JOURNAL OF BACTERIOLOGY, May 1991, p. 3041–3046
0021-9193/91/103041-06$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 173, No. 10

The N-Terminal and C-Terminal Portions of NifV Are Encoded by Two Different Genes in *Clostridium pasteurianum*

SHU-ZHEN WANG, DENNIS R. DEAN, JIANN-SHIN CHEN, AND JOHN L. JOHNSON*

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received 2 November 1990/Accepted 8 March 1991

The *nifV* gene products from *Azotobacter vinelandii* and *Klebsiella pneumoniae* share a high level of primary sequence identity and are proposed to catalyze the synthesis of homocitrate. While searching for potential *nif* (nitrogen fixation) genes within the genomic region located downstream from the *nifN-B* gene of *Clostridium pasteurianum*, we observed two open reading frames (ORFs) whose deduced amino acid sequences exhibit nonoverlapping sequence identity to different portions of the *nifV* gene products from *A. vinelandii* and *K. pneumoniae*. Conserved regions were located between the C-terminal 195 amino acid residues of the first ORF and the C-terminal portion of the *nifV* gene product and between the entire sequence of the second ORF (269 amino acid residues) and the N-terminal portion of the *nifV* gene product. We therefore designated the first ORF *nifVω* and the second ORF *nifVα*. The deduced amino acid sequences of *nifVω* and *nifVα* were also found to have sequence similarity when compared with the primary sequence of the *leuA* gene product from *Salmonella typhimurium*, which encodes α-isopropylmalate synthase. Marker rescue experiments were performed by recombining *nifVω* and *nifVα* from *C. pasteurianum*, singly and in combination, into the genome of an *A. vinelandii* mutant strain which has an insertion and a deletion mutation located within its *nifV* gene. A NifV$^+$ phenotype was obtained only when both the *C. pasteurianum nifVω* and *nifVα* genes were introduced into the chromosome of this mutant strain. These results suggest that the *nifVω* and *nifVα* genes encode separate domains, both of which are required for homocitrate synthesis in *C. pasteurianum*.

Biological nitrogen fixation is catalyzed by nitrogenase. Molybdenum-containing nitrogenase consists of two separable component proteins called the MoFe protein and the Fe protein. The Fe protein is a dimer of identical subunits, and it serves as an ATP-dependent electron donor to the MoFe protein. The MoFe protein has an $\alpha_2\beta_2$ tetrameric structure and contains the site for N_2 binding and reduction. The substrate reduction site on the MoFe protein is believed to reside in or include an iron-molybdenum cofactor (FeMoco). Isolated FeMoco contains five to eight Fe atoms, eight or nine S atoms, and one homocitrate molecule per molybdenum atom (5, 12–14, 17).

In *Klebsiella pneumoniae* and *Azotobacter vinelandii*, at least six nitrogen fixation (*nif*) genes are required for FeMoco biosynthesis (see, for example, reference 7). They include *nifH*, *nifE*, *nifN*, *nifB*, *nifQ*, and *nifV*. Inactivation of the *K. pneumoniae nifV* gene results in accumulation of a nitrogenase having altered substrate specificity and inhibitor susceptibility patterns (8, 10, 11). This alteration is associated with an altered form of FeMoco which contains citrate rather than homocitrate as its organic constituent (5, 9). Hoover et al. (6) have proposed that *nifV* encodes a homocitrate synthase.

The *nifV* gene products from both *K. pneumoniae* and *A. vinelandii* share considerable sequence similarity between their deduced polypeptide sequences (2). In the present study, we show that in *C. pasteurianum*, the function assigned to the *nifV* genes from *K. pneumoniae* and *A. vinelandii* is split into two cistrons. We propose to call these *C. pasteurianum* genes *nifVω* and *nifVα*, respectively.

* Corresponding author.

MATERIALS AND METHODS

DNA biochemistry and strain constructions. Cloning and sequencing of the *C. pasteurianum* W5 chromosomal region located downstream from *nifN-B* were performed as described previously (21).

Attempts to complement the *A. vinelandii* NifV$^-$ phenotype by using *C. pasteurianum* DNA were performed in two steps. In step 1, purified DNA fragments with known sequences from *C. pasteurianum* which contain the *nifVω* and *nifVα* genes, singly or in combination, were ligated to the *A. vinelandii nifF* gene contained within a hybrid plasmid. To accomplish this, blunt-ended *C. pasteurianum* DNA fragments (see below) were ligated with *Hin*cII-digested pDB93, which contains the *A. vinelandii nifF* gene and some flanking sequences cloned into pUC7 (1). There are three *Hin*cII sites within the *A. vinelandii nifF* coding region. When ligated in the proper orientation, such constructions place *nifVω* and *nifVα* singly, or in combination, under the control of the *nifF* promoter. In step 2, each DNA ligation mixture was used to transform a mutant *A. vinelandii* strain which has a deletion

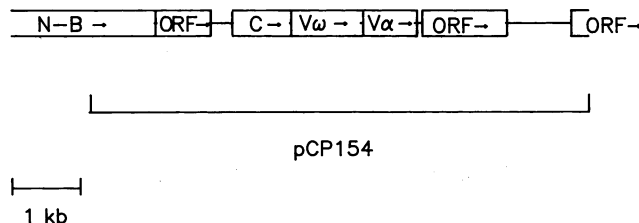

FIG. 1. Organization of *nif* genes and ORFs of *C. pasteurianum* located on the *Sst*I-*Bam*HI fragment cloned in pCP154. The boxes mark the coding regions of the *nif* genes and ORFs. The arrows indicate the direction of translation.

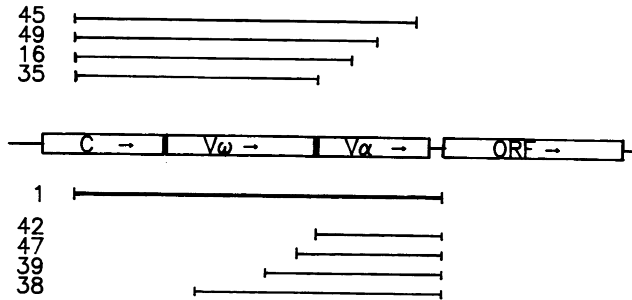

FIG. 2. A diagram of DNA fragments used to complement an *A. vinelandii* mutant (strain DJ388; Δ*nifV*::Km) having a deletion and an insertion in its *nifV* gene. Boxes mark the coding regions of the *nif* genes and ORFs. The arrows indicate the direction of translation. On the left are the construct numbers. The region covered by each construct is indicated by a line.

and a Km^r-encoding gene cassette placed within its *nifV* gene. This strain, designated DJ388, bears the same deletion as described for strain DJ71 (7). Procedures for such strain constructions are described in detail elsewhere (7). Appropriate reciprocal recombination events which can occur during transformation permit incorporation of *C. pasteurianum* sequences into the *A. vinelandii* chromosome at the *nifF* locus. An intact *nifF* allele is not required for nitrogen fixation in *A. vinelandii* (1). Transformations were performed as described by Page and von Tigerstrom (15). Immediately following transformation, cells were plated on Burk's minimal medium containing 0.5 μg of kanamycin per ml. Strain DJ388 is capable of only very slow diazotrophic growth, owing primarily to inactivation of the *nifV* gene but also, possibly, to polar effects upon *nifW* and *nifZ* gene expression (7). Thus, DJ388 transformants which have the NifV$^-$ phenotype corrected by incorporation of *C. pasteurianum* DNA can be detected by their larger colony size. Inclusion of kanamycin in the growth medium ensured that the original *nifV* deletion-Km^r insertion remained intact.

Blunt-ended *C. pasteurianum* DNA fragments were prepared as follows. A *Hin*dIII-*Cla*I fragment including both *nifV*ω and *nifV*α (construct 1) was obtained from pCP154 (Fig. 1). DNA fragments having portions of *nifV*α (constructs 45, 49, 16, and 35 in Fig. 2) or *nifV*ω (constructs 38, 39, 47, and 42 in Fig. 2) deleted were generated by *Exo*III and *Exo*VII nuclease digestion of construct 1 from the appropriate end (23). DNA fragments obtained as described above (Fig. 2) were blunt ended with the Klenow fragment of *Escherichia coli* DNA polymerase in the presence of deoxynucleoside triphosphates as previously described (23).

High-molecular-weight DNAs isolated from both wild-type *A. vinelandii* and the complementation-positive strain,

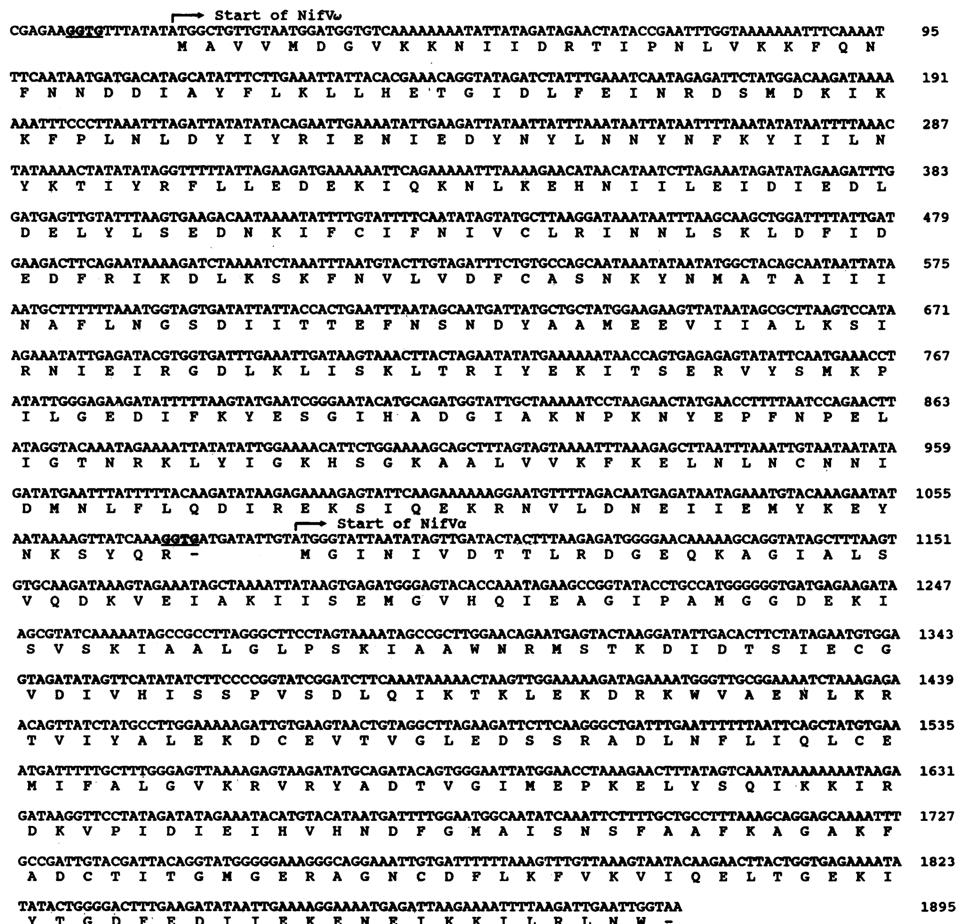

FIG. 3. Nucleotide sequence of and amino acids encoded by *nifV*ω and *nifV*α and the flanking regions of *C. pasteurianum*. Amino acids are indicated below the nucleotide sequence by the single-letter code.

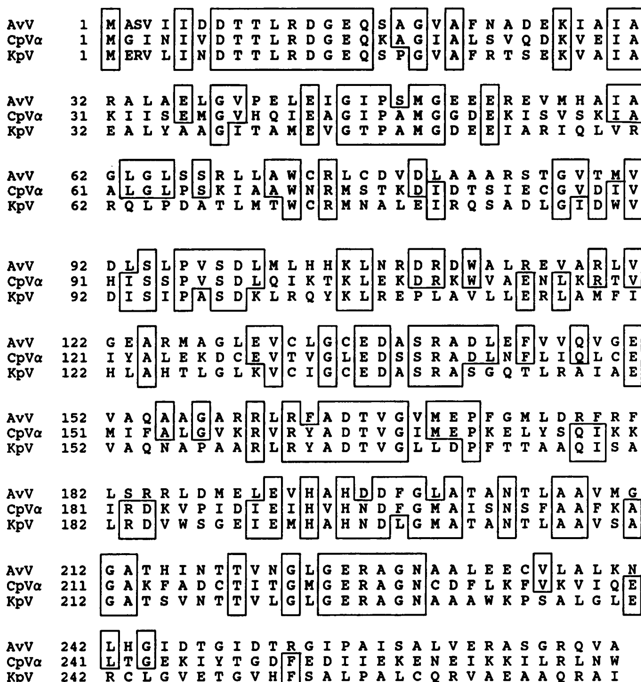

FIG. 4. Comparison of the amino acid sequences encoded by *nifVα* from *C. pasteurianum* (CpVα) and *nifV* from *A. vinelandii* (AvV) and *K. pneumoniae* (KpV). The entire deduced sequence encoded by *nifVα* (CpVα) is shown here, whereas only the N-terminal 270 residues of AvV (of 385 residues) and KpV (of 381 residues) are shown. Conserved residues are boxed.

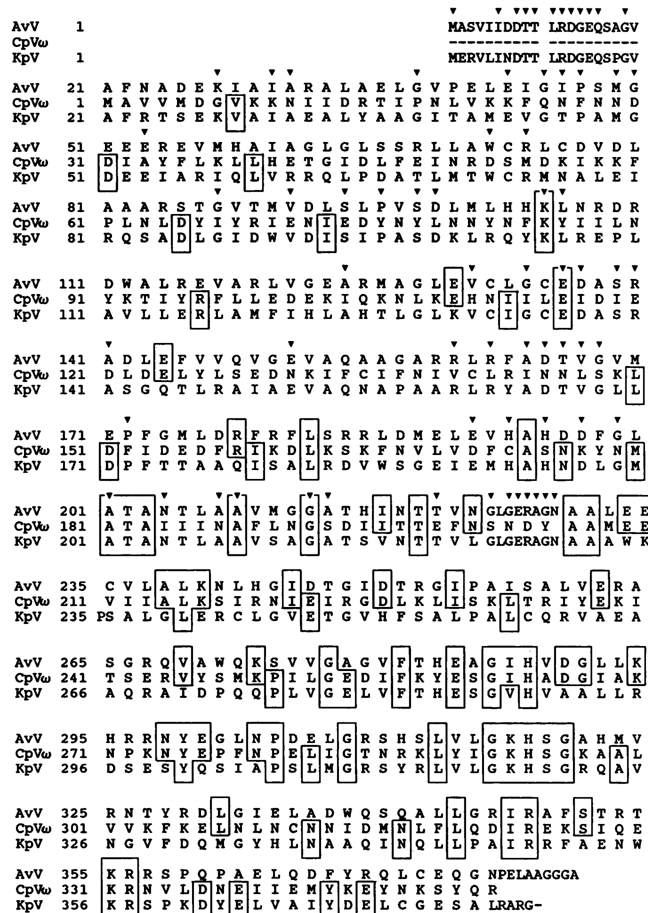

FIG. 5. Comparison of the amino acid sequences encoded by *nifVω* from *C. pasteurianum* (CpVω) and *nifV* from *A. vinelandii* (AvV) and *K. pneumoniae* (KpV). Conserved amino acid residues are boxed. Positions where conservation is observed among CpVα, AvV, and KpV are indicated by inverted triangles.

designated DJ391 (Δ*nifV*::Km *nifF*::*nifVω*$_{Cp}$ *nifVα*$_{Cp}$, where Cp indicates a *C. pasteurianum* gene) were digested with *Xho*I and individually separated by agarose gel electrophoresis. Southern analysis was performed by using peroxidase-labeled *nifVω* and *nifVα* as probes. The ECL Gene Detecting System (RPN 2101; Amersham, Arlington Heights, Ill.) was used as described by the manufacturer.

RESULTS

Cloning and sequencing. A 7.4-kb *Sst*I-*Bam*HI fragment located downstream from the *C. pasteurianum nifN-B* locus was cloned into pUC19 and designated pCP154. DNA sequence analysis revealed five potential complete open reading frames (ORFs) and another incomplete one in this region (Fig. 1). A description of the organization and genotypic assignments of the ORFs within this region was previously reported (21). In Fig. 3, the nucleotide sequence for the genes labeled *nifVω* (encoding 352 amino acid residues; M_r, 41,574; pI, 5.5) and *nifVα* (encoding 269 amino acid residues; M_r, 29,862; pI, 5.28) is presented.

Similarities between *C. pasteurianum* sequences and *nifV* of *A. vinelandii* and *K. pneumoniae*. The deduced amino acid sequence encoded by *nifVα* has sequence similarity to the deduced amino acid sequence of the *nifV* products from both *A. vinelandii* and *K. pneumoniae* (Fig. 4). Identical amino acids account for 38 and 32% of the 269 residues of NifVα in comparison with the first 270 amino acid residues of the NifV proteins of *A. vinelandii* and *K. pneumoniae*, respectively. The conserved region extends throughout the sequence of NifVα, except for the C-terminal 41 residues, in which no extensive similarity is observed. The deduced amino acid sequence encoded by *nifVω* also has sequence similarity to the deduced amino acid sequences of the *nifV* products from both *A. vinelandii* and *K. pneumoniae* (Fig. 5). This similarity is, however, restricted to the C-terminal portions of the corresponding sequences. A discrete boundary between the nonconserved N-terminal portion and the conserved C-terminal portion is difficult to identify because the transition is gradual. As a result, we arbitrarily designated position 158 of NifVω as the start of the conserved portion. The conserved region from NifVω bears 27% identity to the *A. vinelandii* NifV sequence (residues 178 to 385) and 23% identity to the *K. pneumoniae* NifV sequence (residues 178 to 381).

Sequence similarities between NifVω-NifVα of *C. pasteurianum* and α-isopropylmalate synthase of *S. typhimurium*. The *nifV* gene is proposed to encode a homocitrate synthase (6). A homocitrate synthase also catalyzes the first step in the lysine biosynthetic pathway in *Saccharomyces cerevisiae*, in which acetyl coenzyme A and α-ketoglutarate are condensed to form homocitrate (3, 19). An analogous reaction exists in the leucine biosynthetic pathway, in which α-isopropylmalate synthase (the *leuA* gene product) condenses acetyl coenzyme A and α-ketoisovalerate to form α-isopropylmalate (18, 20). Collett and Orme-Johnson (4) previously recognized a low level of primary sequence identity between

```
            ▼   ▼ ▼▼▼▼ ▼▼▼▼▼  ▼ ▼      ▼  ▼▼       ▼     ▼ ▼▼▼ ▼▼   ▼
CpVα      1 MGINIVDTTL RDGEQKAGIA LSVQDKVEIA KIISEMGVHQ IEAGIPAMGG DEKISVSKIA
                * **** *****      **   *  **      ***    * * *             **
LeuA    1 MSQQVIIFDTTL RDGEQALQAS LSAKEKLQIA LALERMGVDV MEVGFPVSSP GDFESVQTIA

                       ▼ ▼                 ▼    ▼  ▼ ▼  ▼▼     ▼▼
CpVα     61 ALGLPSKIAA WNRMSTKDID T----SIECG VDIVHISSPV SDLQIKTKLE KDRKWVAENL
                 *   *   *   ****                *      *   *  **       * *
LeuA     63 RTIKNSRVCA LARCVEKDID VAAQALKVAD AFRIHTFIAT SPMHIATKLR RTLDEVIERA

                  ▼       ▼  ▼ ▼▼  ▼▼▼          ▼         ▼ ▼ ▼▼▼▼ ▼   ▼
CpVα    120 KRTVIYALEK DCEVTVGLED SSRADLNFLI QLCEMIFALG VKRVRYADTV GIMEPKELYS
               *  *       *    **   *     *     *     *        *** *   * *
LeuA    123 VYMVKRARNY TDDVEFSCED AGRTPVDDLA RVVEAAINAG ARTINIPDTV GYTMPFEFAG

                           ▼  ▼ ▼    ▼ ▼ ▼   ▼  ▼▼   ▼▼      ▼  ▼  ▼▼▼▼▼▼
CpVα    177 QIKKIRDKVP -IDIEI-HVH --NDFGMAIS NSFAAFKAGA KFADCTITG MGERAGNCDFL
             *      **  **  *  **    * * *   ** **  ***         *  *******
LeuA    183 IISGLYERVP NIDKAIISVH THDDLGIAVG NSLAAVHAGA RQVEGAMNG IGERAGNCALE

CpVα    233 KFVKVIQELT GEKIYTGDFE DIIEKENEIK KILRLNW
                 *
LeuA    243 EVIMAIKVRK DIMNVHTNIN HHETGAPARP SVQICNI
```

FIG. 6. Comparison of the amino acid sequence encoded by *nifV*α from *C. pasteurianum* (CpVα) with that encoded by *leuA* from *S. typhimurium* (LeuA). Identical amino acids are indicated by asterisks. Inverted triangles mark the positions where conservation among CpVα, AvV, and KpV (Fig. 5, legend) was observed. *leuA* has a coding capacity of 522 amino acid residues, and only part of it is shown here.

α-isopropylmalate synthase from *S. cerevisiae* and the *nifV* gene product from *K. pneumoniae*. In Fig. 6 and 7, the primary sequence of α-isopropylmalate synthase from *Salmonella typhimurium* (16) is compared with the deduced *nifV*ω and *nifV*α gene product sequences, respectively. The conservation between NifVα and α-isopropylmalate synthase (indicated by asterisks in Fig. 6) generally correlates with the conservation among NifVα of *C. pasteurianum* and NifV of *A. vinelandii* and *K. pneumoniae* (indicated by inverted triangles in Fig. 6). These sequence conservations support the hypothesis that the *nifV* product catalyzes condensation of acetyl coenzyme A and α-ketoglutarate to form homocitrate in a reaction similar to α-isopropylmalate synthesis.

Complementation between *nifV*ω-*nifV*α of *C. pasteurianum* and *nifV* of *A. vinelandii*. Fragments of DNA covering both *nifV*ω and *nifV*α or with deletions in one of them (Fig. 2) were placed under the control of the *A. vinelandii nifF* promoter and then used to transform a *nifV* deletion mutant of *A. vinelandii* (strain DJ388, Δ*nifV*::Km). The NifV$^+$

```
CpVω    1  MAVVMDGVKK NIIDRTIPNL VKKFQNFNND DIAYFLKLLH ETGIDLFEIN RDSMDKIKKF
                  *          *       *              *                      *
LeuA   35  LERMGVDVME VGFPVSSPGD FESVQTIART IKNSRVCALA RCVEKDIDVA AQALKVADAF

                                     ▼                                 ▼
CpVω   61  PLNLDYIYRI ENIEDYNYLN NYNFKYIILN YKTIYRFLLE DEKIQKNLKE HNIILEIDIE
                        *                        *     *
LeuA   95  RIHTFIATSP MHIATKLRRT LDEVIERAVY MVKRARNYTD DVEFSCEDAG RTPVDDLARV

                                                        ▼            ▼
CpVω  121  DLDELYLSED NKIFCIFNIV CLRINNLSKL DFIDEDFRIK DLKSKFNVLV DFCASNKYNM
                                                *      *          *       *
LeuA  155  VEAAINAGAR TINIPDTVGY TMPFEFAGII SGLYERVPNI DKAIISVHTH DDLGIAVGN-

           ▼▼▼    ▼    ▼     ▼        ▼▼        ▼
CpVω  181  ATAIIINAFL NGSDIITTEF NSNDYAAMEE VIIALKSIRN IEIRGDLKLI SKLTRIYEKI
             *                         * ** ** * *     *
LeuA  214  SLAAVHAGAR QVEGAMNGIG ERAGNCALEE VIMAIKVRKD IMNVHTNINH HETGAPARPSVQI

                        ▼   ▼  ▼  ▼ ▼           ▼    ▼    ▼    ▼   ▼▼▼▼▼
CpVω  241  TSERVYSMKP ILGEDIFKYE SGIHADGIAK NPKNYEPFNP ELIGTNRKLY IGKHSGKAAL
                 *          *    **** **  *  *  ***   * * **
LeuA  277  CNIADPSQQS DCRQRRFRHS SGIHQDGVLK NRENYEIMTP ESIGSEPDTA EPDLPLWPCR

                               ▼   ▼▼       ▼▼
CpVω  301  VVKFKELNLN CNNIDMNLFL QDIREKSIQE KRNVLDNEII EMYKEYNKSY QR
                                            *  * *
LeuA  337  RETSHGRDGL QGHRLQHGPP VRRVPEAGDK KGQVFDYDLE ALAFINKQQE EP
```

FIG. 7. Comparison of the amino acid sequence encoded by *nifV*ω from *C. pasteurianum* (CpVω) with that encoded by *leuA* from *S. typhimurium* (LeuA). Identical amino acids are indicated by asterisks. Inverted triangles mark the positions where conservation among CpVω, AvV, and KpV (Fig. 5, legend) was observed. *leuA* has a coding capacity of 522 amino acid residues, and only part of it is shown here.

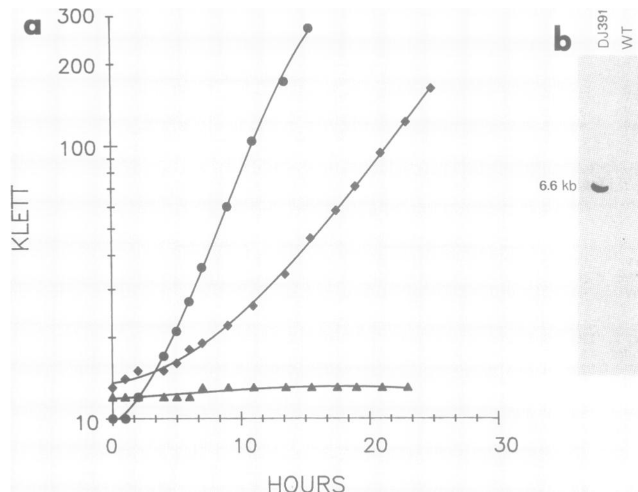

FIG. 8. (a) Restoration of the Nif phenotype of a mutant strain of *A. vinelandii* (having a deletion and an insertion in *nifV*) by *nifV*ω and *nifV*α from *C. pasteurianum*. Shown here are growth curves of the wild type (●), *nifV* deletion strain DJ388 (▲), and rescued strain DJ391 (◆) of *A. vinelandii* in nitrogen-fixing medium containing 10 μM Na_2MoO_4. (b) Southern hybridization analysis of DNAs from the wild type (WT) and rescued strain DJ391 of *A. vinelandii*. The hybridization probe was peroxidase-labeled *C. pasteurianum* DNA containing *nifV*ω and *nifV*α. A 6.6-kb hybridization-positive DNA band was identified only in strain DJ391, illustrating the presence of *C. pasteurianum* DNA in DJ391.

phenotype was observed only when DNA containing both *nifV*ω and *nifV*α (construct 1) was introduced into DJ388. Constructs having portions of *nifV*ω or *nifV*α deleted yielded no Nif$^+$ colonies on NH_3-free plates, indicating an inability of *nifV*ω or *nifV*α to complement the *A. vinelandii* NifV$^-$-producing mutation individually. We compared the diazotrophic growth rates of wild-type *A. vinelandii*, mutant strain DJ388 (Δ*nifV*::Km), and rescued strain DJ391 (Δ*nifV*::Km *nifF*::*nifV*ω$_{Cp}$*nifV*α$_{Cp}$) of *A. vinelandii* (Fig. 8a) in nitrogen-fixing medium containing 10 μM Na_2MoO_4 for suppression of the alternative nitrogen fixation system. Whether the slower growth rate of strain DJ391 than that of the wild type reflects incomplete complementation of the NifV$^-$ phenotype or polarity upon *nifW* and *nifZ* gene expression in the mutant construct is not known. The presence of *C. pasteurianum nifV*ω and *nifV*α DNAs in strain DJ391 was verified by Southern hybridization analysis as shown in Fig. 8b.

DISCUSSION

There are several distinct features in the organization of genes found within the major *nif* cluster of *C. pasteurianum* compared with *nif* gene clusters from other organisms. For example, homologs to the *nifN* and *nifB* genes present in other organisms are fused to form a single cistron in *C. pasteurianum* (22). The existence of *nifV*ω and *nifV*α in *C. pasteurianum* represents the opposite situation. Namely, these two separate genes from *C. pasteurianum* are both required to supply the functionality provided by a single gene product (the *nifV* product) from other organisms. To rule out the possibility that this finding reflects a cloning artifact, *C. pasteurianum* W5 genomic DNA was digested with a variety of restriction enzymes and analyzed by Southern hybridization using *nifV*ω- and *nifV*α-specific

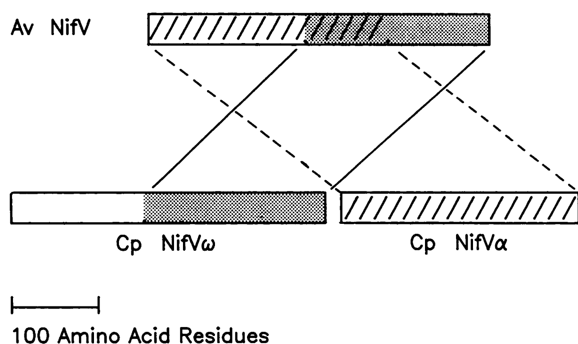

FIG. 9. Schematic illustration of regions of NifVω and NifVα of *C. pasteurianum* corresponding to NifV of *A. vinelandii*.

probes. This analysis (data not shown) indicated that the organization of *nifVω* and *nifVα* recognized by DNA sequence analysis of the cloned DNA fragment is the same as for the *C. pasteurianum* genome. The requirement of both *nifVω* and *nifVα* for complementation of an *A. vinelandii nifV* mutation provides additional evidence that the structural arrangement of *nifVω* and *nifVα* is functional.

CO-sensitive H_2 evolution and a high ratio of acetylene-reducing to nitrogen-fixing activities are characteristics of nitrogenase produced in *nifV* mutants of *K. pneumoniae*. It has been shown that in *K. pneumoniae*, these properties can be attributed to incorporation of citrate in place of homocitrate within FeMoco (9, 11). Thus, it might be predicted that nitrogenase in extracts of DJ388 would exhibit CO-sensitive H_2 evolution activity while nitrogenase in extracts from the rescued strain, DJ391, would exhibit CO-insensitive H_2 evolution activity. However, in the present study, such experiments would not be conclusive for several reasons. (i) Crude extracts of *A. vinelandii* contain a very active hydrogenase activity which is also CO sensitive. (ii) It is not known whether citrate is the organic acid which replaces homocitrate in *nifV*-deficient strains of the obligate aerobe *A. vinelandii*. (iii) The *nifV* insertion mutation within DJ388 could have a polar effect upon *nifW* and *nifZ* gene expression, and the products of both of these genes are known to be required for full MoFe protein activity (7).

Comparison of the deduced amino acid sequences for NifVα and NifVω reveals no obvious sequence similarity between them. This includes regions in both genes that have limited sequence similarities to NifV from *A. vinelandii* and *K. pneumoniae*. The correspondence of NifVω and NifVα from *C. pasteurianum* to NifV from *A. vinelandii* is shown schematically in Fig. 9. Therefore, it does not appear likely that *nifVω* and *nifVα* emerged as the consequence of a gene duplication event.

The ability of *nifVω*-*nifVα* to complement a *nifV* deletion mutation of *A. vinelandii* is remarkable. The DNA G+C contents of these two organisms represent the extremes among bacteria, with *C. pasteurianum* at 28 to 30% and *A. vinelandii* at 68 to 70%. The positive result also shows that, together, the products of *nifVω* and *nifVα* are functionally similar to the product of *nifV*. Thus, the NifV proteins from *A. vinelandii* and *K. pneumoniae* appear to have at least two separate functional domains. Sequence similarities among the *nifV* gene products and α-isopropylmalate synthase also indicate that these separate domains are likely to include acetyl coenzyme A- and α-keto acid-binding regions.

ACKNOWLEDGMENTS

This work was supported by USDA competitive grant 88-37262-3774 and by projects 6127250 and 6126900 from the Commonwealth of Virginia. Work in the laboratory of D.R.D. was supported by a grant from the National Science Foundation (NSF-DMB-8917371).

REFERENCES

1. **Bennett, L. T., M. R. Jacobson, and D. R. Dean.** 1988. Isolation, sequencing, and mutagenesis of the *nifF* gene encoding flavodoxin from *Azotobacter vinelandii*. J. Biol. Chem. **263:**1364–1369.
2. **Beynon, J., A. Ally, M. Cannon, F. Cannon, M. Jacobson, V. Cash, and D. Dean.** 1987. Comparative organization of nitrogen fixation-specific genes from *Azotobacter vinelandii* and *Klebsiella pneumoniae*: DNA sequence of the *nifUSV* genes. J. Bacteriol. **169:**4024–4029.
3. **Broquist, B. H., and J. S. Trupin.** 1966. Amino acid metabolism. Annu. Rev. Biochem. **35:**231–269.
4. **Collett, T. A., and W. H. Orme-Johnson.** Personal communication.
5. **Hoover, T. R., J. Imperial, J. Liang, P. W. Ludden, and V. K. Shah.** 1989. Homocitrate is a component of the iron-molybdenum cofactor of nitrogenase. Biochemistry **28:**2768–2771.
6. **Hoover, T. R., A. D. Robertson, R. L. Cerny, R. N. Hayes, J. Imperial, V. K. Shah, and P. W. Ludden.** 1987. Identification of the V factor needed for synthesis of the iron-molybdenum cofactor of nitrogenase as homocitrate. Nature (London) **329:**855–857.
7. **Jacobson, M. R., V. L. Cash, M. C. Weiss, N. F. Laird, W. E. Newton, and D. R. Dean.** 1989. Biochemical and genetic analysis of the *nifUSVWZM* cluster from *Azotobacter vinelandii*. Mol. Gen. Genet. **219:**49–57.
8. **Liang, J., and R. H. Burris.** 1989. N_2O reduction and HD formation by nitrogenase from a *nifV* mutant of *Klebsiella pneumoniae*. J. Bacteriol. **171:**3176–3180.
9. **Liang, J., M. Madden, V. K. Shah, and R. H. Burris.** 1990. Citrate substitutes for homocitrate in nitrogenase of a *nifV* mutant of *Klebsiella pneumoniae*. Biochemistry **29:**8577–8581.
10. **McLean, P. A., and R. A. Dixon.** 1981. Requirement of *nifV* gene for production of wild-type nitrogenase enzyme in *Klebsiella pneumoniae*. Nature (London) **292:**655–656.
11. **McLean, P. A., B. E. Smith, and R. A. Dixon.** 1983. Nitrogenase of *Klebsiella pneumoniae nifV* mutants. Biochem. J. **211:**589–597.
12. **McLean, P. A., D. A. Wink, S. K. Chapman, A. B. Hickman, D. M. McKillop, and W. H. Orme-Johnson.** 1989. A new method for extrusion of iron-molybdenum cofactor (FeMoco) from nitrogenase adsorbed to DEAE-cellulose. 1. Effects of anions, cations, and preextraction treatments. Biochemistry **28:**9402–9406.
13. **Nelson, M. J., M. A. Levy, and W. H. Orme-Johnson.** 1983. Metal and sulfur composition of iron-molybdenum cofactor of nitrogenase. Proc. Natl. Acad. Sci. USA **80:**147–150.
14. **Newton, W. E., S. F. Gheller, B. J. Feldman, W. R. Dunham, and F. A. Schultz.** 1989. Isolated iron-molybdenum cofactor of nitrogenase exists in multiple forms in its oxidized and semi-reduced states. J. Biol. Chem. **264:**1924–1927.
15. **Page, W. L., and M. von Tigerstrom.** 1979. Optimal conditions for transformation of *Azotobacter vinelandii*. J. Bacteriol. **139:**1058–1061.
16. **Ricca, E., and J. M. Calvo.** 1990. The nucleotide sequence of *leuA* from *Salmonella typhimurium*. Nucleic Acids Res. **18:**1290.
17. **Shah, V. K., and W. J. Brill.** 1977. Isolation of an iron-molybdenum cofactor from nitrogenase. Proc. Natl. Acad. Sci. USA **74:**3249–3253.
18. **Strassman, M., and L. N. Ceci.** 1963. Enzymatic formation of α-isopropylmalic acid, an intermediate in leucine biosynthesis. J. Biol. Chem. **238:**2445–2452.
19. **Strassman, M., and L. N. Ceci.** 1964. Enzymatic formation of homocitric acid, an intermediate in lysine biosynthesis. Biochem. Biophys. Res. Commun. **14:**262–267.
20. **Umbarger, H. E.** 1987. Biosynthesis of the branched chain

amino acids, p. 352–367. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.

21. **Wang, S.-Z., J.-S. Chen, and J. L. Johnson.** 1990. A nitrogen-fixation gene (*nifC*) in *Clostridium pasteurianum* with sequence similarity to *chlJ* of *Escherichia coli*. Biochem. Biophys. Res. Commun. **169:**1122–1128.
22. **Wang, S.-Z., J.-S. Chen, and J. L. Johnson.** Unpublished data.
23. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC19 vectors. Gene **33:**103–119.