic conditions in Mo- and V-deficient medium (Fig. 1), indicating that the *orf3* product is not significantly involved in nitrogen fixation under these conditions. On the other hand, strains CA114 (*anfO*::*kan*), CA115 (Δ*anfO*), and CA116 (Δ*anfR*) are unable to grow under the same conditions. However, in the presence of Mo or V, these strains grew normally. To ascertain whether the observed phenotype is maintained in strains that carry mutations in the structural genes for nitrogenases 1 and 2, strains CA120 (Δ*nifHDK* Δ*vnfDGK* Δ*anfO*) and CA121 (ΔnifHDK ΔvnfDGK ΔanfR) were constructed. Both strains were unable to grow under Mo- and V-deficient diazotrophic conditions (Fig. 1). It should be mentioned that after long periods of incubation (~50 h), slow diazotrophic growth was observed with strain CA121. This growth could be attributed to secondary mutations that arise in the population, allowing diazotrophic growth in the absence of *anfR*.

In a previous report (11) it was noted that the predicted translation product of *anfO* contains a site that exhibits similarity to the presumed heme-binding sites of P-450 cytochromes. In AnfO, Cys-158 appears to be conserved among the putative heme-binding sites of these cytochromes (21, 26). Thus, substitutions of this residue were performed to test its role in the function of the predicted *anfO* product. Strains CA122 (C158A) and CA123 (C158S) exhibited an intermediate phenotype with respect to diazotrophic growth (Fig. 1). Both mutant strains grew better than CA120 (Δ*anfO*) but much more slowly than wild-type strain CA. In the presence of Mo and/or V, these mutants grew as well as the wild type under nitrogen-fixing conditions. Hence, it appears that Cys-158 is important for a fully functional *anfO* product.

Incorporation of $^{15}N_2$. The inability of CA115 and CA116 to fix nitrogen in medium lacking Mo and V was confirmed by the absence of an increase in the $^{15}N/^{14}N$ ratio when these strains were grown in the presence of $^{15}N_2$. The atom percent enrichment was 4% for the wild-type cells grown in the presence of $^{15}N_2$, while the atom percent enrichment for CA115 and CA116 was 0% over the same growth period (40 h).

In summary, the results presented above indicate that both

TABLE 2. Acetylene reduction by *anfO* and *anfR* deletion mutants

Strain	Whole-cell acetylene reduction[a] (% of wild type)
CA (wild type)	0.0872 (100.0)
CA115 (Δ*anfO*)	0.0980 (112.3)
CA116 (Δ*anfR*)	0.0750 (86.0)
CA11.71 (Δ*nifHDK* Δ*vnfDGK*::*kan*)	0.0090 (10.3)

[a] Units are nanomoles of C_2H_4 reduced · 60 min^{-1} · Klett $unit^{-1}$.

anfO and *anfR* are involved in the nitrogen fixation process under Mo-deficient conditions.

Two-dimensional gel electrophoresis of protein extracts from mutant cells derepressed for nitrogenase under Mo-deficient conditions. Since strains CA114, CA115, CA116, CA120, and CA121 were observed to have an Anf^- phenotype, it was of interest to see whether the subunits of nitrogenase 3 accumulated under Mo- and V-deficient conditions. After derepression in Mo-deficient medium (12 h) nitrogenase 3 subunits accumulated to approximately the same levels in the mutant strains as in the wild type (data not shown). Thus, the accumulation of nitrogenase 3 subunits does not seem to be affected by the absence of *anfO* or *anfR*.

Acetylene reduction experiments. In previous studies (18, 22) $NifV^-$ mutants of *Klebsiella pneumoniae* and *A. vinelandii* were found to reduce acetylene levels even though they are unable to fix nitrogen. Thus, strains CA115 and CA116 were tested for acetylene reduction capability and strains CA and CA11.71 were used as positive and negative controls, respectively. CA115 and CA116 reduced acetylene levels at 112 and 86%, respectively, of the rate observed with strain CA derepressed in Mo-deficient medium (Table 2). The reduction of acetylene by strains CA115 and CA116 coupled with their inability to fix N_2 indicates that the *anfO* and *anfR* products are required for the reduction of N_2 but not for acetylene reduction. Previous studies show that the reduction of N_2 to NH_3 requires more specific cofactor-polypeptide interactions than does the reduction of other substrates (8, 29, 34, 35). Thus, the *anfO* and *anfR* products could be involved in cofactor-polypeptide interactions in dinitrogenase 3.

DISCUSSION

In this study we have shown that in-frame deletions of *anfO* and/or *anfR* result in an Anf^- phenotype. Thus, on the basis of the results of diazotrophic growth and $^{15}N_2$ incorporation experiments, it is clear that the products of these genes are required for N_2 reduction by nitrogenase 3. Because of this and the location of *anfO* and *anfR* in the same operon as *anfHDGK*, we propose designating *orf1* and *orf2 anfO* and *anfR*, respectively.

We previously reported (6) that purification of nitrogenase 3 from *A. vinelandii* resulted in a less-active enzyme compared with the Mo and V nitrogenases. We indicated that this characteristic might be due to a number of factors, including the following: (i) nitrogenase 3 is by nature a less efficient enzyme, (ii) the optimum conditions for maximum enzymatic activity may not have been found, and (iii) the absence of trace elements or protein subunits (lost during purification) that are required for maximal activity are absent or depleted. With regard to the third possibility, these proteins could be the products of *anfO* and *anfR*.

Genes that appear to be homologs of *anfO* and *anfR* also appear necessary for nitrogenase 3-dependent diazotrophic growth in *R. capsulatus* (16). In *R. capsulatus*, the organization of the *anf* genes is the same as that in *A. vinelandii* (33). Two ORFs were found immediately downstream of *anfK*. Insertion mutagenesis in these ORFs resulted in an Anf^- phenotype. The predicted products of these ORFs show a high degree of similarity between the two organisms. AnfO from *A. vinelandii* has 34.06% identity with ORF1 from *R. capsulatus*, while AnfR has 30.97% identity with its counterpart in *R. capsulatus*. From this it appears that these products could serve the same function in these organisms.

Previously (11) we reported that AnfO has a site that exhibits some identity to the heme-binding domain of P-450 cytochromes. These cytochromes are found in most eukaryotes and in a range of prokaryotes (24–36). They are oxidative enzymes involved in detoxification. One region of the P-450 primary sequence that is uniquely associated with the heme at the enzyme's active site has been identified. This segment is located near the carboxyl end of the protein (21, 26) and contains a conserved cysteine residue that serves as the fifth thiolate ligand to the heme iron. Sequence comparisons among a number of P-450 cytochromes revealed that they share a high degree of similarity and that they all have a conserved cysteine residue at the heme-binding site of the enzyme (21). This cysteine is also conserved in AnfO. Since alanine and serine substitutions for Cys-158 in AnfO lead to greatly reduced nitrogenase 3-dependent diazotrophic growth, we can conclude that this cysteine residue is required for a fully functional AnfO protein.

To determine if other ORFs located downstream of *anfR* are involved in diazotrophic growth, the nucleotide sequence of this region was obtained. One ORF (*orf3*) was found 334 nucleotides downstream of *anfR*. Insertion mutagenesis of *orf3* showed that this ORF is dispensable for diazotrophic growth under all conditions. A putative ρ-independent transcription termination site was found in the genomic region located immediately downstream of *anfR*. Previous studies (31) have shown that the *anfHDGKOR* operon is transcribed as a single polycistronic message that undergoes posttranscriptional processing. On the basis of the size of the *anf* transcripts and the fact that *orf3* is dispensable for diazotrophic growth, it appears that the identified ρ-independent transcription termination site may define the 3′ end of the *anfHDGKOR* operon.

In conclusion, our studies show that *anfO* and *anfR* are required for nitrogenase 3-dependent reduction of N_2 but not for the reduction of acetylene.

ACKNOWLEDGMENTS

We thank R. Richards for the ^{15}N determinations.

This work was the result of a cooperative study between the Agricultural Research Service, U.S. Department of Agriculture, and the North Carolina Agricultural Research Service. This investigation was supported by U.S. Department of Agriculture competitive grant 92-37305-7722 and by NATO travel grant 880532. P.V.M. has been supported by the State Scholarship Foundation of Greece.

REFERENCES

1. **Benson, D., D. J. Lipman, and J. Ostell.** 1993. GenBank. Nucleic Acids Res. **21:**2963–2965.
2. **Bishop, P. E., M. E. Hawkins, and R. R. Eady.** 1986. Nitrogen fixation in Mo-deficient continuous culture by a strain of *Azotobacter vinelandii* carrying a deletion of the structural genes for nitrogenase (*nifHDK*). Biochem. J. **238:** 437–442.
3. **Bishop, P. E., D. M. L. Jarlenski, and D. R. Hetherington.** 1980. Expression of an alternative nitrogen fixation system in *Azotobacter vinelandii*. J. Bacteriol. **150:**1244–1251.
4. **Bishop, P. E., and R. Premakumar.** 1992. Alternative nitrogen fixation systems, p. 737–762. *In* G. Stacey, R. H. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman & Hall, New York.

5. **Brendel, V., and E. N. Trifonov.** 1984. A computer algorithm for testing potential prokaryotic terminators. Nucleic Acids Res. **12:**4411–4427.
6. **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.
7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
8. **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.
9. **Eady, R. R.** 1991. The Mo-, V- and Fe-based nitrogenase systems of *Azotobacter*. Adv. Inorg. Chem. **36:**77–102.
10. **Jacobson, M. R., K. E. Brigle, L. T. Bennett, R. A. Setterquist, M. S. Wilson, V. L. Cash, J. Beynon, W. E. Newton, and D. R. Dean.** 1989. Physical and genetic map of the major *nif* gene cluster from *Azotobacter vinelandii*. J. Bacteriol. **171:**1017–1027.
11. **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.
12. **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.
13. **Johnston, R. E., J. MacKenzie, Jr., and W. G. Dougherty.** 1986. Assembly of overlapping DNA sequences by a program written in BASIC for 64K CP/M and MS-DOS IBM-compatible microcomputers. Nucleic Acids Res. **14:**517–527.
14. **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.
15. **Kiyoshi, K., S. R. Van Doren, D. M. Kramer, A. R. Crofts, and R. B. Gennis.** 1991. Preparation and characterization of water-soluble heme binding domain of cytochrome c_1 from the *Rhodobacter sphaeroides* bc_1 complex. J. Biol. Chem. **266:**14270–14276.
16. **Klipp, W.** Personal communication.
17. **Luque, F., and R. N. Pau.** 1991. Transcriptional regulation by metals of structural genes for *Azotobacter vinelandii* nitrogenases. Mol. Gen. Genet. **227:**481–487.
18. **Madden, M. S., T. D. Paustian, P. W. Ludden, and V. K. Shah.** 1991. Effects of homocitrate lactone and fluorohomocitrate on nitrogenase in $NifV^-$ mutants of *Azotobacter vinelandii*. J. Bacteriol. **173:**5403–5405.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Martin, F. H., and I. Tinoco.** 1980. DNA-RNA duplexes containing oligo(dU:dA) sequences are exceptionally unstable and may facilitate termination of transcription. Nucleic Acids Res. **8:**2295–2299.
21. **Matsunaga, E., M. Umero, and F. J. Gonzalez.** 1990. The rat P450IID subfamily: complete sequences of four closely linked genes and evidence that gene conversion maintained sequence homogeneity at the heme-binding region of the cytochrome P450 active site. J. Mol. Evol. **30:**155–169.
22. **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.
23. **Messing, J. B., B. Gronenborn, B. Muller-Hill, and P. H. Hofschneider.** 1977. Filamentous coliphage M13 as a cloning vehicle: insertion of a *Hind*II fragment of the *lac* regulatory region in M13 replicative form in vivo. Proc. Natl. Acad. Sci. USA **76:**3642–3646.
24. **Nebert, D. W., M. Adesnik, M. J. Coon, R. W. Eastbrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman.** 1987. P450 gene superfamily: recommended nomenclature. DNA **6:**1–11.
25. **Nebert, D. W., and F. J. Gonzalez.** 1985. Cytochrome P-450 gene expression and regulation. Trends Pharmacol. Sci. **6:**160–164.
26. **Nebert, D. W., and F. J. Gonzalez.** 1987. P450 genes: structure, evolution and regulation. Annu. Rev. Biochem. **56:**943–993.
27. **O'Farrell, P. H.** 1975. High resolution two dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
28. **Page, W. J., and M. von Tigerstrom.** 1979. Optimal conditions for transformation of *Azotobacter vinelandii*. J. Bacteriol. **139:**1058–1061.
29. **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.
30. **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.
31. **Premakumar, R., M. R. Jacobson, T. M. Loveless, and P. E. Bishop.** 1992. Characterization of transcripts expressed from nitrogenase-3 structural genes of *Azotobacter vinelandii*. Can. J. Microbiol. **38:**929–936.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
33. **Schneider, K., A. Muller, U. Schramm, and W. Klipp.** 1991. Demonstration of molybdenum- and vanadium-independent nitrogenase in *nifHDK*-deletion mutant of *Rhodobacter capsulatus*. Eur. J. Biochem. **195:**653–661.
34. **Scott, D. J., H. D. May, W. E. Newton, K. E. Brigle, and D. R. Dean.** 1990. Role of the nitrogenase Mo-Fe protein α-subunit in FeMo-cofactor binding and catalysis. Nature (London) **343:**188–190.
35. **Smith, B. E., R. R. Eady, D. J. Lowe, and C. Gormel.** 1988. The vanadium-iron protein of vanadium nitrogenase from *Azotobacter chroococcum* contains an iron-vanadium cofactor. Biochem. J. **250:**299–302.
36. **Strandberg, G. W., and P. W. Wilson.** 1968. Formation of the nitrogen-fixing enzyme system in Azotobacter vinelandii. Can. J. Microbiol. **14:**25–31.
37. **Straus, E. C., J. A. Kobori, G. Siu, and L. E. Hood.** 1986. Specific primer directed DNA sequencing. Anal. Biochem. **154:**353–360.
38. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
39. **Waugh, S. I., D. M. Paulsen, P. V. Mylona, R. H. Maynard, R. Premakumar, and P. E. Bishop.** 1995. The genes encoding the delta subunits of dinitrogenases 2 and 3 are required for Mo-independent diazotrophic growth by *Azotobacter vinelandii*. J. Bacteriol. **177:**1505–1510.
40. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene **33:**103–109.
41. **Zinoni, F. R., R. M. Robson, and R. L. Robson.** 1993. Organisation of potential alternative nitrogenase genes from *Clostridium pasteurianum*. Biochim. Biophys. Acta **174:**83–86.