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

ROLF D. JOERGER, MARTY R. JACOBSON,‡ R. PREMAKUMAR, ELIZABETH D. WOLFINGER, AND PAUL E. BISHOP*

Agricultural Research Service, U.S. Department of Agriculture, and Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615

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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 μM 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_rs of the subunits were determined to be 58,000 and 50,000 (14).

In this paper, we present the nucleotide sequence of the

* Corresponding author.

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‡ Present address: Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

TABLE 1. Bacterial strains, plasmids, and phages

Strain, phage, or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
HB101	F⁻ *hsd-20* (r_B^- m_B^-) *proA2 lacYI rpsL20 supE44*	36
NM539	*supF hsdR* (P2 *cox3*)	20
K-12 71-18	Δ(*lac-pro*) F′ lac^q *lacZ* M15 pro^+ *supE*	40
JM101	Δ(*lac-proAB*) *supE thi* (r_K^+ m_K^+)/F′ *traD36 proAB* $lacI^q$ *ZM15*	67
GM33	*dam-3* mutation in prototrophic *E. coli* W3110	37
A. vinelandii		
CA	Wild type	12
CA11	Δ*nifHDK*	6
CA70[a]	Kan^r *Kpn*I fragment (positions 856-1687) replaced by Kan^r cartridge	This study
CA71[a]	Kan^r *Bcl*I fragment (positions 1721-3533) replaced by Kan^r cartridge	This study
CA72[a]	Kan^r *Bcl*I fragment (positions 3437-3533) replaced by Kan^r cartridge	This study
Phages		
λEMBL-3A-H3-3	Recombinant λEMBL-3A phage (20) containing *anfH* and upstream sequences	This study
λEMBL-3A-H3-10	Recombinant λEMBL-3A phage (20) containing the *A. vinelandii anfHDGK* region	This study
Plasmids		
pUC9	Amp^r	65
pHDK3	Amp^r 11.8-kbp *Sal*I fragment (containing *anfHDGK* region) in pUC9	This study
pHDK3-1	Amp^r Kan^r *Kpn*I fragment (positions 856-1687) of pHDK3 replaced by Kan^r cartridge	This study
pJWD3	Amp^r 3-kbp *Eco*RI fragment (positions 1000-4078) in pUC9	This study
pJWD3-1	Amp^r Kan^r *Bcl*I fragment (positions 1721-3533) of pJWD3 replaced by Kan^r cartridge	This study
pJWD3-2	Amp^r Kan^r *Bcl*I fragment from pJWD3 (positions 3537-3533) replaced by Kan^r cartridge	This study
pKISS	pUC4 with Kan^r cartridge derived from Tn*903*	Pharmacia
pKIXX	pUC4 with Kan^r cartridge derived from Tn*5*	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. λEMBL3-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 $CaCl_2$ 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 *Sau*3A, 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 λEMBL-3A digested with *Bam*HI to construct the library as described by the suppliers of λEMBL-3A (Promega Biotec).

DNA sequencing and sequence analysis. The 3.8-kbp *Eco*RI fragment (isolated from λEMBL-3A-H3; Table 1) which hybridized to *nifH* was cloned into M13 mp18 and mp19. Two recombinant M13 phages containing the *Eco*RI fragment inserted in opposite orientations were chosen for the generation of phage with deletions in the *Eco*RI fragment insert by the method of Misra (41). The deletions were created by *Bal*31 digestion of phage DNA previously cut with *Sal*I, followed by cleavage with *Eco*RI, treatment with Klenow fragment of DNA polymerase to generate blunt ends, and ligation into M13 mp18 replicative-form DNA that had been cut with *Eco*RI and *Sal*I. (The *Sal*I 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 *Eco*RI fragment hybridizing to *nifH*. The region downstream

from *anfH* was isolated from λEMBL-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 [^{35}S]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 λEMBL-3A with a *nifH*-specific probe (28) yielded a phage (λEMBL-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 μg/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 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.

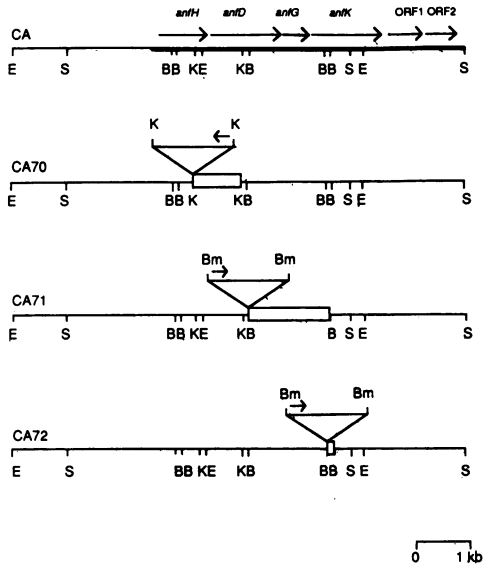
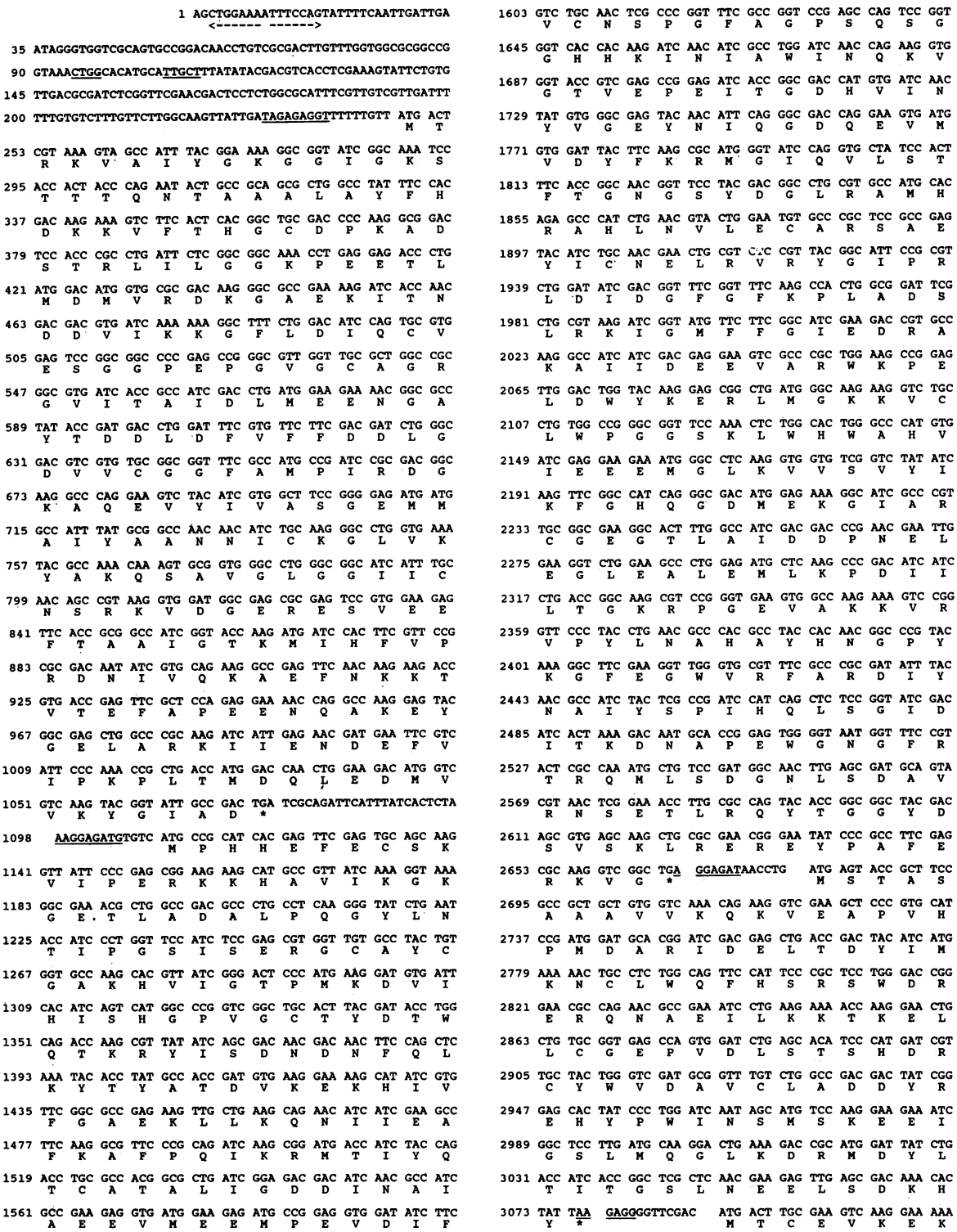


FIG. 1. Restriction map of the *anfHDGK* region from *A. vinelandii* strains CA, CA70, CA71, and CA72. The region whose nucleotide sequence has been determined (Fig. 2) is indicated by the heavier line. Restriction sites: E, *Eco*RI; S, *Sma*I; K, *Kpn*I; B, *Bcl*I. ▽, Kan^r cartridge insertion; →←, 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. □, Deletion.

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



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.*

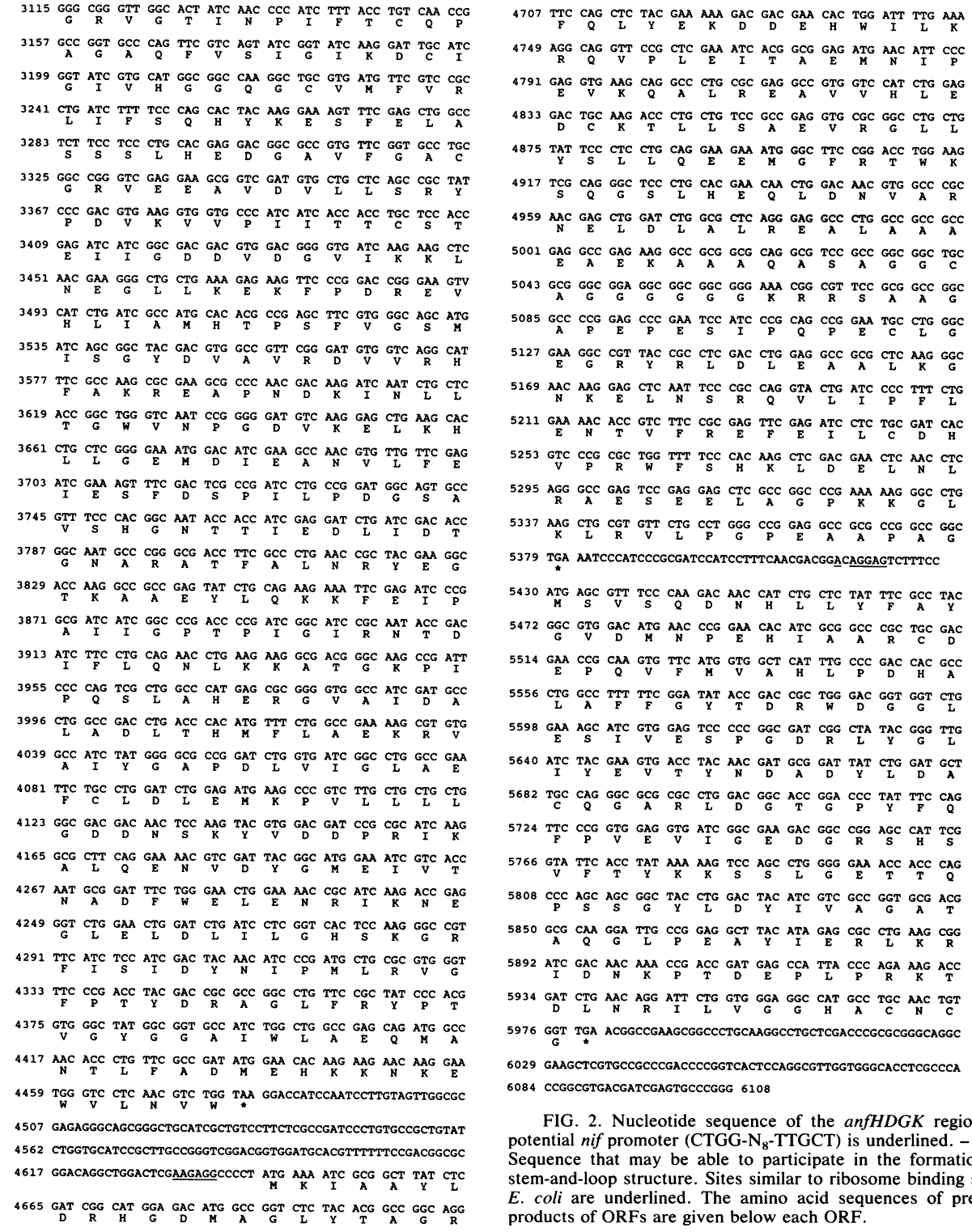


FIG. 2. Nucleotide sequence of the *anfHDGK* region. The potential *nif* promoter (CTGG-N_8-TTGCT) is underlined. – – – →, Sequence that may be able to participate in the formation of a stem-and-loop structure. Sites similar to ribosome binding sites of *E. coli* are underlined. The amino acid sequences of presumed products of ORFs are given below each ORF.

chroococcum (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. However, 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

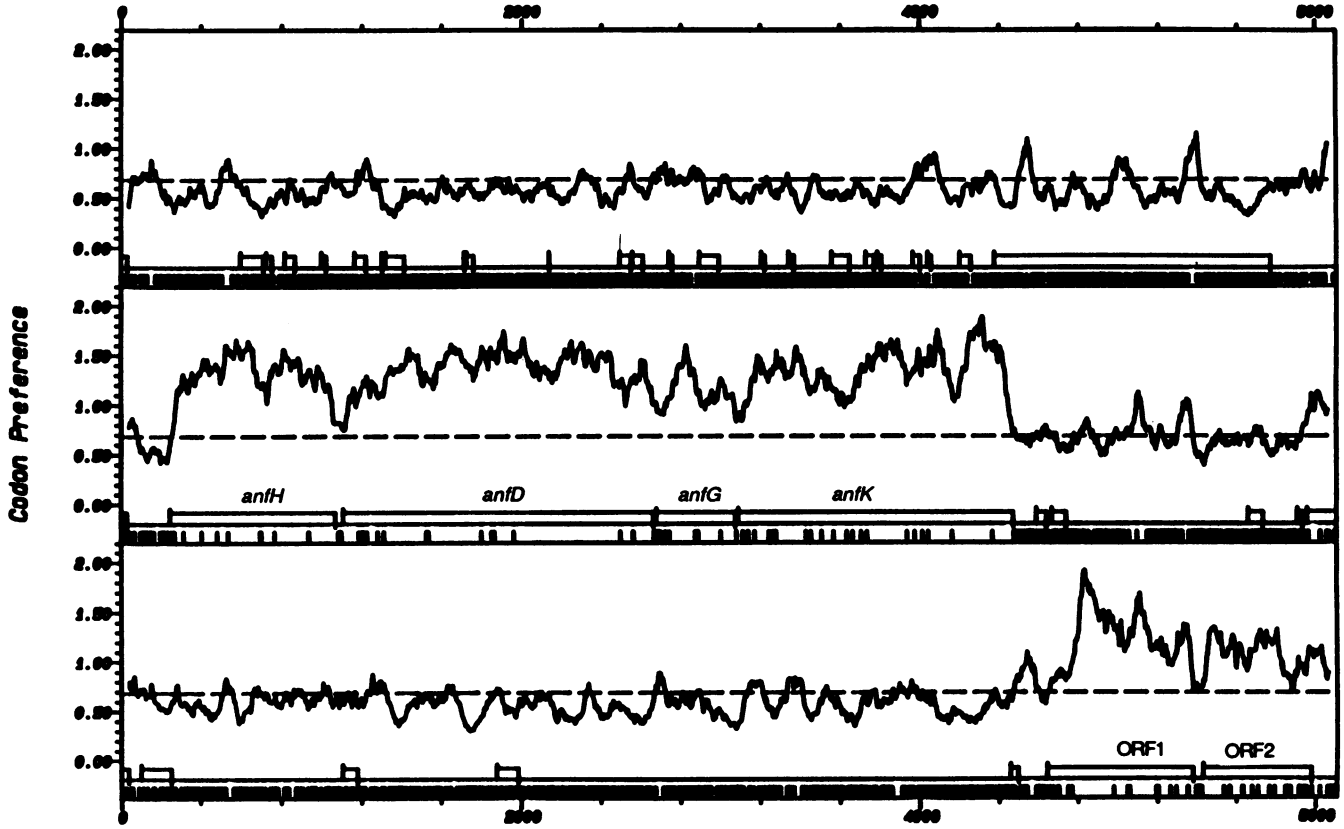


FIG. 3. Codon preference analysis of the nucleotide sequence available for the *anfHDGK* region (positions 1 to 6108). The codon usage in each of the three reading frames is compared by using a codon frequency table established from the *A. vinelandii nifHDK* genes. The probability for the regions of 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 MAMRQCAIYGKGGIGKSTTTQNLVAALAEM.GKKVMIVGCDPKADSTRLILHSKAQNTIM
     :: :::::::::::::::::::::::::::  :::::::::::::::::::::::::::::: :
AcH2 MALRQCAIYGKGGIGKSTTTQNLVAALAEA.GKKVMIVGCDPKADSTRLILHSKAQNTVM
     :  : ::::::::::::::::  ::::  :::  :::::::::::::::  :  :  : :
AvH3  MTRKVAIYGKGGIGKSTTTQNTAAALAYFHDKKVFTHGCDPKADSTRLILGGKPEETLM
      :::: :::::::::::::: ::: :: : : ::::: ::::::::::::::::::: : :::
CpH3  MTRKIAIYGKGGIGKSTTQQNT.AAMAHFYDKKVFIHGCDPKADSTRLILGGMPQKTLM

     60                  *          *                    119
AvH1 EMAAEAGTVEDLELEDVLKAGYGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYEDD
     :::: ::  :::::::::  :::::::::::::::::::::::::::::::::::::: ::
AcH2 EMAASAGSGEDLELEDVLQIGYGGVKCVESGGPEPGVGCAGRGVITAINFLEEEGAYSDD
      :   :  :   ::  :   :::::::::::::::::::::::::   :: ::: ::
AvH3 DMVRDKG.AEKITNDDVIKKGFLDIQCVESGGPEPGVGCAGRGVITAIDLMEENGAYTDD
     :: :: :  ::::       :  :: ::::::::::::::::::::::::::::::: ::::: :
CpH3 DMLRDEG..EKITTENIVRVGYEDIRCVESGGPEPGVGCAGRGVITAIDLMEKNGAYTED

     120         *                                        179
AvH1 LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNISKGIVKYANSGSVR
     ::::::::::::::::::::::::::::::::::::::::::::::::::: ::::::: :::::
AcH2 LDFVFYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNIAKGIVKYAHSGSVR
     ::::: : ::::::::::::::  :::: ::: :::::: :::::: :: ::::    :
AvH3 LDFVFFDDLGDVVCGGFAMPIRDGKAQEVYIVASGEMMAIYAANNICKGLVKYAKQSAVG
     :::::::: :::::::::::::::::::::::::::::::::::::::::::::::: :: : :
CpH3 LDFVFFDVLGDVVCGGFAMPIRDGKAQEVYIVASGEMMAVYAANNICKGLVKYANQSGVR

     180  *                                              239
AvH1 LGGLICNSRNTDREDELIIALANKLGTQMIHFVPRDNVVQRAEIRRMTVIEYDPKAKQAD
     ::::::::: :::::::: ::: : ::::::::::::::: ::::::::::::::::::::
AcH2 LGGLICNSRKTDREDELIMALAAKIGTQMIHFVPRDNVVQHAEIRRMTVIEYDPKAKQAD
     ::: :::::: : : :      : ::: :::::::::: :: ::    :: :  :   ::
AvH3 LGGIICNSRKVDGERESVEEFTAAIGTKMIHFVPRDNIVQKAEFNKKTVTEFAPEENQAK
     :::::::::: :: :::  ::: : ::: ::::: :::::::::::::: :: ::    ::::
CpH3 LGGIICNSRMVDLEREFIEEFAASIGTQMIHFMPRDNIVQKAEFNKQTVIEFDDTCNQAK

     240                                              290
AvH1 EYRALARKVVDNKLLVIPNPITMDELEELLMEFGIMEVEDESIVGKTAEEV
     :::::: :   :: :::::  :  ::::::::::::: :::::::   :
AcH2 EYRALAQKILNNK.LVIPNPGSMEDLEELLMEFGIMEAEDESIVGKAGAEG
     ::  :: ::  :   ::: : :  ::        ::
AvH3 EYGELARKIIENDEFVIPKPLTMDQLEDMVVKYGIAD
     ::::::::::::  :::: :: :: :: ::::::  :
CpH3 EYGELARKIIENEMFVIPTPLKMDDLEAMVVKYGMTD
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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.

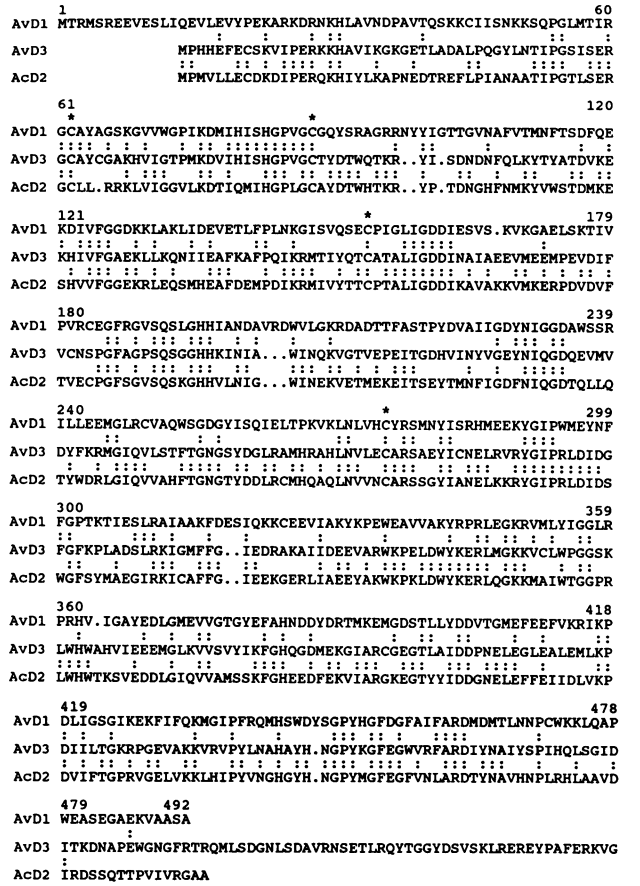

FIG. 5. Comparison of the predicted amino acid sequence of *anfD* (AvD3) with the products of *nifD* from *A. vinelandii* (AvD1) (9) and *vnfD* from *A. chroococcum* (AcD2) (Robson et al., manuscript submitted). *, Conserved Cys residue.

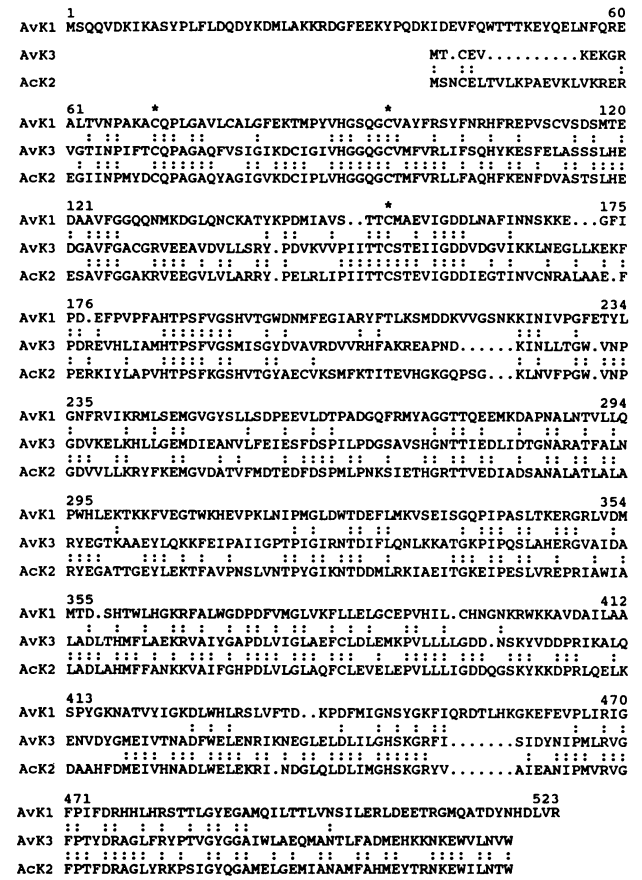

FIG. 7. Comparison of the amino acid sequence deduced from *anfK* (AvK3) with the products of *nifK* (AvK1) (9) and *vnfK* (AcK2) (Robson et al., manuscript submitted). *, Conserved Cys residue.

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 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.

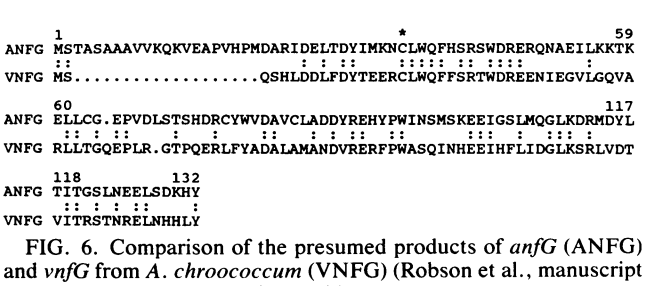

FIG. 6. Comparison of the presumed products of *anfG* (ANFG) and *vnfG* from *A. chroococcum* (VNFG) (Robson et al., manuscript submitted). *, Conserved Cys residue.

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

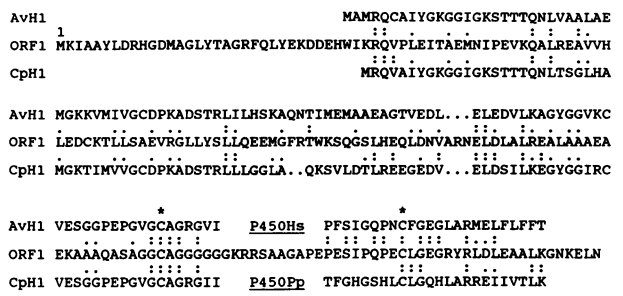

FIG. 8. Comparison of the presumed product of ORF1 (ORF1) with portions of the *nifH* product from *A. vinelandii* (amino acids 1 to 104) (AvH1) and the product of *nifH1* from *C. pasteurianum* (CpH1) (66) and of two proposed heme-binding sites from human cytochrome P-450 (phenobarbital induced) (P450Hs) (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 *anfHDGK* operon is not located within the cloned and sequenced fragments.

Growth of mutants carrying deletion or insertion mutations in the *anfDGK* region. The mutant strains CA70, CA71, and CA72 (Table 1, Fig. 1) are able to grow under N_2-fixing conditions in liquid Burk medium containing 1 μM Na_2MoO_4 or 1 μM V_2O_5 but not in Mo-deficient medium. Strains that also carry the *nifHDK* deletion of strain CA11 in addition to the mutations in the *anfHDGK* 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 *Kpn*I 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 *anfHDGK* 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_rs of protein products of the ORFs similar to *nifH*, *nifD*, and *nifK* correlate well with the M_rs 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 *anfHDGK* 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 *anfHDGK* 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

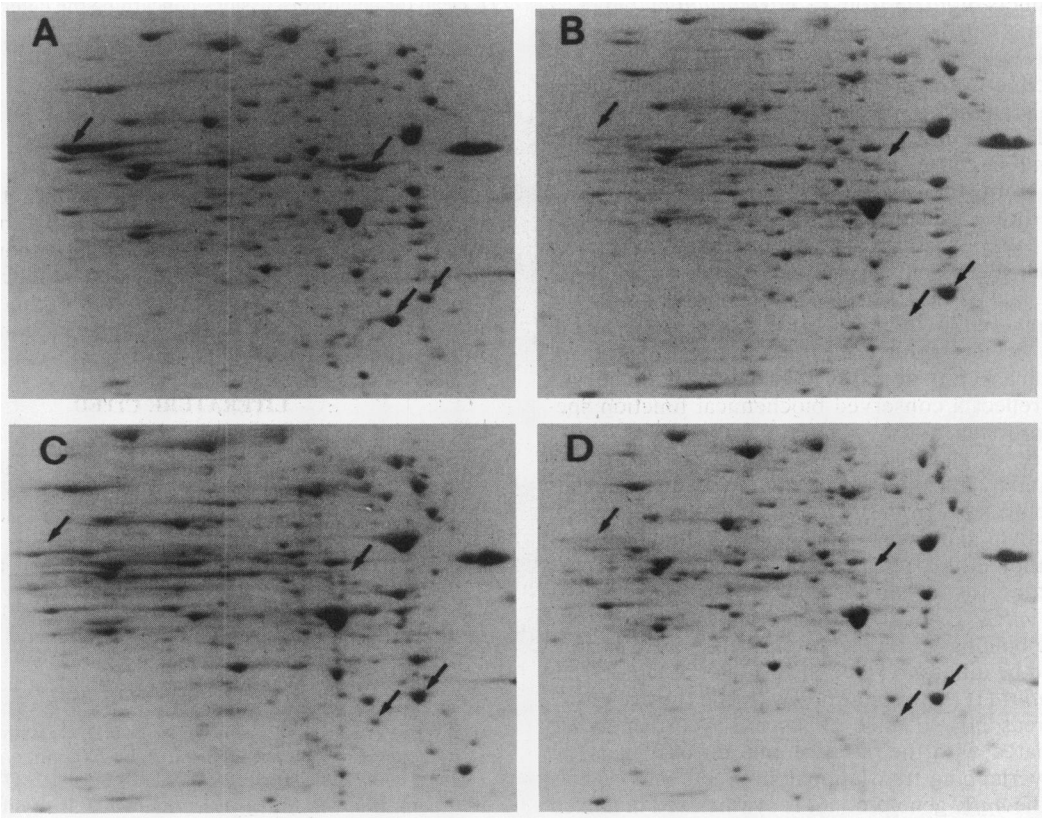

FIG. 9. Two-dimensional gels of protein extracts from cells derepressed in N-free, Mo-deficient medium for 18 h. ↙, Migration position of the nitrogenase 3 subunits. Clockwise from the left upper corner of the gels, these arrows point to the presumed products of *anfD* (α subunit), *anfK* (β subunit), *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 N_2 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 N_2 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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