

Nucleotide Sequence and Mutational Analysis of the Structural Genes (*anfHDGK*) for the Second Alternative Nitrogenase from *Azotobacter vinelandii*†

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The nucleotide sequence of a region of the *Azotobacter vinelandii* genome exhibiting sequence similarity to *nifH* has been determined. The order of open reading frames within this 6.1-kilobase-pair region was found to be *anfH* (alternative nitrogen fixation, *nifH*-like gene), *anfD* (*nifD*-like gene), *anfG* (potentially encoding a protein similar to the product of *vnfG* from *Azotobacter chroococcum*), *anfK* (*nifK*-like gene), followed by two additional open reading frames. The 5'-flanking region of *anfH* contains a *nif* promoter similar to that found in the *A. vinelandii* *nifHDK* gene cluster. The presumed products of *anfH*, *anfD*, and *anfK* are similar in predicted M_r and pI to the previously described subunits of nitrogenase 3. Deletion plus insertion mutations introduced into the *anfHDGK* region of wild-type strain *A. vinelandii* CA resulted in mutant strains that were unable to grow in Mo-deficient, N-free medium but grew in the presence of 1 μ M Na_2MoO_4 or V_2O_5 . Introduction of the same mutations into the *nifHDK* deletion strain CA11 resulted in strains that grew under diazotrophic conditions only in the presence of vanadium. The lack of nitrogenase 3 subunits in these mutant strains was demonstrated through two-dimensional gel analysis of protein extracts from cells derepressed for nitrogenase under Mo and V deficiency. These results indicate that *anfH*, *anfD*, and *anfK* encode structural proteins for nitrogenase 3.

Azotobacter vinelandii and *Azotobacter chroococcum* are capable of reducing N_2 to NH_4^+ by different nitrogenases whose expression is regulated by the molybdenum (Mo) or vanadium (V) content of the culture medium (3-6, 14, 18, 23, 24, 28, 47, 51, 53). Nitrogenase 1, which is expressed by both organisms in the presence of Mo, is an enzyme complex composed of two components, dinitrogenase reductase 1 and dinitrogenase 1 (11, 68). Dinitrogenase reductase 1 is made up of two identical subunits encoded by *nifH* (9) with a single 4Fe-4S cluster bridged between the two subunits (21). Dinitrogenase 1 is a tetramer of two pairs of nonidentical subunits (34) encoded by *nifD* and *nifK* (8, 9, 33). Dinitrogenase contains two types of metal centers: P centers that might be organized as four 4Fe-4S clusters (for a review, see reference 46) and two identical FeMo cofactors (58). These metal centers are thought to be involved in the redox reactions of the N_2 reduction process. In *A. vinelandii*, *nifHDK* are organized in an operon that might contain four additional genes located downstream from *nifK* (57). Nitrogenase 2 from *A. vinelandii* and *A. chroococcum* is present in cells grown in N-free medium containing V (5, 18, 23, 24, 51, 53). This nitrogenase also consists of two components. Dinitrogenase reductase 2 is a dimer (18, 23) whose subunits are thought to be encoded by *vnfH* (previously designated *nifH2* [30] and *nifH** [55]). Dinitrogenase 2 has been purified from *A. vinelandii* (24) and *A. chroococcum* (18) as a

tetramer of two pairs of subunits. However, a third type of subunit with an M_r of approximately 14,000 appears to be present in dinitrogenase 2 from *A. chroococcum* (17, 18). Dinitrogenase 2 contains Fe and V (18, 24), and a FeV cofactor analogous to the FeMo cofactor has been reported for *A. chroococcum* (1, 60). The genetic basis of the V nitrogenase from *A. vinelandii* is not as well understood as it is for the V nitrogenase from *A. chroococcum*, but extensive similarities appear to exist within the two organisms (49, 55; our unpublished results). The *vnfH* genes in both organisms are organized in an operon also containing a ferredoxin-like gene downstream from *vnfH* (28, 54). The *vnfH*-ferredoxin operon is separated from an operon containing the structural genes (*vnfD* *vnfK*) for dinitrogenase 2 by approximately 2 kilobase pairs (kbp) in *A. chroococcum* and by 1 kbp in *A. vinelandii* (55 and our unpublished results). In *A. chroococcum*, a gene (*vnfG*) is located between *vnfD* and *vnfK* and encodes a protein now thought to be a third type of subunit (δ subunit) of V nitrogenase (17, 55). The function of the δ subunit is not known.

A third nitrogenase (nitrogenase 3) is present in *A. vinelandii*. It is expressed only in the absence of both Mo and V and has been purified as an enzyme complex of two components that does not appear to contain Mo or V (14). Dinitrogenase reductase 3 consists of two identical subunits with an M_r of 32,500 as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (14). Dinitrogenase 3 has been purified in two active configurations: $\alpha_2\beta_2$ and $\alpha_1\beta_2$. The component, as a tetramer (M_r 216,000), contained approximately 24 Fe and 18 acid-labile S^{2-} atoms, while the trimer contained approximately 11 Fe and 9 acid-labile S^{2-} atoms. The M_r s of the subunits were determined to be 58,000 and 50,000 (14).

In this paper, we present the nucleotide sequence of the

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, phage, or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i>		
HB101	F ⁻ <i>hsd-20</i> (<i>r_B</i> ⁻ <i>m_B</i> ⁻) <i>proA2 lacYI rpsL20 supE44</i>	36
NM539	<i>supF hsdR</i> (P2 <i>cox3</i>)	20
K-12 71-18	Δ (<i>lac-pro</i>) F' <i>lac^a lacZ M15 pro⁺ supE</i>	40
JM101	Δ (<i>lac-proAB</i>) <i>supE thi</i> (<i>r_K</i> ⁺ <i>m_K</i> ⁺)/F' <i>traD36 proAB lacI^a ZM15</i>	67
GM33	<i>dam-3</i> mutation in prototrophic <i>E. coli</i> W3110	37
<i>A. vinelandii</i>		
CA	Wild type	12
CA11	Δ <i>nifHDK</i>	6
CA70 ^a	Kan ^r <i>KpnI</i> fragment (positions 856-1687) replaced by Kan ^r cartridge	This study
CA71 ^a	Kan ^r <i>BclI</i> fragment (positions 1721-3533) replaced by Kan ^r cartridge	This study
CA72 ^a	Kan ^r <i>BclI</i> fragment (positions 3437-3533) replaced by Kan ^r cartridge	This study
Phages		
λ EMBL-3A-H3-3	Recombinant λ EMBL-3A phage (20) containing <i>anfH</i> and upstream sequences	This study
λ EMBL-3A-H3-10	Recombinant λ EMBL-3A phage (20) containing the <i>A. vinelandii</i> <i>anfHDKG</i> region	This study
Plasmids		
pUC9	Amp ^r	65
pHDK3	Amp ^r 11.8-kbp <i>Sall</i> fragment (containing <i>anfHDKG</i> region) in pUC9	This study
pHDK3-1	Amp ^r Kan ^r <i>KpnI</i> fragment (positions 856-1687) of pHDK3 replaced by Kan ^r cartridge	This study
pJWD3	Amp ^r 3-kbp <i>EcoRI</i> fragment (positions 1000-4078) in pUC9	This study
pJWD3-1	Amp ^r Kan ^r <i>BclI</i> fragment (positions 1721-3533) of pJWD3 replaced by Kan ^r cartridge	This study
pJWD3-2	Amp ^r Kan ^r <i>BclI</i> fragment from pJWD3 (positions 3537-3533) replaced by Kan ^r cartridge	This study
pKISS	pUC4 with Kan ^r cartridge derived from Tn903	Pharmacia
pKIXX	pUC4 with Kan ^r cartridge derived from Tn5	Pharmacia

^a Strains designated CA11.70, CA11.71, and CA11.72 carry the deletion plus insertion mutations as described for the strains CA70, CA71, and CA72 in addition to the *nifHDK* deletion of strain CA11.

third genomic region of *A. vinelandii* previously shown to hybridize to *nifH* (28). In addition, we provide genetic evidence indicating that this genomic region contains the genes encoding the subunits of the components of nitrogenase 3. We also propose that the genes encoding the structural proteins for nitrogenase 3 be designated *anf* (alternative nitrogen fixation).

(Some preliminary results from this study were presented at the 7th International Congress on Nitrogen Fixation [7].)

MATERIALS AND METHODS

Maintenance and growth of bacteria and bacteriophages.

The *A. vinelandii* strains (Table 1) used for this study were maintained and cultured in Burk medium as previously described (4, 31). For growth studies under Mo-deficient conditions and in the presence of V, precautions were taken to minimize contamination by metals as previously described (29). *Escherichia coli* HB101, GM33, and NM539 were grown in TYE or LB medium. *E. coli* K-12 71-18 and JM101 were maintained in M9 minimal medium (39) but were grown in TYE medium for transformation or phage infection experiments. λ EMBL3-A phages were grown and maintained by the procedures outlined by Silhavy et al. (59). M13 mp18 and mp19 (67) were grown and maintained as described by Messing (38, 39).

DNA manipulations. Plasmid DNA was isolated as described by Norgard (44) or by the rapid boiling procedure of Holmes and Quigley (26). Large-scale isolation of phage lambda DNA was accomplished by following the procedure described by Silhavy et al. (59). The isolation procedure for M13 single-stranded DNA has been published by International Biotechnologies, Inc. (IBI), New Haven, Conn. Restriction enzymes, alkaline phosphatase, and T4 DNA ligase

were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., or from Brisco Ltd., Winthrop, Mass. Transformation of competent *E. coli* cells by using the CaCl₂ method (35) was done as described by Maniatis et al. (36). *A. vinelandii* was transformed with plasmid or chromosomal DNA by the methods of Page and von Tigerstrom (48). Southern hybridization analyses were conducted as previously described (8).

Construction of an *A. vinelandii* genomic library. Large-molecular-weight total genomic DNA was isolated from *A. vinelandii* CA11, partially digested with *Sau3A*, and fractionated by centrifugation through a sucrose gradient (36). Fractions containing DNA fragments ranging between 10 and 20 kbp in size were pooled and used for ligation into λ EMBL-3A digested with *BamHI* to construct the library as described by the suppliers of λ EMBL-3A (Promega Biotec).

DNA sequencing and sequence analysis. The 3.8-kbp *EcoRI* fragment (isolated from λ EMBL-3A-H3; Table 1) which hybridized to *nifH* was cloned into M13 mp18 and mp19. Two recombinant M13 phages containing the *EcoRI* fragment inserted in opposite orientations were chosen for the generation of phage with deletions in the *EcoRI* fragment insert by the method of Misra (41). The deletions were created by *Bal31* digestion of phage DNA previously cut with *Sall*, followed by cleavage with *EcoRI*, treatment with Klenow fragment of DNA polymerase to generate blunt ends, and ligation into M13 mp18 replicative-form DNA that had been cut with *EcoRI* and *Sall*. (The *Sall* end was converted into a blunt end by treatment with the Klenow fragment of DNA polymerase.) DNA from phages carrying inserts generated in the manner described above was used for the sequence determination of the region of the 3.8-kbp *EcoRI* fragment hybridizing to *nifH*. The region downstream

from *anfH* was isolated from λ EMBL-3A-H3-10 (Table 1) as three overlapping restriction fragments (*Kpn*I, positions 856 to 1687; *Eco*RI, positions 1000 to 4078; *Sma*I, positions 3794 to 6103). Each of these fragments was cleaved with *Alu*I, *Hae*III, *Tha*I, or *Rsa*I, and the resulting fragments were ligated into M13 mp18 cleaved with *Sma*I. In addition, the *Kpn*I, *Eco*RI, and *Sma*I fragments were digested with *Sau*3A, and the fragments resulting from this digestion were cloned into M13 mp18 cleaved with *Bam*HI. The recombinant M13 mp18 phages were used to prepare templates for sequencing. Sequencing was carried out by the method of Sanger et al. (56) by using [³⁵S]dATP (Dupont, NEN Research Products, Boston, Mass.). All nucleotides (7-deaza-dGTP was substituted for dGTP) were purchased from Boehringer Mannheim Biochemicals. The Klenow fragment was purchased from IBI. The M13 single-stranded primer (17 bases), as well as a number of site-specific primers, was synthesized by using a Pharmacia gene assembler. The site-specific primers were used to determine the sequence of regions for which no suitable subfragments, generated through the use of the four restriction enzymes listed above, were available. Individual sequences were analyzed for overlaps and were organized into a contiguous sequence with the aid of a sequence alignment program (32).

Determination of restriction sites and amino acid sequences was accomplished by using the programs written by Mount and Conrad (42). The DNA sequence was analyzed for base and codon preference by using the UWGCG computer programs (16), which are based on the work of Fickett (19) and Gribskov et al. (22). Amino acid sequences were compared with other sequences by using the XFASTP program of the BIONET National Computer Resource for Molecular Biology. Amino acid sequences were aligned, and the percentage of identical amino acid residues was determined by the GAP program of the UWGCG computer programs (16).

Construction of *A. vinelandii* mutants carrying *Kan*^r cartridge insertions plus deletions. Screening of the *A. vinelandii* genomic library in λ EMBL-3A with a *nifH*-specific probe (28) yielded a phage (λ EMBL-3A H3-10) that carried DNA sequences extending approximately 5 kbp downstream from *anfH*. These downstream sequences, as well as *anfH* and upstream sequences, are contained on an 11.8-kbp *Sal*I fragment. This fragment was ligated into *Sal*I-cut pUC9, resulting in plasmid pHDK3 (Table 1). Plasmid pHDK3 was cut with *Kpn*I, and the resulting 13.7-kbp fragment was ligated in the presence of the *Kan*^r cartridge isolated from pKISS following cleavage with *Kpn*I. The ligation mixture was used to transform competent *E. coli* HB101, and *Kan*^r *Amp*^r transformants were selected. The presence of plasmids that contained a *Kan*^r cartridge in place of the 800-bp *Kpn*I fragment was confirmed by restriction analysis, and one plasmid preparation (pHDK3-1) was used to transform competent *A. vinelandii* strains CA, CA11, and CA11.6. *Kan*^r transformants which were *Amp*^s were transferred at least four times on medium containing kanamycin (10 μ g/ml) to ensure segregation of the *Kan*^r marker prior to tests for the *Nif* phenotype of presumed mutants. Plasmid pJWD3 containing the 3-kbp *Eco*RI fragment (positions 1000 to 4078; Fig. 1) was isolated from *E. coli* GM33 (*dam*-3) and used to construct pJWD3-1 by replacing the *Bcl*I fragment with the *Kan*^r cartridge (*Bam*HI-cut) from pKIXX. Plasmid pJWD3-2 (Table 1) was obtained after pJWD3 was partially digested with *Bcl*I, and the linearized plasmid was ligated in the presence of the *Bam*HI-*Kan*^r cartridge from pKIXX. Cleavage of pJWD3-2 with *Bcl*I showed that during the partial

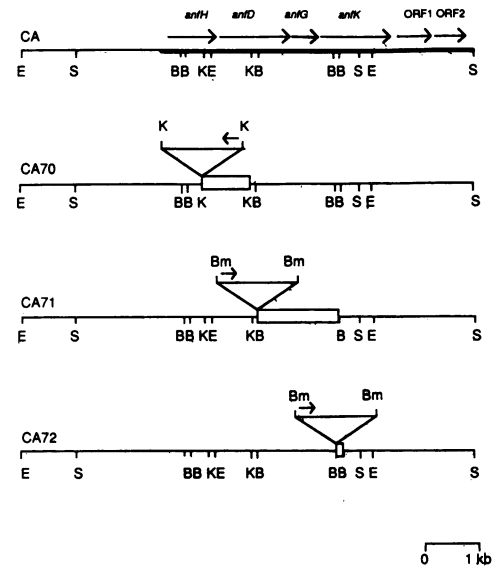


FIG. 1. Restriction map of the *anfHDGK* region from *A. vinelandii* strains CA, CA70, CA71, and CA72. The region whose nucleotide sequence has been determined (Fig. 2) is indicated by the heavier line. Restriction sites: E, *Eco*RI; S, *Sma*I; K, *Kpn*I; B, *Bcl*I. ∇ , *Kan*^r cartridge insertion; \rightarrow , \leftarrow , directions of transcription of the *Kan*^r genes of the cartridges. The cartridges used for strain CA71 and CA72 were *Bam*HI fragments (Bm) which were ligated into *Bcl*I-cut *A. vinelandii* DNA. \square , Deletion.

digestion step this restriction endonuclease had cleaved pJWD3 at positions 3437 and 3533. Thus, in pJWD3-2 the *Kan*^r cartridge replaces the *Bcl*I fragment (positions 3437 to 3533) of pJWD3. Plasmid pJWD3-1 and pJWD3-2 were used to construct mutants of *A. vinelandii* in the manner described above. The presence of the *Kan*^r cartridges and the deletions in these mutant strains was verified through Southern hybridization experiments involving various DNA probes and purified chromosomal DNA cleaved with appropriate restriction enzymes.

Two-dimensional gel electrophoresis. *A. vinelandii* CA11, CA11.70, CA11.71, and CA11.72 (Table 1) were derepressed for nitrogenase in N-free Mo-deficient Burk medium for 18 h. Cell-free protein extracts were obtained as previously described (4), and the isoelectric-focusing and SDS-polyacrylamide gel electrophoresis steps were conducted as described by O'Farrell (45) with modifications as described by Bishop et al. (4).

RESULTS

Nucleotide sequence analyses. The nucleotide sequence of a 6,108-bp region of the *A. vinelandii* genome (containing the third *nifH*-hybridizing region) has been determined and is indicated as a dark horizontal bar in Fig. 1. This nucleotide sequence (Fig. 2) was analyzed for base and codon preferences (Fig. 3). Six complete open reading frames (ORFs) (designated *anfH*, *anfD*, *anfG*, *anfK*, ORF1, and ORF2) were found (Fig. 1 to 3). The codon preference analysis (Fig. 3) indicates that the codon usage of the last ORF differs to some extent from that of the other ORFs, since the plot in that area does not rise as high above background level as the plot for the other ORFs. In addition, the plot does not return to background level at the end of the ORF. A *nif* consensus

1 AGCTGGAAAATTTCCAGTATTTTCAATTGATTGA
 <----->

35 ATAGGGTGGTCCGACGTCGGGACACCTGTCGGACTTGTGGTGGCGCGGGCCG
 90 GTAAACTGGCACATGCAITGGCTTTATATACGACGTCACCTCGAAAAGTATTCTGTG
 145 TTGACGCGATCTCGGTTGGAACGACTCTCTGCGCGCATTTCTGTTGCTGTTGATTT
 200 TTTGTCTCTTTGTTCTTGGCAAGTTATGATAGAGAGGTTTTTTGTGTT ATG ACT
 M T

253 CGT AAA GTA GCC ATT TAC GGA AAA GGC GGT ATC GGC AAA TCC
 R K V A I Y G K G G I G K S

295 ACC ACT ACC CAG AAT ACT GCC GCA GCG CTG GCC TAT TTC CAC
 T T M A N T A A A L A Y F H

337 GAC AAG AAA GTC TTC ACT CAC GGC TGC GAC CCC AAG GGG GAC
 D K K V F T H G A A C P K A D

379 TCC ACC CGC CTG ATT CTC GGC GGC AAA CCT GAG GAC ACC CTG
 S T R L I L G G K P E E T L

421 ATG GAC ATG GTG CGC GAC AAG GGC GCC GAA AAG ATC ACC AAC
 M D M V T D L M E K I T N

463 GAC GAC GTG ATC AAA AAA GGC TTT CTG GAC ATC CAG TGC GTG
 D D V I K K G F L D I C Q C V

505 GAG TCC GGC GGC CCC GAG CCG GGC GTT GGT TGC GCT GGC CGC
 E S G G P E P G V G C A G R

547 GGC GTG ATC ACC GCC ATC GAC CTG ATG GAA GAA AAG GGC GCC
 G V T A I D L M E E N G A

589 TAT ACC GAT GAC CTG GAT TTC GTG TTC TTC GAC GAT CTG GGC
 Y T D D L G G F V F T C D D L G

631 GAC GTC GTG TGC GCG GGT TTC GCC ATG CCG ATC CGC GAC GGC
 D V V C G G F A M P I R D G

673 AAG GCC CAG GAA GTC TAC ATC GTG GCT TCC GGG GAG ATG ATG
 K A Q E V I V A S G G E M H

715 GCC ATT TAT GCG GCC AAC AAC ATC TGC AAG GGC CTG GTG AAA
 A I Y A A N I C A K G L V K

757 TAC GCC AAA CAA AGT GCG GTG GGC CTG GGC GGC ATC ATT TGC
 Y A K Q S A V G L G G I I C

799 AAC AGC CGT AAG GTG GAT GGC GAG CGC GAG TCC GTG GAA GAG
 N S R K V D G E R E S V E E

841 TTC ACC GCG GCC ATC GGT ACC AAG ATG ATC CAC TTC GTT CCG
 F T A A I G T K M I H F P P

883 CGC GAC AAT ATC GTG CAG AAG GCC GAG TTC AAC AAG AAG ACC
 R D N I V Q K A E F N K K T

925 GTG ACC GAG TTC GCT CCA GAG GAA AAC CAG GCC AAG GAG TAC
 V T E F A P E E N Q A K E Y

967 GGC GAG CTG GCC AAG ATC ATT GAG AAC GAT GAA TTC GTG
 G E L A R C I I E N D E F V

1009 ATT CCC AAA CCG CTG ACC ATG GAC CAA CTG GAA GAC ATG GTG
 I P K P L T M D C L E D M V

1051 GTC AAG TAC GGT ATT GCC GAC TGA TCGCAGATTCATTATCACTCTA
 V K Y G I A D *

1098 AAGGAGATGTGTC ATG CCG CAT CAC GAG TTC GAG TGC AGC AAG
 M P H E F E C S K

1141 GTT ATT CCC GAG CCG AAG AAG CAT GCC GTT ATC AAA GGT AAA
 V I P E R K H A V I K G K

1183 GGC GAA ACG CTG GCC GAC GCC CTG CCT CAA GGG TAT CTG AAT
 G E T L A D A L P Q G Y L N

1225 ACC ATC CCT GGT TCC ATC TCC GAG CGT GGT TGT GCC TAC TGT
 T I P F S A E R G C A Y C

1267 GGT GCC AAG CAC GTT ATC GGG ACT CCC ATG AAG GAT GTG ATT
 G A K H V I G T P M K D V I

1309 CAC ATC AGT CAT GGC CCG GTC GGC TGC ACT TAC GAT ACC TGG
 H I S H G P V G C T Y D T W

1351 CAG ACC AAG CGT TAT ATC AGC GAC AAC GAC AAC TTC CAG CTC
 Q T K R Y I S D N D N F Q L

1393 AAA TAC ACC TAT GCC ACC GAT GTG AAG GAA AAG CAT ATC GTG
 K Y T Y A T D V K E K H I V

1435 TTC GGC GCC GAG AAG TTG CTG AAG CAG AAC ATC ATC GAA GCC
 F G A E K L K Q N I I E A

1477 TTC AAG GCG TTC CCG CAG ATC AAG CGG ATG ACC ATC TAC CAG
 F K A F P Q I K R M T I Y Q C

1519 ACC TGC GCC ACT GCG CTG ATC GGA GAC GAC ATC AAC GCC ATC
 T C A A T L I G D A I N A I

1561 GCC GAA GAG GTG ATG GAA GAG ATG CCG GAG GTG GAT ATC TTC
 A E E V M E E M P E V D I F

1603 GTC TGC AAC TCG CCC GGT TTC GCC GGT CCG AGC CAG TCC GGT
 V C N S P G F A G G P S Q S G

1645 GGT CAC CAC AAG ATC AAC ATC GCC TGG ATC AAC CAG AAG GTG
 G H C H K I N I A W I N Q K V

1687 GGT ACC GTC GAG CCG GAG ATC ACC GGC GAC CAT GTG ATC AAC
 G T V E P E I T G D H V I N

1729 TAT GTG GGC GAG TAC AAC ATT CAG GGC GAC CAG GAA GTG ATG
 Y V G E Y N I Q G D Q E V M

1771 GTG GAT TAC TTC AAG CGC ATG GGT ATC CAG GTG CTA TCC ACT
 V D Y F K R M G I Q V L S T

1813 TTC ACC GGC AAC GGT TCC TAC GAC GGC CTG CGT GCC ATG CAC
 T T G N A G T C A Y D M H

1855 AGA GCC CAT CTG AAC GTA CTG GAA TGT GCC CGC TCC GCG AAG
 R A H L N V L E C C R S A E

1897 TAC ATC TGC AAC GAA CTG CGT CAC CGT TAC GGC ATT CCG CGT
 Y I C N E L R V R Y G I P R

1939 CTG GAT ATC GAC GGT TTC GGT TTC AAG CCA CTG GCG GAT TCG
 L D I D G F G A P L G S

1981 CTG CGT AAG ATC GGT ATG TTC TTC GGC ATC GAA GAC CGT GCC
 L R K I G M F F G E D C R A

2023 AAG GCC ATC ATC GAC GAG GAA GTC GCC CGC TGG AAG CCG GAG
 K A I I D E E V A R W K P E

2065 TTG GAC TGG TAC AAG GAG CCG GTG ATG GGC AAG GAG GTC TCC
 L D W Y K E R L M G A K K V C

2107 CTG TGG CCG GGC GGT TCC AAA CTC TGG CAC TGG GCC CAT GTG
 L W P G G S K L W H A E V G

2149 ATC GAG GAA GAA ATG GGC CTC AAG GTG GTG TCG GTC TAT ATC
 I E E E M G L K V V S V Y I

2191 AAG TTC GGC CAT CAG GGC GAC ATG GAG AAA GGC ATC CCG GT
 K T F G H Q G D M E K G I A R

2233 TGC GGC GAA GGC ACT TTG GCC ATC GAC GAC CCG AAC GAA TTG
 C G E G T L A I D A N S L

2275 GAA GGT CTG GAA GCC CTG GAG ATG CTC AAG CCC GAC ATC ATC
 E G L E A L E M L K P D I I

2317 CTG ACC GGC AAG CGT CCG GGT GAA GTG GCC AAG AAA GTC CCG
 L T G K R P G E V A K K V R

2359 GTT CCC TAC CTG AAC GCC CAC GCC TAC CAC AAC GGC CCG TAC
 V P Y L N A H A A N G C P Y

2401 AAA GGC TTC GAA GGT TGG GTG CGT TTC GCC AC CCG GAT ATT TAC
 K G F E G W V R F A R D I Y

2443 AAC GCC ATC TAC TCG CCG ATC CAT CAG CTC TCC GGT ATC GAC
 N A I Y S P I H Q L S G I D

2485 ATC ACT AAA GAC AAT GCA CCG GAG TGG GGT AAT GGT TTC CGT
 I T K D N A P E W A F R

2527 ACT CGC CAA ATG CTG TCC GAT GGC AAC TTG AGC GAT GCA GTA
 T R Q M L S D G A V

2569 CGT AAC TCG GAA ACC TTG CGC CAG TAC ACC GGC GGC TAC GAC
 R N S E T L R C Y T G G Y D

2611 AGC GTG AGC AAG CTG CGC GAA CCG GAA TAT Y CCC GTC GAG E
 S V S K L R E R E P A F

2653 CGC AAG GTC GGC TGA GAGATAACCTG ATG AGT ACC GCT TCC
 R K V G G * M S T A S

2695 GCC GCT GCT GTG GTC AAA CAG AAG GTC GAA GCT CCC GTG CAT
 A A A V V K Q K V E A P V H

2737 CCG ATG GAT GCA CCG ATC GAC CTG ACC GAC ATC TAC ATG ATG
 P M D A R I D E L T D Y I M

2779 AAA AAC TGC CTC TGG CAG TTC CAT TCC CCG TCC TGG GAC CCG
 K N C L W Q F H S R S W D R

2821 GAA CGC CAG AAC GCC GAA ATC CTG AAG AAA ACC AAG GAA CTG
 E R Q N A E I L K K T T K E L

2863 CTG TGC GGT GAG CCA GTG GAT CTG AGC ACA TCC CAT GAT CGT
 L C G E P V D L S T S H D R

2905 TGC TAC TGG GTC GAT GCG GTT TGT CTG GCC GAC GAT TAC CGG
 C Y W V D A V C L A D Y R

2947 GAG CAC TAT CCC TGG ATC AAT AGC ATG TCC AAG GAA GAA ATC
 E H Y P W I N S M S K E E I

2989 GGC TCC TTG ATG CAA GGA CTG AAA GAC CGC ATG GAT TAT CTG
 G S L M Q G L K D R M D Y L

3031 ACC ATC ACC GGC TCG CTC AAC GAA GAG TTG AGC GAC AAA CAC
 T I T G S L N E E T S D K H

3073 TAT TAA GAGGGTTCGAC ATG ACT TGC GAA GTC AAG GAA AAA
 Y * M T C E V K E K

promoter sequence (2) is located 132 nucleotides upstream from the putative translation initiation site of the first ORF (*anfH*). No *nifA* activator sequence (10) was observed in the region between *anfH* and an ORF upstream from it (our unpublished results). Between -244 and -230 nucleotides upstream from the initiation codon (positions 3 to 17 in Fig. 2) there is a possibility for the formation of a stem-and-loop

structure (Fig. 2). However, it is not known whether this feature is part of a transcription termination signal (13, 27) for a preceding operon.

Comparison of the predicted amino acid sequences of the protein products of the ORFs. A comparison of the amino acid sequences deduced from the nucleotide sequences of *nifH* (9) and *anfH* from *A. vinelandii*, of *vnfH* from *A.*

3115 GGG CGG GTT GGC ACT ATC AAC CCC ATC TTT ACC TGT CAA CCG
 G R V I N P I F T C C Q A P

3157 GCC GGT GCC CAG TTC GTC AGT ATC GGT ATC AAG GAT TGC ATC
 A G A Q F V S I G I K D C I

3199 GGT ATC GTG CAT GGC GGC CAA GGC TGC GTG ATG TTC GTC CGC
 G I V H M G C Q V F V M F V C R

3241 CTG ATC TTT TCC CAG CAC TAC AAG GAA AGT TTC GAG CTG GCC
 L I F S L H E D G A V F G A C

3283 TCT TCC TCC CTG CAC GAG GAC GGC GGC ATC TTC GGT GCC TGC
 S S S L H E D G A V F G A C

3325 GGC CGG GTC GAG GAA GGC GTC GAT GTG CTG CTC AGC CGC TAT
 G R V E A V D V L S L A C G C Y

3367 CCC GAC GTG AAG GTG GTG CCC ATC ATC ACC ACC TGC TCC ACC
 P D V K V E A V D V L S L A C G C Y

3409 GAG ATC ATC GGC GAC GAC GTG GAC GGG GTG ATC AAG AAG CTC
 E I I G D D V D G V I K K L

3451 AAC GAA GGG CTG CTG AAA GAG AAG TTC CCG GAC CGG GAA GTV
 N E G G L L K E K P P D G R E V

3493 CAT CTG ATC GCC ATG CAC ACG CCG AGC TTC GTG GGC AGC ATG
 H L I F S L H E D G A V F G A C

3535 ATC AGC GGC TAC GAC GTG GCC GTT CGG GAT GTG GTC AGG CAT
 I S G Y D V A V D V L S L A C G C Y

3577 TTC GCC AAG GGC GAA GGC GAC AAC ATC AAT CTG CTC
 F A K R E A C C P N D A K I A T L L

3619 ACC GGC TGG GTC AAT CCG GGG GAT GTC AAG GAG CTG AAG CAC
 T G W V K V P I I T V G S R Y

3661 CTG CTC GGG GAA ATG GAC ATC GAA GCC AAC GTG TTG TTC GAG
 L L G E M D I E A N V L F E

3703 ATC GAA AGT TTC GAC TCG CCG ATC CCG GAT GGC AGT GCA
 I E S T F D C S P I L P D G G S A

3745 GTT TCC CAC GGC AAT ACC ACC ATC EAG GAT CTG ATC GAC ACC
 V S H G N T T A T I E D L I D C T

3787 GGC AAT GCC RCG GCG ACC TTC GCC CTG AAC CGC TAC GAA GGC
 G N A R A T F A L N R Y E G

3829 ACC AAG GCC GCC GAG TAT CTG CAG AAG AAA TTC GAG ATC CCG
 T K A A E Y L Q K K F E I P

3871 GCG ATC ATC GGC CCG ACC CCG ATC GGC ATC CGC AAT ACC GAC
 A I I G C T P I R N T D

3913 ATC TTC CTG CAG AAC CTG AAG AAG GCG ACG GGC AAG CCG ATT
 I F L Q N L K K A T G G K P I

3955 CCC CAG TCG CTG GCC CAT GAG CGC GGG GTG GCC ATC GAT GCC
 P Q S L A H E R G V A I D A

3996 CTG GCC GAC CTG ACC CAC ATG TTT CTG GCC GAA AAG CGT GTG
 L A D L A T H F L A E K R V

4039 GCC ATC TAT GGG GCG CCG GAT CTG GTG ATC GGC CTG GCC GAA
 A I Y G A P D L V I G L A E

4081 TTC TGC CTG GAT CTG GAG ATG AAG CCC GTC TTG CTG CTG CTG
 F C L D L E M K P V L L L L

4123 GGC GAC GAC AAC TCC AAG TAC GTG GAC GAT CCG CGC ATC AAG
 G D D N S A K Y V D C P R I K

4165 GCG CTT CAG GAA AAC GTC GAT TAC GGC ATG GAA ATC GTC ACC
 A L Q E N V D Y M E I V T

4267 AAT GCG GAT TTC TGG GAA CTG GAA AAC CGC ATC AAG ACC GAG
 N A D F W E L E N R I K N E

4249 GGT CTG GAA CTG GAT CTG ATC CTC GGT CAC TCC AAG GGC CGT
 G L E L D L I L G H S K G R

4291 TTC ATC TCC ATC GAC TAC AAC ATC CCG ATG CTG CGC GTG GGT
 F I S I D Y N I P M L R V G

4333 TTC CCG ACC TAC GAC CGC GCC GGC CTG TTC CGC TAT CCC ACG
 F P T Y D R A G L F R Y P T

4375 GTG GGC TAT GCG GGC ATC TGG CTG GGC CAG CAG ATG GCC
 V G Y G G A I W L A E Q M A

4417 AAC ACC CTG TTC GCC GAT ATG GAA CAC AAG AAG AAC AAG GAA
 N T L F A D M E H K N K E

4459 TGG GTC CTC AAC GTC TGG TAA * GGACCATCAACTCTGTAGTTGGCCG
 W V L N V W *

4507 GAGAGGGCAGCGGGCTGCATCGCTCTCCTTCTCGCCGATCCCTGTGCGCGTGTAT
 4562 CTGGTGATCCCGTTGCCGGTTCGGACGGTGGATGCACGTTTTTTCGACGCGCGC
 4617 GGACAGGCTGGACTCGAAGAGGCCCTT ATG AAA ATC GCG GCT TAT CTC
 M K I A A Y L

4665 GAT CGG CAT GGA GAC ATG GCC GGT CTC TAC ACG GCC GGC AAG
 D R H G D M A G L Y T A G R

4707 TTC CAG CTC TAC GAA AAA GAC GAC GAA CAC TGG ATT TTG AAA
 F Q L Y E K D D E H W I L K

4749 AGG CAG GTT CCG CTC GAA ATC ACG GCG GAG ATG AAC ATT CCC P
 R Q V F L E I T A E M N I P

4791 GAG GTG AAG CAG GCC CTG CGC GAG GCC GTG GTC CAT CTG GAG
 E V K Q A L R E A C V V H L E

4833 GAC TGC AAG ACC CTG CTG TCC GCC GAG GTG CGC GGC CTG CTG
 D C K T L L S A E V R G L L

4875 TAT TCC CTC CTG CAG GAA GAA ATG GGC TTC CGG ACC TGG AAG
 Y S L L Q E E M G F R ACC T W K

4917 TCG CAG GGC TCC CTG CAC GAA CAA CTG GAC AAC GTG GCC CGC
 S Q G S L H E D G A V F G A C

4959 AAC GAG CTG GAT CTG GCG CTC AGG GAG GCC CTG GCC GCC GCC
 N E L D L A A C A L A L A A A

5001 GAG GCC GAG AAG GCC GCG CAG GCG TCC CGC GGC GGC AAG
 E A E K A A A Q A S A G G C

5043 GCG GGC GGA GGC GGC GGC GGC AAA CCG CGT TCC GCC GCC GCC
 A G G G G G G G G G G G G A A A

5085 GCC CCG GAG CCC GAA TCC ATC CCG CAG CCG GAA TGC CTG GGC
 A P E F S I P Q G R R E A A A

5127 GAA GGC CGT TAC CGC CTC GAG GAG GAG GGC CTC AAG GGC
 E G R Y R L C D L E A A L K G

5169 AAC AAG GAG CTC AAT TCC CGC CAG GTA CTG ATC CCC TTT CTG
 N K E L N S R C G A T A A A A

5211 GAA AAC ACC GTC TTC CGC GAG TTC GAG ATC CTC TGC GAT CAC
 E N T V F R E F I L C D H

5253 GTC CCG CGC TGG TTT TCC CAC AAG CTC GAG AAC CTA ACC CTC
 V P R W F S H K L D E L N L

5295 AGG GCC GAG TCC GAG GAG CTC GCC CCG AAA AAG GGC CTG
 R A E S E E L A G G C A A K G L

5337 AAG CTG CGT GTT CTG CCT GGG CCG P G E GCC GGC CCG GCC GGC
 K L R V L P G P A A G C C A A G

5379 TGA AATCCCATCCCGATCCATCCCTTCAACGACGGACAGGAGCTTTCC
 *

5430 ATG AGC GTT TCC CAA GAC AAC CAT CTG CTC TAT TTC GCC TAC
 H S V S C A A D N H L T L Q Y F A Y

5472 GGC GTG GAC ATG AAC CCG GAA CAC ATC GCG GCC CGC TGC GAC
 G V D M N P E H I A A R C D

5514 GAA CCG CAA GTG TTC ATG GTG GCT CAT TTG CCC GAC CAC GCC
 E P Q V F M V A H L P D H A

5556 CTG GCC TTT TTC GGA TAT ACC GAC CGC TGG GAC GGT GGT CTG
 L A T F F G A T A C D R W G G L

5598 GAA AGC ATC GTG GAG TCC CCG GGC GAT CCG CTA TAC GGC TTG
 E S I V E S P C G D R L Y G L

5640 ATC TAC GAA GTG ACC TAC AAC GAT GCG GAT TAT CTG GAT GCT
 I Y E V T Y N D A D Y L D A

5682 TGC CAG GGC GCG CGC CTG GAC ACC GAC CCC TAT TTC CAG
 C Q G A R L C D G T G P Y F Q

5724 TTC CCG GTG GAG GTG ATC GGC GAA GAC GGC CGG AGC CAT TCG
 F P V E V I G D E L R S H S

5766 GTA TTC ACC TAT AAA AAG TCC AGC CTG GGG GAA ACC ACC CAG
 V F T Y K K S A C G L G E T T Q

5808 CCC AGC AGC GGC TAC CTG GAC TAC ATC TGC GCC GGT GCG ACG
 P S S G Y L D Y I V A G A T

5850 GCG CAA GGA TTG CCG GAG GCT TAC ATA GAG CGC CTG AAG CGG
 A Q G L P E A Y I E L R K R

5892 ATC GAC AAC AAA CCG ACC GAT GAG CCA TTA CCC AGA AAG ACC
 I D N K P T D E P C L P A R K T

5934 GAT CTG AAC AGG ATT CTG GTG GGA GGC CAT GCC TGC AAC TGT
 D L N R I L V G G C H A C N C

5976 GGT TGA ACGGCCAAGCGGCCCTGCAAGGCCCTGCTCGACCCCGCGGGCAGGC
 G *

6029 GAAGCTCGTGCCGCCGACCCCGGTCACCCAGGCGTTGGTGGGCACCTCGCCCA
 6084 CCGGCGTACGATCGAGTGCCCGGG 6108

FIG. 2. Nucleotide sequence of the *anfH* HDGK region. The potential *nif* promoter (CTGG-N₈-TTGCT) is underlined. --->, Sequence that may be able to participate in the formation of a stem-and-loop structure. Sites similar to ribosome binding sites of *E. coli* are underlined. The amino acid sequences of presumed products of ORFs are given below each ORF.

chromococcum (54), and of *nifH3* from *Clostridium pasteurianum* (66) is shown in Fig. 4. The region of the amino acid sequence (residues 80 to 185) that contains four of the five conserved cysteinyl residues is highly conserved in all four cases. There is a 63% overall amino acid sequence identity between the presumed *anfH* and *nifH* gene products. How-

ever, the highest degree of similarity exists between the predicted amino acid sequences of the *anfH* product (AvH3) and the *nifH3* product (CpH3) from *C. pasteurianum* (66) (Fig. 4). Of the 273 amino acids of CpH3, 223 amino acids are exact matches with those of AvH3. The *M_r* and the pI of the putative product of *anfH* as determined by the IBI Pustell

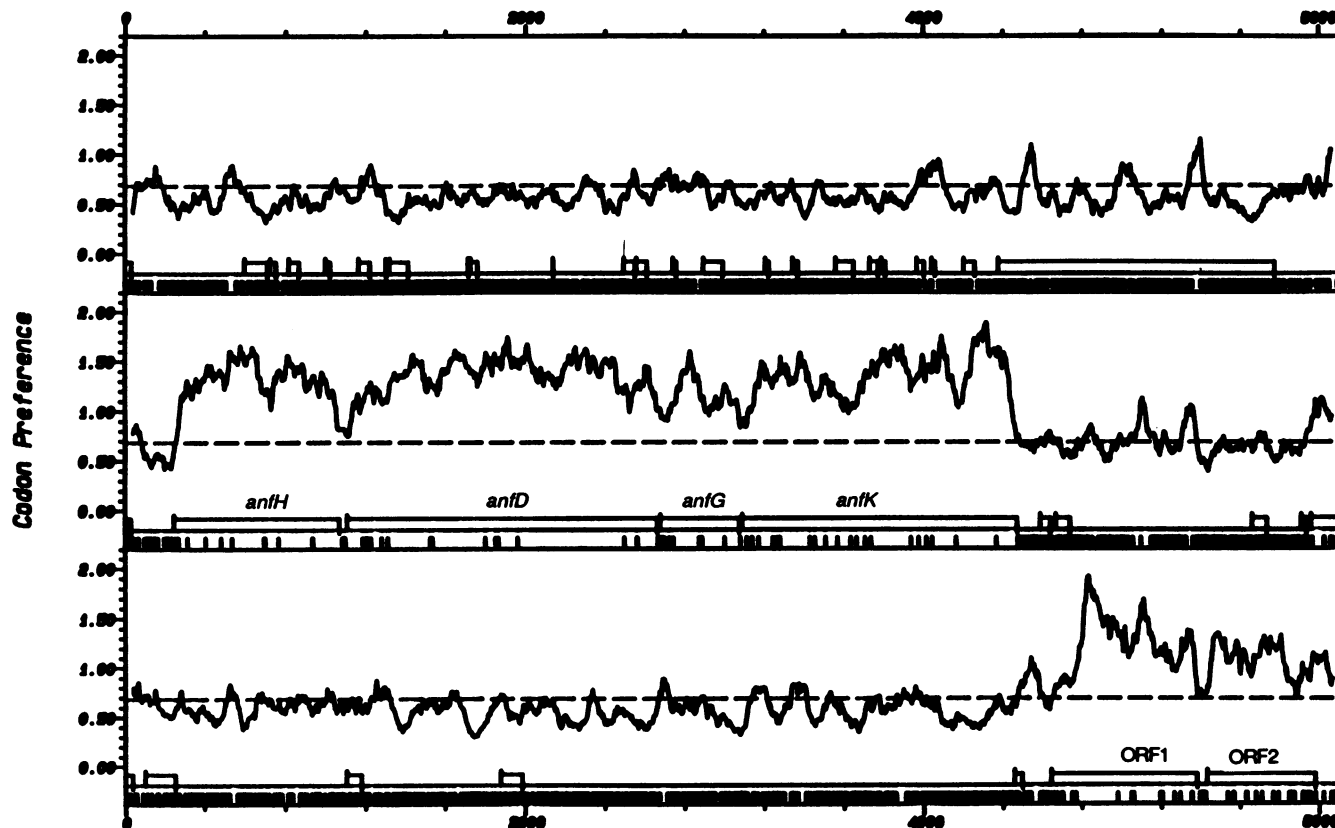


FIG. 3. Codon preference analysis of the nucleotide sequence available for the *anfHDK* region (positions 1 to 6108). The codon usage in each of the three reading frames is compared by using a codon frequency table established from the *A. vinelandii nifHDK* genes. The probability for the regions of the ORFs being coding regions rises significantly above the background level (---).

DNA sequence analysis program were 29,865 and 5.7, respectively.

The ORF immediately downstream from *anfH* potentially encodes a product which is similar to the product of *nifD* and the predicted product of *vnfD* (R. L. Robson, P. R. Woodley, R. N. Pau, and R. R. Eady, submitted for publication) (Fig. 5) and was therefore designated *anfD*. Of the amino acids of the predicted product of *anfD*, 30 and 54% are identical with those of the products of *nifD* from *A. vinelandii* and *vnfD* from *A. chroococcum*, respectively. Cysteine residues corresponding to Cys-62, -88, -154, and -275, as well as the histidine residues His-80, -83, -195, -196, and -362 from the *nifD* product of *A. vinelandii*, are conserved. Cys-183 from the *nifD* gene product is conserved in the product of *vnfD*. A serine residue is present at the corresponding position in the predicted product of *anfD*. However, a cysteine residue is located near this serine residue at a position corresponding to Val-178 from the *nifD* gene product. The M_r of the predicted product of *anfD* is 58,391, and the estimated pI is 6.6. The predicted M_r of this protein is in agreement with a previous estimation of the M_r of the α subunit of dinitrogenase 3 on the basis of SDS-polyacrylamide gel electrophoresis of purified dinitrogenase 3 (14). This subunit also migrated to a position where the pH of the isoelectric focusing gel was estimated to be between 6.5 and 7.0.

As shown in Fig. 6, the presumed translation product of the ORF found downstream from *anfD* is similar to that of the *vnfG* (Robson et al., manuscript submitted) product from *A. chroococcum*. Thirty-nine percent of the amino acid

	1		59
AvH1	MAMRQCAIYKGGIGKSTTTQNLVAAALAE. GKKVMIVGCDPKADSTRILHLSKAQNTIM	*	
AcH2	MALRQCAIYKGGIGKSTTTQNLVAAALAE. GKKVMIVGCDPKADSTRILHLSKAQNTVM		
AvH3	MTRKVAIYKGGIGKSTTTQNTAAALAYFHKVFTTHGCDPKADSTRILHLSKAQNTIM		
CpH3	MTRKIAIYKGGIGKSTTQNT. AAMAHFYDKVFIHGCDPKADSTRILHLSKAQNTIM		
	60		119
AvH1	EMAAEAGTVDELELDVLRKAGYGGVCKVESGGPEPGVGCAGRGVITAINFLEEAGAYEDD	*	*
AcH2	EMAASAGSGDELELDVLRKAGYGGVCKVESGGPEPGVGCAGRGVITAINFLEEAGAYSD		
AvH3	DHVRDYG. AEKITNDVIRKGFLLDIQCVESGGPEPGVGCAGRGVITAINFLEEAGAYTDD		
CpH3	DMLRDEG. .EKITTENIVRVGVEDIRCVESGGPEPGVGCAGRGVITAINFLEEAGAYTED		
	120		179
AvH1	LDVFVYDVLGDVCGGFAMPPIRENKAQEIYIVCSGEMMAYANNISKGIKVIKANGSGSVR	*	
AcH2	LDVFVYDVLGDVCGGFAMPPIRENKAQEIYIVCSGEMMAYANNIAKGIKVIKANGSGSVR		
AvH3	LDVFVYDVLGDVCGGFAMPPIRENKAQEIYIVCSGEMMAYANNIAKGIKVIKANGSGAVG		
CpH3	LDVFVYDVLGDVCGGFAMPPIRENKAQEIYIVCSGEMMAYANNIAKGIKVIKANGSGSVR		
	180		239
AvH1	LGGLICNSRNTDREDELIILANKLGTQMIHFVPRDNVQRAEIRRTVIEYDPKAKQAD		
AcH2	LGGLICNSRKTDRDELIMALAAKIGTQMIHFVPRDNVQRAEIRRTVIEYDPKAKQAD		
AvH3	LGGLICNSRKTDRGERESVEEFTAAIGTKMIHFVPRDNVQRAEIRRTVIEYDPKAKQAD		
CpH3	LGGLICNSRNVDLEREFIEEFAASIGTQMIHFVPRDNVQRAEIRRTVIEYDPKAKQAD		
	240		290
AvH1	EYRALARKVVDNKLIVIPNPTMDELELLMEFGIMEVEDESIVGKTAEV		
AcH2	EYRALAQKILNNK. LVIPNPGSMEDLELLMEFGIMEVEDESIVGKTAEV		
AvH3	EYELARKIIEENDEFVLPKPLTMDQLEDVYKGIAD		
CpH3	EYELARKIIEENEMFVIPTPLKMDLEAMVYKGMTD		

FIG. 4. Comparison of the predicted amino acid sequence of the product of *anfH* (AvH3) with the predicted products of *A. vinelandii nifH* (9) (AvH1), *A. chroococcum vnfH* (54) (AcH2), and *C. pasteurianum nifH3* (66) (CpH3). *, Conserved cysteine residue.

```

1          60
AvD1 MTRMSREEVESLIQEVLEVYPEKARKDRNKLHNAVNDPAVTQSKKCIISNKKSQPLGMLTIR
AvD3      MPHHEFECISKVI PERKHAVIKGGETLADALPQGVYNTIPGSI SER
AcD2      MPMVLLECDKDI PERQKH IYLKAPNEDTREFLPIANAATIPGTSER

61          120
AvD1 GCAYAGSKGVVWGPDKMHIHSHGPGVCGQYSRAGRNNYVIGTGVNAFVTHMFTSDFQE
AvD3      GCAYCGAKHVIGTPMKDVIHSHGPGVCTYDTWQTKR. .YI. SDNDFOLKTYVATDVKE
AcD2      GCLL. RRKLVIGGVLKDTIQMIHGLPGCAVDTWHTKR. .YP. TDNGHFNMKYVWSTDMKE

121          179
AvD1 KDIVFGGDKLAKLIDEVETLFPNLKGISVQSECPGLIGDDIESVS. KVKGAELSKTIV
AvD3      KHIVFGAEKLLKQNIIEAFKAPQIKRMTIYQCATALIGDDINAI AEVEEMPEVDIF
AcD2      SHVVFGEKRLQESMHEAFDEMPDIKRMIVVYTCPTALIGDDIKAVAKVMKERPDVDF

180          239
AvD1 PVRCEGFRGVSQSLGHHIANDAVRDVWLKGRDADTTFASTPYDVAIIGDYNIGGDAWSSR
AvD3      VCNPCGAFGPSQSGHHKINIA. .WINKVGTVEPEITGDHVINYVGEYKQDQEVVM
AcD2      TYECPGFSVGSQSGHHVNLIG. .WINEKVTMEKEITSEYTMNFIDGNFQGTQLQ

240          299
AvD1 ILLEEMGLRCVAQWSDGQYISQIELTPVKLNLVHCYRSMNYISRHMEEKYGI PMWMEYFN
AvD3      DYFKRMGIQVLSTPTNGSYDGLRAMHRAHLNVLCEARSAEYICNELRVRYGIPRLDIDG
AcD2      TYWDLRGIQVAHFTNGFYDDLRCMQAQLNVVNCARSSGYIANELKRYGIPRLDIDS

300          359
AvD1 FGPTKTIESLRAIAAKFDESIOKKEEVI AKYKPEWEAVVAKYRPRLEGKRVMLYIGGLR
AvD3      FGFKPLADSLRRKIGMFFG. .EDRAKAIIDEVARWKPELDWYKERLMGKVCVLPWGGSK
AcD2      WGFSYMAEGIRKICAFFG. .TEEKERLIAEYAKWPKLDWYKERLQGGKMAIWTGGPR

360          418
AvD1 PRHV. IGAYEDLGHVEVVTGYEFAHNDYDRTHKEMGDSTLLVDVDTGMEFEFVKRIKP
AvD3      LWHWAHVIEEEMGLKVVSVYIKFGHQGMKEGIARCEGTLAIDDPNEGLELALEMLKP
AcD2      LWHWTKSVEDDDLGIVVAMSSKFGHEEDEFVKIARGKEGTYYIDGNELEFPEIIDLVKP

419          478
AvD1 DLLIGSIIKEKFIQKMGIFPRQMHSWDYSGPYHGFDFGFAIFARDMDMTLNNPKWKKLQAP
AvD3      DIILGTRKPGVEAKKRVVYPLNAHAYH. NGPYKGFEGWVRFARDIYNAIYSPHQLSGID
AcD2      DVIFTPGRVGLVKLKHIPVYVNGHYH. NGPYMGFEGVFNLRDVTYAVHNPLRLHAAVD

479          492
AvD1 WEASEGAEKVAASA
AvD3      ITKDNAPEWNGFRTRQMLSDGNLSDAVRNSETLRQYTGGYDSVSKLREYPAFERKVG
AcD2      IRDSSQTPVIVRGA
    
```

FIG. 5. Comparison of the predicted amino acid sequence of *anfD* (AvD3) with the products of *nifD* from *A. vinelandii* (AvD1) (9) and *vnfD* from *A. chroococcum* (AcD2) (Robson et al., manuscript submitted). *, Conserved Cys residue.

residues are conserved in both translation products. Only one of the four cysteine residues in *anfG* is conserved in *vnfG*. The molecular weight of the predicted product of *anfG* is 15,324, and the pI was estimated to be 5.79.

The presumed translation product of the ORF immediately downstream from *anfG* is similar to the products of *nifK* from *A. vinelandii* (9) and *vnfK* from *A. chroococcum* (Robson et al., manuscript submitted). The degree of conservation of amino acid residues is 30 and 56%, respectively. As observed with the product of *anfD*, the Cys residues in the N-terminal section of the protein are conserved in the three amino acid sequences compared in Fig. 7. The predicted product of *anfK* has an M_r of 51,157 and an estimated pI of 5.79. The β subunit of purified dinitrogenase 3 migrated

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1          59
ANFG MSTASAAAVVKQKVEAPVHPMDARIDELTDYIMKNCLWQFHSRSDWRERQNAEILKKTK
VNFG MS. . . . . QSHLDDLDFDYTEERCLWQFFSRTWDRREENIEGVLGQVA

60          117
ANFG ELLCG. EPVDLSTSHDRCYVWDAVCLADDYREHPWINSMSKEEIGSLMQGLKDRMDYL
VNFG RLLTGQEPLR. GTPQERLFYADALAMANDVRRFPWASQINHEEIHFLIDGLKSRVLVD

118          132
ANFG TITGSLNEELSDKHV
VNFG VITRSTNRELNHHLV
    
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FIG. 6. Comparison of the presumed products of *anfG* (ANFG) and *vnfG* from *A. chroococcum* (VNFG) (Robson et al., manuscript submitted). *, Conserved Cys residue.

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1          60
AvK1 MSQQVDKIKASYPLFLDQDYKDMLAKKRDGFEEKYPODKIDEVFWTMTTKEYQELNMFQRE
AvK3      MT.CEV. . . . . KEKGR
AcK2      MSNCELTVLKPAEVKLVKRE

61          120
AvK1 ALTVNPAKACQPLGAVLALGFEKTMPIVHGSQGCVAIFRSYFNRFREPVSVCVSDSMT
AvK3      VGTINPIFTCQPAQAQFVSIKICDCIGIVHGGQCVMFVRLIFSQHYKESFELASSLSHE
AcK2      EGIINPMYDCQPAQAQYAGIVKDCIPLVHGGQCTMFVRLLAQHFKENFDVASTLSHE

121          175
AvK1 DAAVFGGQNMKDLGNCKATYKPDMAV. .TTCMAEVI GDDLNAFINNSKKE. .GFI
AvK3      DGAVFGACGRVEEAVDVLVLSRY. PDVKVPIITTCSTEIIGDDVDGVKIKLNEGLLKEKF
AcK2      ESAVFGGAKRVEEGLVLLARRY. FELRLIPIITTCSTEVI GDDIEGTINVCNRLAAE. F

176          234
AvK1 PD. EFFVPFAHTPSFVSGSHVTGWDMFEGIARYFTLKSMDKVVGSNKINIVPGFEYTL
AvK3      PDREVHLIAMHTPSFVSGSMISGYDVAVRDVRHFAKREAPND. . . . . KINLLTGW. VNP
AcK2      PERKIYLAHVHTPSFVSGSHVTGYAECVKSMTITTEVHGKQPSG. . . . . KLVVFPW. VNP

235          294
AvK1 DAFRVIKRMLSEMVGYSLLSDPEVLDTPADGQFRMYAGGTQTEEMKDAPNALNVLQ
AvK3      GDVKELKHLGEMDIEANVLEIPEISFDSPIPLDGSVAVSHGNTTIEDLIDTGNARATFALN
AcK2      GDVLLKRYFKEMGDVATVFMDEDFDSMPLFNKSIETHGRITVEDIADSANALATLALA

295          354
AvK1 PWHLEKTKKFEVGTWKHEVPKLNIPMLDWDTEFLMKVSEISGQPIASLTKERGRVDM
AvK3      RYEGTAAEYLVKQKFEIPAIIGPTPIGIRNTDIFLQNLKATGKPIQSLAHERGVAIDA
AcK2      RYEGATTGEYLEKTFVAVNSLVNTPYGINKTDDMLRKAIEITGKEIPESLVREPRIAWIA

355          412
AvK1 MTD. SHTWLHGKRFALMGDPDFVGMGLVKFLLLELGCPEVHIL. CHNGNKRKKAVIDAIIAA
AvK3      LADLTHMFLAEKRVAVYGAPDLVIGLAEFCLEDMKPVLLLLGDD. NSKYVDDPRKALQ
AcK2      LADLAHMFANKKVAIFGHPDLVGLGAQFCLEVELEFVLLIGDQGSYKPKDPRQLQELK

413          470
AvK1 SPYGNATVYIGKDLWHLRSLVFTD. .KPDFMIGNSYKGIQRDTLHKGKEFEVPLIRIG
AvK3      ENVDYGHIEVITNADFWLENRIKNEGLEDDLILGHSGKRF. . . . . SIDYNI FMLRVG
AcK2      DAAHFDMIEI VHNADLWLEKRI. NDGLQLDLIMGHSGKRYV. . . . . AIEANI PMVRVG

471          523
AvK1 FPIFDRHHLRSTTLGEGAMQILTTLVNSILERLDEETRMQATDYNHDLVR
AvK3      FPTYDRAGLFRYPTVGYGAIWLAEQMANTLFDAMEHKKNKEWLVNVW
AcK2      FPTDRAGLVKRSIGYQGAEMELGEMIANAMFAHMEYTRNKEWILMTW
    
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FIG. 7. Comparison of the amino acid sequence deduced from *anfK* (AvK3) with the products of *nifK* (AvK1) (9) and *vnfK* (AcK2) (Robson et al., manuscript submitted). *, Conserved Cys residue.

as a 50-kilodalton protein on SDS-polyacrylamide gels (14), and two-dimensional gel analysis showed that this subunit migrated as a protein with a pI between 5.5 and 6.0. The agreement between the physical measurements of the molecular weight and pI of the subunits of dinitrogenase 3 and the estimated values obtained for the predicted products of *anfD* and *anfK* suggests that *anfD* and *anfK* encode the α and β subunits of dinitrogenase 3, respectively.

The amino acid sequence of the putative translation product of the ORF downstream from *anfK* (ORF1) (predicted M_r , 26,943; pI, 5.99) was compared with other amino acid sequences by using the XFASTP program of the BIONET National Computer Resource for Molecular Biology. No amino acid sequence was found that matched the entire sequence of the product of ORF1. However, two matches appear to be noteworthy. A portion of the translation product of ORF1 (amino acids 37 to 136) exhibits some degree of identity with the N-terminal part of dinitrogenase reductases (Fig. 8). In addition, a 19-amino-acid sequence (amino acids 150 to 168) exhibits 57.9% identity to a segment of cytochrome P-450 (phenobarbital induced) (50). This segment is presumed to be involved in the binding of heme groups (43). A comparison with two presumed heme-binding domains of P-450 cytochromes is also shown in Fig. 8.

The putative product of ORF2 (predicted M_r , 20,241; pI, 5.38) has also been subjected to a search by using the XFASTP programs. This search did not yield any interesting similarities with other protein products. The sequence data

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AvH1      MAMRQCAIYKGGIGKSTTTQNLVAALAE
ORF1 1  MKIAAYLDRHGMAGLYTAGRFQLYEKDDEHWIKRQVPLEITAEMNIPEVKQALREAVVH
CpH1      MRQVAIYKGGIGKSTTTQNLTSGLHA

AvH1  MGKMKVMHVGCDPKADSTRLLHLSKAQNTIMEMAAEAGTVEDL...ELEDVLKAGYGVKQC
ORF1  LEDCKTLLSAEVGRLLYSLLQEEMGFRTWKSQGSLHEQLDNVARNELDLALREALAAAEA
CpH1  MGKTMVVGCDPKADSTRLLHLSGLA...QKSVLDTLREEGEDV...ELDSILKEGYGGIRC

AvH1  VESGGPEPGVGCAGRGVI  P450Hs  PFSIGQPNCFGEGGLARMELFLFFT
ORF1  EKAAQAASAGGGCAGGGGGKRRSAAAGAPEPESIPQPECLGEGRYRLDLAALKGNKELN
CpH1  VESGGPEPGVGCAGRGII  P450Pp  TFGHSHLCLGQHLARREIIVTLK

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FIG. 8. Comparison of the presumed product of ORF1 (ORF1) with portions of the *nifH* product from *A. vinelandii* (amino acids 1 to 104) (AvH1) and the product of *nifH1* from *C. pasteurianum* (CpH1) (66) and of two proposed heme-binding sites from human cytochrome P-450 (phenobarbital induced) (P450Hs) (50) and cytochrome P-450 from *Pseudomonas putida* (P450Pp) (64). Symbols: ·, Identical amino acid residues; ·, conservative substitutions (I, L, V, M; D, E; K, R, H; S, T; G, A; and F, Y); *, conserved cysteine residue.

available for the region downstream from ORF2 are not sufficient to allow a definitive conclusion as to the presence or absence of another ORF downstream from ORF2. Transcription termination sequences or promoter sequences, which would indicate the end of a transcriptional unit or the beginning of another one, have not been found downstream from ORF2. We conclude, therefore, that the end of the *anfH* operon is not located within the cloned and sequenced fragments.

Growth of mutants carrying deletion or insertion mutations in the *anfD* region. The mutant strains CA70, CA71, and CA72 (Table 1, Fig. 1) are able to grow under N₂-fixing conditions in liquid Burk medium containing 1 μM Na₂MoO₄ or 1 μM V₂O₅ but not in Mo-deficient medium. Strains that also carry the *nifH* deletion of strain CA11 in addition to the mutations in the *anfH* region grew only in the presence of vanadium in N-free medium.

Two-dimensional gel electrophoresis of protein extracts from mutant cells derepressed for nitrogenase under Mo-deficient conditions. In previous studies (5 and unpublished results), it was shown on two-dimensional gels that spots representing the subunits of nitrogenase 3 were only observable when cells were either grown in N-free, Mo-deficient medium or derepressed in Mo-deficient medium for at least 12 h. In the case of strain CA11, spots representing nitrogenase 2 (5) are seen after 3 h of derepression in Mo-deficient medium, and traces of these spots are still visible on two-dimensional gels of extracts from cells derepressed for 12 h. (The dinitrogenase reductase 2 spot, however, persists under Mo-deficient conditions.) After about 12 h of derepression, spots representing nitrogenase 3 are clearly visible on two-dimensional gels, and at 18 h after the start of derepression, these spots are even more intense. In light of these observations, strains CA11.70, CA11.71, CA11.72, and CA11 were derepressed in Mo-deficient, N-free medium for 18 h. Two-dimensional gels of protein extracts from these strains are shown in Fig. 9. Only strain CA11 appeared to have expressed all three subunits of nitrogenase 3. In the gel of protein extracts from strain CA11.70 (which carries a Kan^r cartridge in place of the *KpnI* fragment [positions 856 to 1687]), none of the spots representing nitrogenase 3 can be seen. The spot representing dinitrogenase reductase 2, however, is present in this strain and in strain CA11. In the gels of extracts from strains CA11.71 and CA11.72, the spot representing dinitrogenase reductase 3 can be seen along

with the spot representing dinitrogenase reductase 2. The mutations affecting the *anfH* region in strains CA11.71 and CA11.72 are located downstream from *anfH*, and the synthesis of the product of *anfH* could be expected in both strains. However, for both strains, the intensity of the spot representing dinitrogenase reductase 3 is less than that of the dinitrogenase reductase 3 spot found on gels of protein extracts from strain CA11. Strain CA11.72 carries a deletion or insertion mutation downstream from *anfD* and would therefore be expected to synthesize the product of *anfD* in addition to the product of *anfH*. However, the spot representing the α subunit of dinitrogenase 3 could not be detected with certainty on two-dimensional gels.

DISCUSSION

The presence of alternative nitrogenases in *A. vinelandii* has been demonstrated by a number of genetic and biochemical studies over the last few years. This study characterizes the genomic region of *A. vinelandii* that contains one of the *nifH*-like genes first identified through Southern hybridization experiments (28). The following lines of evidence support the idea that this genomic region contains the structural genes of nitrogenase 3. (i) The predicted *M_r*s of protein products of the ORFs similar to *nifH*, *nifD*, and *nifK* correlate well with the *M_r*s of the nitrogenase 3 subunits as determined by Chisnell et al. (14). The predicted pIs of the products of these ORFs are also close to the pIs of the purified nitrogenase 3 subunits (14). (ii) Mutations introduced into the *anfH* genomic region result in a Nif⁻ phenotype under Mo- and V-deficient conditions, and these mutants lack the nitrogenase 3 subunits. (iii) Preliminary RNA blot analysis (data not shown) indicates that only under conditions where nitrogenase 3 is expressed, *anfH*-hybridizing transcripts accumulate that are up to 6.6 kb in length. The nucleotide sequence data presented here indicate that a transcript of this size could be transcribed from the *anfH* operon.

A comparison of the amino acid sequence of the predicted *anfH* product with the sequence of the *nifH* product (9) (dinitrogenase reductase 1) indicates that 63% of the amino acid residues are identical. Amino acids 7 through 22 are completely conserved in the predicted *nifH* and *anfH* products. This region contains a Gly-X-Gly-XX-Gly motif characteristic of nucleotide-binding domains (62). Other conserved regions in the predicted protein products of *nifH* and *anfH* are those surrounding residues Cys-39, -86, -98, -133, and -185 (residues are numbered as in reference 9). Hausinger and Howard (25) examined the thiol reactivity of dinitrogenase reductase 1 and concluded that Cys-98 and -133 were the probable ligands for the Fe-S center which is bound symmetrically between the dinitrogenase reductase 1 subunits. Cysteine residue Cys-86 was suggested to be part of the nucleotide-binding site. Thus it appears that dinitrogenase reductase 3 may be very similar to dinitrogenase 1 with respect to the nucleotide-binding site and ligands for the Fe-S center. However, in a complementation reaction with dinitrogenase 1, dinitrogenase reductase 3 gave only 0.8% of the activity obtained when dinitrogenase reductase 1 and dinitrogenase 1 were paired (14; R. Premakumar, J. R. Chisnell, and P. E. Bishop, Can. J. Microbiol., in press). The inability of dinitrogenase reductase 3 to form an active complex with dinitrogenase 1 suggests that different structures for the interaction of the nitrogenase components are present in dinitrogenase reductases 1 and 3. The amino acid sequence of the dinitrogenase reductase 1 subunit and the

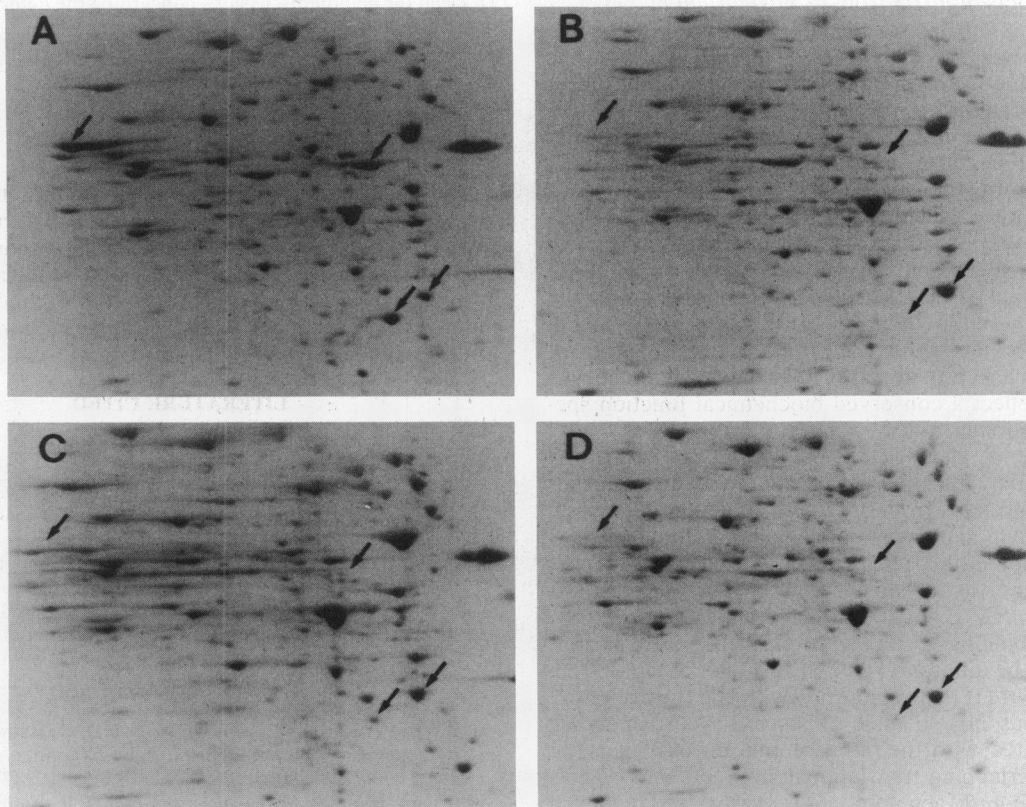


FIG. 9. Two-dimensional gels of protein extracts from cells derepressed in N-free, Mo-deficient medium for 18 h. ↙, Migration position of the nitrogenase 3 subunits. Clockwise from the left upper corner of the gels, these arrows point to the presumed products of *anfD* (α subunit), *anfK* (β subunit), *vnfH* (dinitrogenase reductase 2), and *anfH* (dinitrogenase reductase 3). The strains used are CA11 (all four subunits present) (panel A), CA70 (only dinitrogenase reductase 2 present) (panel B), CA71 (dinitrogenase reductase 2 and 3 present) (panel C), and CA72 (dinitrogenase reductase 2 and 3 present) (panel D).

predicted sequence of the *anfH* gene product differ considerably in the region between amino acids 50 and 85, as well as in the carboxy-terminal regions. However, it is not known whether these regions are involved in the interaction of the nitrogenase components. Dinitrogenase reductase 1 is required for the synthesis of FeMo cofactor (52). Whether dinitrogenase reductase 3 plays an analogous role is not known. However, any differences between the two dinitrogenase reductases in this function might also be reflected in their amino acid sequences.

The presumed protein products of *anfD* and *anfK* contain Cys and His residues which are conserved in virtually all dinitrogenases examined to date. These residues are considered candidates for Fe-S center ligands. In addition, carboxy or amino groups might also interact with these centers. Site-directed mutagenesis experiments have indicated that Cys-62, -88, -154, and -275 from the *nifD* gene product and Cys-70 and -95 from the *nifK* gene product are essential for activity (15), but it has not been determined which of these Cys residues might be ligands for FeMo cofactor or the P centers. Further site-directed mutagenesis experiments that will result in replacements of conserved residues in the dinitrogenase 1 subunits are in progress (15), and comparisons of the *nifDK*, *vnfDK*, and *anfDK* gene products might suggest additional residues that could be targeted for mutagenesis.

The similarity of the amino acid sequences of the α and β subunits of dinitrogenases has led to the suggestion that the two genes coding for these subunits have evolved from a

common ancestral gene (63). The similarity is 16% between the *nifD* and *nifK* products, 19% between the *vnfD* and *vnfK* products, and 20% between the products of *anfD* and *anfK*. This supports the hypothesis of a common ancestral gene and suggests that the structural genes for the subunits of dinitrogenase 1 may have diverged earlier during evolution than the genes encoding the subunits of dinitrogenases 2 and 3.

The amino acid sequences of dinitrogenase 2 from *A. chroococcum* (and also from *A. vinelandii* [our preliminary results]) and dinitrogenase 3 from *A. vinelandii* show more similarities to each other than either does to that of dinitrogenase 1. This could suggest that the structural genes for dinitrogenases 2 and 3 may have diverged from common ancestral subunit genes relatively recently in evolutionary time. The evolutionary relationship of the three dinitrogenase reductases, on the other hand, appears to be different. The amino acid sequences of dinitrogenase reductase 1 from *A. vinelandii* and dinitrogenase reductase 2 from *A. chroococcum* are 90% identical. (The predicted *vnfH* products of *A. chroococcum* and *A. vinelandii* are 97% identical [our unpublished results].) The amino acid sequence identity is only 63% when the predicted product of *anfH* is compared with the products of *nifH* or *vnfH*. This suggests that the structural gene (*vnfH*) for dinitrogenase reductase 2 may have arisen from a duplication of *nifH* in recent evolutionary time.

The predicted product of *anfH* appears to belong to a group of similar dinitrogenase reductases that includes the

predicted products of *nifH3* from *C. pasteurianum* (66) (82% identity with the *anfH* product) and ORF*nifH1* from *Methanococcus thermolithotrophicus* (61) (72% identity with the *anfH* gene product). The predicted product of *anfD* from *A. vinelandii* is also more similar to the predicted product of a *nifD*-like ORF from *M. thermolithotrophicus* (L. Sibold, personal communication) (35% identity) than it is to the *nifD* gene product from *A. vinelandii* (9) (30% identity). It has been suggested that the similarity between the predicted *C. pasteurianum nifH3* product and the predicted product of ORF*nifH1* from *M. thermolithotrophicus* could be the result of an ancient lateral transfer of genetic material between *Clostridium* spp. and methanogens (61). On the other hand, the similarity of the predicted amino acid sequences of certain nitrogenase proteins from very diverse organisms might simply reflect a conserved biochemical function specific to these proteins.

The presence of an ORF between *anfD* and *anfK* and between *vnfD* and *vnfK* is a genetic feature that distinguishes the alternative systems from the conventional system. It is, of course, possible that a product is made, under conditions where conventional nitrogenase is synthesized, that serves the same functions as, or similar functions to, the products of *anfG* and *vnfG*. Even if this should be the case, the strength of the binding of such a protein to the nitrogenase 1 subunits would be different, since the product of *vnfG* (from *A. chroococcum*) (17, 18) copurifies with the nitrogenase 2 subunits whereas no evidence exists for a corresponding subunit associated with the conventional nitrogenase. The presence of overlapping translational stop and start signals suggests that the *anfG* gene product is synthesized in a fixed ratio with the products of *anfD* and *anfK*. The role of the products of *vnfG* and *anfG* in the N₂ fixation process still remains to be determined. There is only one conserved cysteine residue present in *anfG* and *vnfG*, and it would be of interest to determine the function of this residue. The availability of nucleotide sequence data should make it possible to generate specific mutations in *anfG* that will answer the question as to whether this gene is essential and what domains might be important. Also, hybridization probes can now be prepared which should aid in the search for the presence of related sequences in organisms other than *Azotobacter* species.

ORFs apparently cotranscribed with the genes encoding the structural proteins of nitrogenases have been observed in *A. vinelandii* and *Klebsiella pneumoniae* (57), but the role of these ORFs is still unknown. The function of the ORFs downstream from *anfK* is also unknown. The apparent relatedness of part of ORF1 and Fe proteins (especially the conservation of Cys-98) and the indication for a possible heme-binding domain suggest that the product of ORF1 might be an iron-containing protein. However, not enough information on the predicted products of ORF1 and of ORF2 is available, at this time, to warrant speculation on the roles of these products. Here, as in the case of *anfG*, mutagenesis procedures will have to be used to determine whether these genes are essential or beneficial to N₂ fixation under Mo-deficient conditions.

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ADDENDUM IN PROOF

Our recent sequence data indicate that the predicted product of *vnfD* from *A. vinelandii* contains the amino acid residues GCAFCGAKL at positions corresponding to residues 61 through 69 of the *nifD* gene product. Thus, this particular region (in contrast to the corresponding region of the *vnfD* gene product from *A. chroococcum*) is very similar in the products of *nifD*, *vnfD*, and *anfD* from *A. vinelandii*.

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