JOURNAL OF BACTERIOLOGY, Dec. 1991, p. 7565–7572
0021-9193/91/237565-08$02.00/0
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Vol. 173, No. 23

Nucleotide Sequence and Mutational Analysis of the *vnfENX* Region of *Azotobacter vinelandii*†

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The nucleotide sequence (3,600 bp) of a second copy of *nifENX*-like genes in *Azotobacter vinelandii* has been determined. These genes are located immediately downstream from *vnfA* and have been designated *vnfENX*. The *vnfENX* genes appear to be organized as a single transcriptional unit that is preceded by a potential RpoN-dependent promoter. While the *nifEN* genes are thought to be evolutionarily related to *nifDK*, the *vnfEN* genes appear to be more closely related to *nifEN* than to either *nifDK*, *vnfDK*, or *anfDK*. Mutant strains (CA47 and CA48) carrying insertions in *vnfE* and *vnfN*, respectively, are able to grow diazotrophically in molybdenum (Mo)-deficient medium containing vanadium (V) (Vnf^+) and in medium lacking both Mo and V (Anf^+). However, a double mutant (strain DJ42.48) which contains a *nifEN* deletion and an insertion in *vnfE* is unable to grow diazotrophically in Mo-sufficient medium or in Mo-deficient medium with or without V. This suggests that NifE and NifN substitute for VnfE and VnfN when the *vnfEN* genes are mutationally inactivated. AnfA is not required for the expression of a *vnfN-lacZ* transcriptional fusion, even though this fusion is expressed under Mo- and V-deficient diazotrophic growth conditions.

Azotobacter vinelandii is able to grow diazotrophically using any of three genetically distinct nitrogenases depending on the presence or absence of molybdenum (Mo) or vanadium (V) in the growth medium. The well-characterized Mo-containing nitrogenase (nitrogenase-1) is synthesized under conditions of Mo sufficiency. Under conditions where V replaces Mo, an alternative V-containing nitrogenase (nitrogenase-2) is expressed, and in the absence of both Mo and V, an alternative nitrogenase (nitrogenase-3) that does not appear to contain either Mo or V is made (18, 23). Each of these nitrogenase complexes is composed of two protein components, dinitrogenase reductase and dinitrogenase. Dinitrogenase reductase-1 is a dimer of two identical subunits with an M_r of approximately 60,000 (10, 11). Dinitrogenase reductase-2 and dinitrogenase reductase-3 are also dimers of two identical subunits (13, 20). Dinitrogenase-1 is a tetramer (M_r, ~240,000) consisting of two pairs of nonidentical subunits ($\alpha_2\beta_2$). Dinitrogenase-2 and dinitrogenase-3, on the other hand, are probably hexamers, each containing three pairs of nonidentical subunits ($\alpha_2\beta_2\delta_2$) (25, 39).

The structural genes encoding nitrogenase-1 and nitrogenase-3 are organized as single operons (*nifHDK* and *anf HDGKorf1orf2*, respectively), while those encoding nitrogenase-2 form two independently regulated operons, *vnfHorf Fd* and *vnfDGK* (8, 25, 26, 39). *nifH*, *vnfH*, and *anfH* encode the subunits for dinitrogenase reductase-1, dinitrogenase reductase-2, and dinitrogenase reductase-3, respectively. The α and β subunits of dinitrogenase-1, dinitrogenase-2, and dinitrogenase-3 are encoded by *nifDK*, *vnfDK*, and *anfDK*, respectively. The δ subunit of dinitrogenase-2 is encoded by *vnfG*, and, in all probability, *anfG* encodes a similar subunit for dinitrogenase-3 (25, 39).

Dinitrogenase-1 contains two types of metal centers: P centers that might be organized as four unusual [4Fe-4S] clusters and two identical FeMo cofactors (FeMoco) which are the sites for binding and reduction of N_2 (42, 46). Recent evidence from X-ray crystallography, however, indicates that the P centers may actually be organized as two eight-iron centers (33). A cofactor (FeVaco) analogous to FeMoco has been extracted from dinitrogenase-2 by using *N*-methylformamide (47). The cofactor for dinitrogenase-3 has not yet been isolated; it is likely, however, that the only metal in this putative cofactor is iron, since dinitrogenase-3 does not appear to contain significant quantities of other metals (13). FeMoco is a complex entity composed of Mo, Fe, S, and homocitrate (43). FeVaco is known to contain V, Fe, and S, and it is likely that this cofactor contains homocitrate, since deletion of *nifV* causes *A. vinelandii* to exhibit a Nif^- Vnf^- Anf^- phenotype (5, 51).

The genes required for FeMoco synthesis are *nifE*, *nifN*, *nifV*, *nifB*, *nifQ*, and *nifH* (43). Since the *nifDK* gene products are not required for FeMoco synthesis, apodinitrogenase-1 apparently does not serve as a scaffold for synthesis of this cofactor (38, 49). From the striking amino acid sequence similarity between the *nifDK* and *nifEN* gene products, Brigle et al. (9, 14) suggested that there may be an important structural and evolutionary relationship between these protein complexes and proposed that FeMoco may be synthesized on the *nifEN* gene product complex prior to incorporation into apodinitrogenase-1. Comparisons between the *nifDK* and *nifEN* gene products from *Klebsiella pneumoniae* (2, 41), *Rhodobacter capsulatus* (32), and *Bradyrhizobium japonicum* (1) have yielded similar results.

In *A. vinelandii*, the structural genes encoding nitrogenase-1 (*nifHDK*) are followed by the operon containing *nifENX* (22). The role of *nifX* in *A. vinelandii* is unknown. On the basis of DNA sequence comparisons of *nifB* and *nifX* from *R. capulatus*, Moreno-Vivian et al. (32) suggested that *nifX* is likely to participate in FeMoco biosynthesis, though experimental confirmation has not yet been published. On the other hand, Gosink et al. (19) have provided evidence that *nifX* acts as a negative regulator of the *nif* regulon in *K. pneumoniae*.

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† Cooperative study between the Agricultural Research Service, U.S. Department of Agriculture, and the North Carolina Agricultural Research Service.

TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant characteristics or genotype	Reference or source
Strains		
E. coli		
HB101	F^- *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^-	29
C600	F^- *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* λ^-	29
S17-1	*thi pro* $HsdR^-$ $HsdM^+$ *recA* RP4 2-Tc::Mu-Km::Tn7 (integrated plasmid)	44
JC5466	*trp his recA56 rplE*	C. Kennedy
K-12 71-18	Δ(*lac-pro*) F′ *lacI*^{q} *lacZ*ΔM15 *pro*$^+$ *supE*	30
A. vinelandii		
CA	Wild type	12
CA11	Δ*nifHDK*	6
CA47	*vnfN47*::Tn*5*-B21 Tc^r	This study
CA48	*vnfE705*::*kan*	This study
CA66	Δ*anfA66*::*kan*	24
CA66.47	Δ*anfA66*::*kan vnfN47*::Tn*5*-B21 Tc^r	This study
DJ42	Δ*nifYorf1orf2ENXorf3orf4*	9
DJ42.48	Δ*nifEN*[a] *vnfE705*::*kan*	This study
Plasmids		
pLWE2	pUC9 containing the 1.4-kbp *Sma*I fragment containing *vnfE*	This study
pLWE2-1	pUC9 containing the 1.4-kbp *Sma*I fragment containing *vnfE* plus the Kan^r cartridge from pKISS at position 750	This study
pLWNX2	pUC18 containing the C terminus of *vnfE* and all of *vnfNX*	This study
pWWB12	pLWNX2 containing Tn*5*-B21 insertion in *vnfENX* region	This study
pUC9 and pUC18	Amp^r	50
pAU48	pUC9 containing a 4.3-kbp *Bam*HI-*Sma*I fragment which includes the *vnfEN* genes	35
Phages		
M13mp18 and M13mp19	M13 cloning vectors	53
λTn*5*-B21	λ*b*221 *c*I857P am80 containing Tn*5*-B21	45

[a] See genotype of DJ42 for complete description of DJ42 mutation.

In this article, we present the complete nucleotide sequence of a second copy of *nifENX*-like genes cloned from *A. vinelandii*. These genes are designated *vnfE*, *vnfN*, and *vnfX* and are located immediately downstream from the regulatory gene *vnfA* (24).

(A preliminary report of this study has been presented elsewhere [52].)

MATERIALS AND METHODS

Plasmid and bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1. The *A. vinelandii* strains were grown at 30°C in modified Burk medium (48). For growth studies under Mo-deficient conditions and in the presence of V, precautions were taken to minimize contamination by metals as previously described (4). Na_2MoO_4 and V_2O_5 were each added to a final concentration of 1 μM. Fixed N was added as ammonium acetate (2.2-mg/ml final concentration). When required, kanamycin and tetracycline were added to a final concentration of 10 μg/ml. The concentration of ampicillin was 50 μg/ml. *Escherichia coli* K-12 71-18 was maintained on M9 minimal medium and grown in tryptone-yeast extract (TYE) or Luria-Bertani (LB) medium for transformation or infection. *E. coli* S17-1 and JC5466 were cultured and maintained in TYE or LB medium.

λTn*5*-B21 (45) was propagated on *E. coli* C600. This strain was grown in Y broth (10 g of Bacto Tryptone per liter, 2.5 g of NaCl per liter, and 0.1 g of yeast extract per liter). Maltose was added to a concentration of 0.2%. When necessary, kanamycin and tetracycline were added to concentrations of 50 and 20 μg/ml, respectively.

DNA manipulations. DNA isolation procedures, ligations, restriction analysis, and transformations were carried out essentially as described by Maniatis et al. (29). *A. vinelandii* cells were made competent and transformed with DNA as described by Page and von Tigerstrom (34).

DNA sequencing and sequence analysis. A 4.5-kbp *Kpn*I fragment contiguous with the 9-kbp *Eco*RI-*Kpn*I fragment containing *vnfA* and the N terminus of the *vnfE* gene (24) was isolated, and the nucleotide sequence was determined by the method of Sanger et al. (40). The sequencing strategy involved the sequence determination of overlapping cloned fragments generated with *Alu*I, *Hae*III, *Rsa*I, *Sau*3A, or *Tha*I. In addition, a 1.4-kbp *Sma*I fragment spanning the *Kpn*I junction was cloned into M13mp18, and a primer consisting of a 15-bp sequence (AATGCGCAGGCCGAG) located near the juncture was used to confirm contiguity at this site. Individual sequences were aligned and organized into a contiguous sequence with the aid of a sequence alignment program (27). Restriction sites, base and codon preference analyses, and amino acid sequence alignments (GAP program) were performed on a VAX computer using the Genetics Computer Group (GCG) programs (17).

Construction of *A. vinelandii* mutants carrying an interposon insertion in *vnfE*. Plasmid pLWE2, which contains a 1.4-kbp *Sma*I fragment (isolated from pAU48 [35]) spanning the *Kpn*I site (position 750 in Fig. 1), was cleaved with *Kpn*I, resulting in linearization of the plasmid. The linearized

plasmid was combined with a kanamycin resistance (Kan^r) cartridge (released from pKISS by *Kpn*I digestion). After ligation and transformation into *E. coli* K-12 71-18, plasmid DNA was purified from Kan^r Amp^r (ampicillin-resistant) colonies. *A. vinelandii* CA was transformed with plasmid DNA (pLWE2-1) from one of these transformants. After selection for Kan^r and screening for Amp^s, one of the *A. vinelandii* transformants was selected for further study and designated strain CA48. Strain DJ42 (Δ*nifEN*) was also transformed with pLWE2-1 plasmid DNA. This double mutant was designated strain DJ42.48. The presence of the interposon insertions in *vnfE* was confirmed by Southern hybridization.

Construction of *A. vinelandii* mutants carrying Tn*5*-B21 insertions. Plasmid pLWNX2 was transformed into *E. coli* S17-1 (suppressor negative). The transformed strain was grown to a cell density of approximately 10^9 cells per ml (A_{600} = 0.8). The cells were harvested and resuspended in 0.01 M $MgSO_4$ solution. Four hundred microliters of λTn*5*-B21 (45) phage suspension (titer, 10^9 PFU/ml) was added to 200 μl of the transformed cell suspension, and the resulting mixture was incubated at 37°C for 2 h. The infected cells were plated onto LB medium containing tetracycline (20 μg/ml). After an overnight incubation, tetracycline-resistant (Tet^r) cells were harvested from the plate cultures, and plasmid DNA was then isolated from these cells. *E. coli* JC5466 was transformed with this plasmid DNA preparation, and Tet^r transformants were selected. Plasmid DNA was isolated from individual colonies. The approximate location and orientation of Tn*5*-B21 insertions in the individual plasmids were determined by restriction enzyme analysis. *A. vinelandii* CA was transformed with one of these plasmids (pWWB12), and Tet^r Amp^s colonies were selected. One of the transformants (strain CA47), which contained Tn*5*-B21 inserted in *vnfN*, was selected for further study. *A. vinelandii* CA66 was transformed with CA47 DNA, and the resulting transformant was designated strain CA66.47.

Nucleotide sequence accession number. The GenBank accession number of the nucleotide sequence of the *vnfENX* region presented in this article is M74768.

RESULTS AND DISCUSSION

Nucleotide sequence analysis. We previously reported the nucleotide sequence of the genomic region encoding the putative regulatory protein VnfA (24). A partial sequence of an open reading frame (ORF) which appeared to be a *nifE*-like gene was found to be located immediately downstream from the *vnfA* gene (Fig. 1). The complete nucleotide sequence of this ORF (*vnfE*) along with those of two additional ORFs (*vnfN* and *vnfX*) is shown in Fig. 2. These three genes appear to be part of an operon with the order promoter-*vnfENX*. The identification and designation of these ORFs as *vnfENX* is based on mutant phenotypes and the similarity of these ORFs and their predicted products with those of the *nifENX* genes from *A. vinelandii*. Comparisons of the *nif* and *vnf* genes at the nucleotide level are as follows: *nifE* (1,416 bp) and *vnfE* (1,410 bp) are 75% identical, *nifN* (1,400 bp) and *vnfN* (1,383 bp) are 65% identical, and *nifX* (478 bp) and *vnfX* (541 bp) are 44% identical. Base and codon preference analyses of the region following *vnfX* indicate the possible presence of another ORF following *vnfENX* which may or may not be a part of the *vnfENX* cluster (data not shown).

Although the gene order of the *vnfENX* operon is the same as that of the *nifENX* operon, these gene clusters differ as to

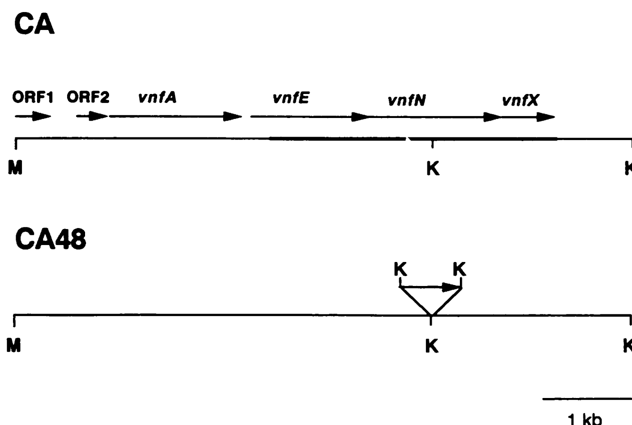

FIG. 1. The *vnfENX* region. The heavier line indicates the region whose nucleotide sequence is given in Fig. 2. Restriction sites: M, *Sma*I; K, *Kpn*I. The triangle in the CA48 map indicates the location of a Kan^r cartridge inserted into the *Kpn*I restriction site; arrows indicate the direction of transcription of the neomycin phosphotransferase gene.

location within their respective regulons. The *nifENX* genes are just downstream of the gene cluster *nifHDKTY*, while the *vnfENX* genes are located about 8 kb upstream from the *vnfHorfFd* operon (35). The *vnfENX* cluster is preceded by a sequence (TTGGN$_8$TTGCT) that conforms to an RpoN-dependent promoter (3), and each gene in the cluster is preceded by a potential ribosome-binding site (Fig. 1). The *vnfE* and *vnfN* genes are separated by one nucleotide, while the stop codon of *vnfN* and start codon of *vnfX* overlap; therefore, translation of mRNA from these genes may be coupled (9). The *nifEN* genes also appear to be translationally coupled (9, 14).

Comparison of predicted amino acid sequences with those of other gene products. The amino acid sequences of the presumed products of *vnfE*, *vnfN*, and *vnfX* show significant identity with those of the *nifE*, *nifN*, and *nifX* gene products, respectively (Fig. 3). The predicted molecular weights and pIs of the *vnf* and *nif* gene products, as calculated by the GCG PeptideMap program, are shown in Table 2. The amino acid sequences of NifE and VnfE are 66% identical, while those of NifN and VnfN are 52% identical. This identity is much less than that observed between NifH and VnfH (91%) but is more than those between NifD and VnfD (33%) and NifK and VnfK (31.1%).

On the basis of the identity between the *nifD* and *nifE* gene products as well as the identity between the *nifK* and *nifN* gene products, it has been suggested that the *nifE* and *nifN* genes share an ancestral relationship with the *nifD* and *nifK* genes (1, 2, 9, 14, 32, 41). When VnfE is compared with NifD, VnfD, and AnfD (30, 27, and 26% identities, respectively), it is found to have slightly more identity with NifD than with the other two products. Comparison of VnfN with NifK, VnfK, and AnfK (27, 28, and 27% identities, respectively) shows that VnfN has slightly more identity with VnfK than with NifK or AnfK. This identity, however, is substantially less than the identity between NifEN and VnfEN. It therefore appears that *vnfE* and *vnfN* are most closely related to *nifE* and *nifN* in an evolutionary sense. The NifE and NifN proteins could share a functional relationship with the NifD and NifK proteins, since it is thought that the NifE-NifN protein complex may be required to bind FeMoco before it is donated to apodinitrogenase-1 (9, 14, 36, 37).

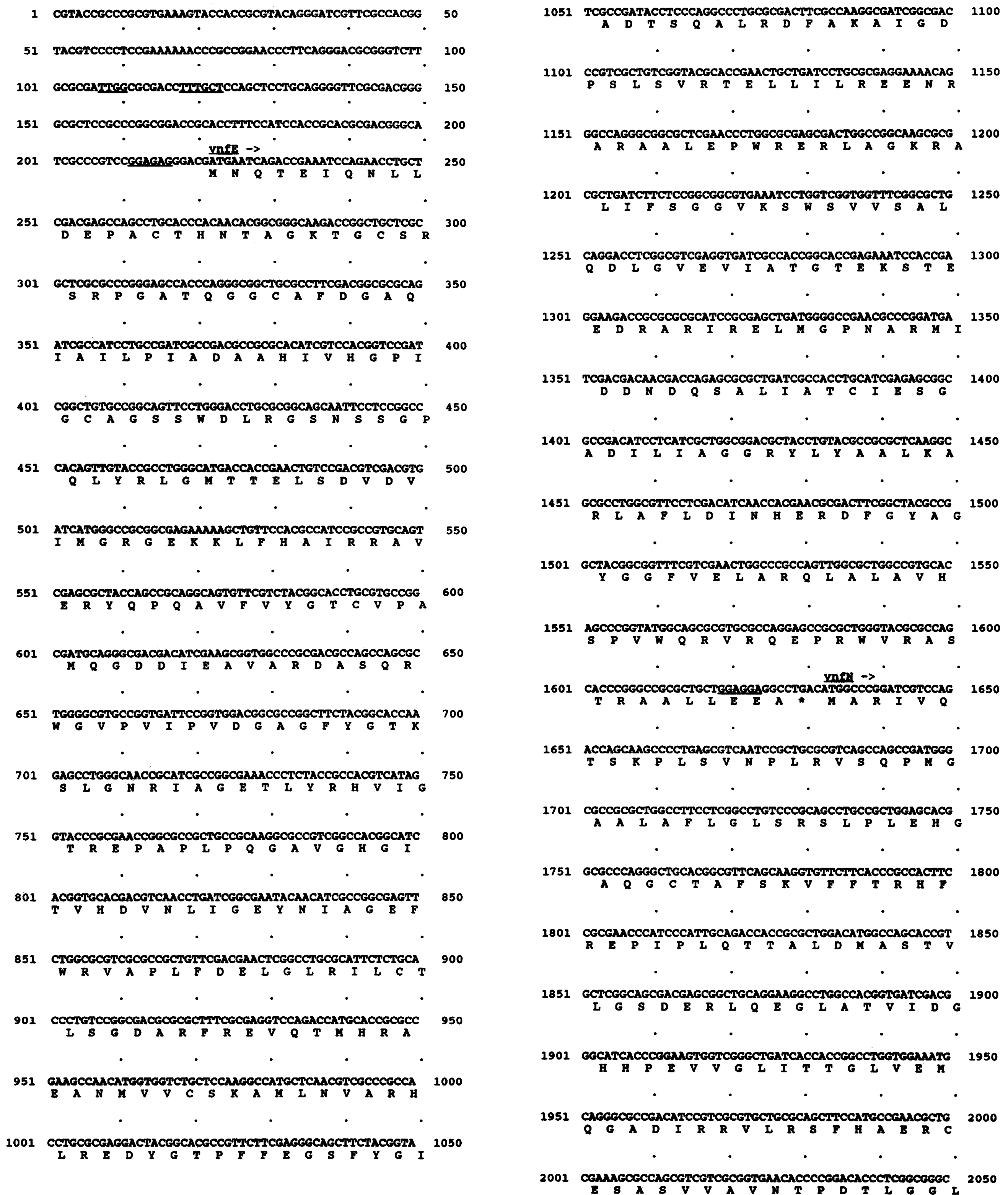

FIG. 2. Nucleotide sequence of the region containing *vnfENX*. The predicted translation product of this gene is given below the nucleotide sequence. Sites similar to ribosome-binding sites from *E. coli*, a potential *ntrA*-dependent promoter, and the stop codon of *vnfN* are underlined. Nucleotide 1 corresponds to nucleotide 2659 in the previously reported sequence of the *vnfA* region (24).

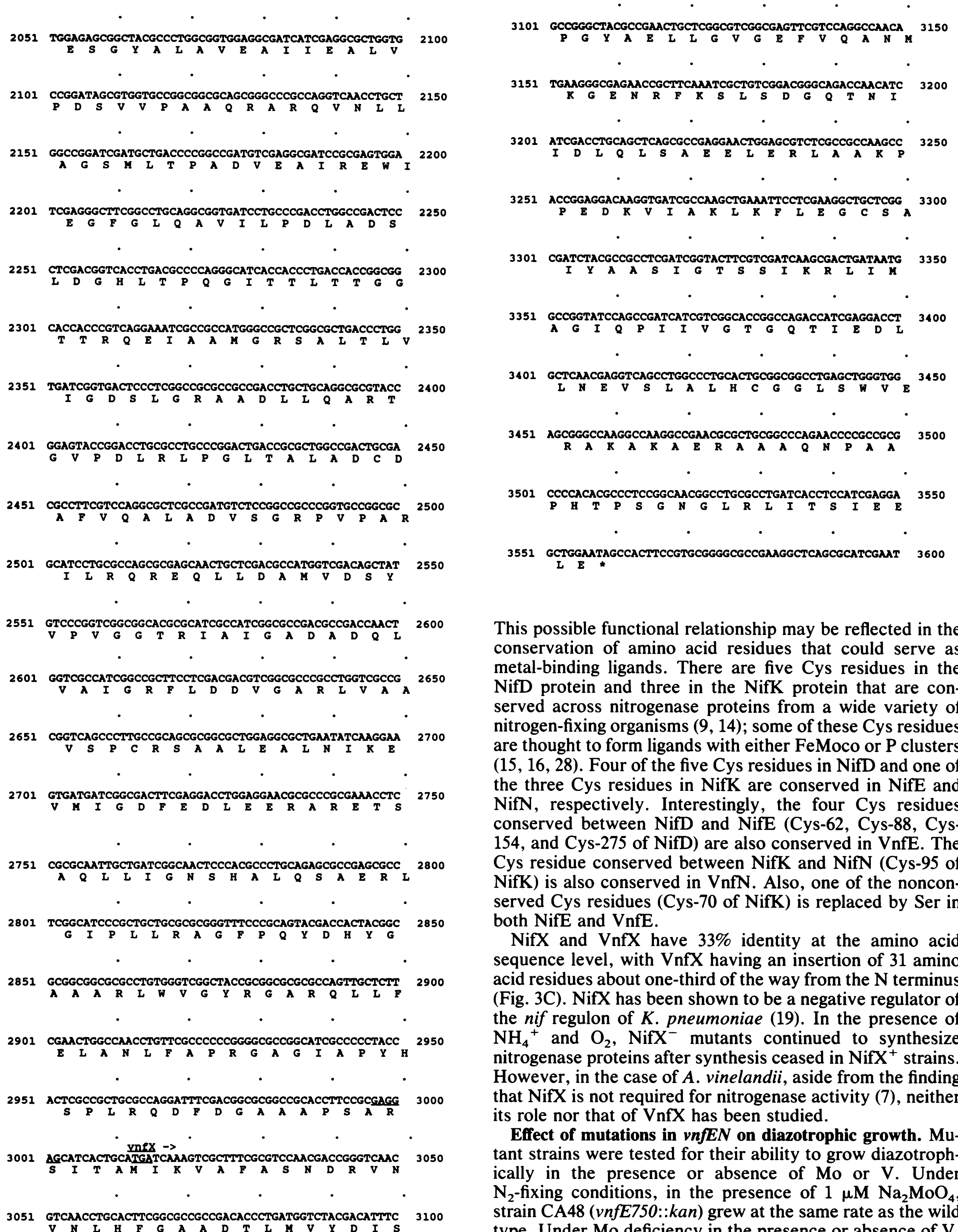

FIG. 2—*Continued.*

This possible functional relationship may be reflected in the conservation of amino acid residues that could serve as metal-binding ligands. There are five Cys residues in the NifD protein and three in the NifK protein that are conserved across nitrogenase proteins from a wide variety of nitrogen-fixing organisms (9, 14); some of these Cys residues are thought to form ligands with either FeMoco or P clusters (15, 16, 28). Four of the five Cys residues in NifD and one of the three Cys residues in NifK are conserved in NifE and NifN, respectively. Interestingly, the four Cys residues conserved between NifD and NifE (Cys-62, Cys-88, Cys-154, and Cys-275 of NifD) are also conserved in VnfE. The Cys residue conserved between NifK and NifN (Cys-95 of NifK) is also conserved in VnfN. Also, one of the nonconserved Cys residues (Cys-70 of NifK) is replaced by Ser in both NifE and VnfE.

NifX and VnfX have 33% identity at the amino acid sequence level, with VnfX having an insertion of 31 amino acid residues about one-third of the way from the N terminus (Fig. 3C). NifX has been shown to be a negative regulator of the *nif* regulon of *K. pneumoniae* (19). In the presence of NH_4^+ and O_2, $NifX^-$ mutants continued to synthesize nitrogenase proteins after synthesis ceased in $NifX^+$ strains. However, in the case of *A. vinelandii*, aside from the finding that NifX is not required for nitrogenase activity (7), neither its role nor that of VnfX has been studied.

Effect of mutations in *vnfEN* on diazotrophic growth. Mutant strains were tested for their ability to grow diazotrophically in the presence or absence of Mo or V. Under N_2-fixing conditions, in the presence of 1 μM Na_2MoO_4, strain CA48 (*vnfE750::kan*) grew at the same rate as the wild type. Under Mo deficiency in the presence or absence of V, strain CA48 exhibited a slightly extended lag period after which the growth rate approached that of the wild type.

```
A
        1                                                50
AvNifE  MKAKDIAELLDEPACSHNKKEKSGCAKPKPGATDGRCSFDGAQIALLPVA
AvVnfE  MNQTEIQNLLDEPACTHNTAGKTGCSRSRPGATQGGCAFDGAQIAILPIA

        51                                              100
AvNifE  DVAHIVHGPIACAGSSWDNRGTRSSGPDLYRIGMTTDLTENDVIMGRAEK
AvVnfE  DAAHIVHGPIGCAGSSWDLRGSNSSGPQLYRLGMTTELSDVDVIMGRGEK

        101                                             150
AvNifE  RLFHAIRQAVESYLPPAVFVYNTCVPALIGDDVDAVCKAAAERFGTPVIP
AvVnfE  KLFHAIRRAVERYQPQAVFVYGTCVPAMQGDDIEAVARDASQRWGVPVIP

        151                                             200
AvNifE  VDSAGFYGTKNLGNRIAGEAMLKYVIGTREPDPLPVGSERPGIRVHDVNL
AvVnfE  VDGAGFYGTKSLGNRIAGETLYRHVIGTREPAPLPQGAVGHGITVHDVNL

        201                                             250
AvNifE  IGEYNIAGEFWHVLPLLDELGLRVLCTLAGDARYREVQTMHRAEVNMMVC
AvVnfE  IGEYNIAGEFWRVAPLFDELGLRILCTLSGDARFREVQTMHRAEANMVVC

        251                                             300
AvNifE  SKAMLNVARKLQETYGTPWFEGSFYGITDTSQALRDFARLLDDPDLTART
AvVnfE  SKAMLNVARHLREDYGTPFFEGSFYGIADTSQALRDFAKAIGDPSLSVRT

        301                                             350
AvNifE  EALIAREEAKVRAALEPWRARLEGKRVLLYTGGVKSWSVVSPLQDLGMKV
AvVnfE  ELLILREENRARAALEPWRERLAGKRALIFSGGVKSWSVVSALQDLGVEV

        351                                             400
AvNifE  VATGTKKSTEEDKARIRELMGDDVKMLDEGNARVLLKTVDEYQADILIAG
AvVnfE  IATGTEKSTEEDRARIRELMGPNARMIDDNDQSALIATCIESGADILIAG

        401                                             450
AvNifE  GRNMYTALKGRVPFLDINQEREFGYGGYDRMLELVRHVCITLECPVWEAV
AvVnfE  GRYLYAALKARLAFLDINHERDFGYAGYGGFVELARQLALAVHSPVWQRV

        451                     475
AvNifE  RRPAPWDIPASQDARPSGGPFGER*
AvVnfE  RQEPRWVRASTRAALLEEA*.....

B
        1                                                50
AvNifN  MAEIINRNKALAVSPLKASQTMGAALAILGLALSMPLFHGSQGCTAFAKV
AvVnfN  MARIVQTSKPLSVNPLRVSQPMGAALAFLGLSRSLPLEHGAQGCTAFSKV

        51                                              100
AvNifN  FFVRHFREPVPLQTTAMDQVSSVMGADENVVEALKTICERQNPSVIGLLT
AvVnfN  FFTRHFREPIPLQTTALDMASTVLGSDERLQEGLATVIDGHHPEVVGLIT

        101                                             150
AvNifN  TGLSETQGCDLHTALHEFRTQYEEYKDVPIVPVNTPDFSGCFESGFAAAV
AvVnfN  TGLVEMQGADIRRVLRSFHA..ERCESASVVAVNTPDTLGGLESGYALAV

        151                                             200
AvNifN  KAIVETLVPERRDQVGKRPRQVNVLCSANLTPGDLEYIAESIESFGLRPL
AvVnfN  EAIIEALVPDSVVPAAQRARQVNLLAGSMLTPADVEAIREWIEGFGLQAV

        201                                             250
AvNifN  LIPDLSGSLDGHLDENRFNALTTGGLSVAELATAGQSVATLVVGQSLAGA
AvVnfN  ILPDLADSLDGHLTPQGITTLTTGGTTRQEIAAMGRSALTLVIGDSLGRA

        251                                                  300
AvNifN  ADALAERTGVPDRRFGMLYGLDAVDAWLMALAEISGNPVPDRYKRQRAQL
AvVnfN  ADLLQARTGVPDLRLPGLTALADCDAFVQALADVSGRPVPARILRQREQL

        301                                                  350
AvNifN  QDAMLDTHFMLSSARTAIAADPDLLLGFDALLRSMGAHTVAAVVPARAAA
AvVnfN  LDAMVDSYVPVGGTRIAIGADADQLVAIGRFLDDVGARLVAAVSPCRSAA

        351                                                  400
AvNifN  LVDSPLPSVRVGDLEDLEHAARAGQAQLVIGNSHALASARRLGVPLLRAG
AvVnfN  LEALNIKEVMIGDFEDLEERARETSAQLLIGNSHALQSAERLGIPLLRAG

        401                                                  450
AvNifN  FPQYDLLGGFQRCWSGYRGSSQVLFDLANLLVEHHQGIQPYHSIYAQKPA
AvVnfN  FPQYDHYGAAARLWVGYRGARQLLFELANLFAPRGAGIAPYHSPLRQDFD

        451     463
AvNifN  TEQPQWRH*
AvVnfN  GAAAPSARSITA*

C
        1                                                50
AvNifX  MSSPTRQLQVLDSEDDGTLLKVAFASSDRELVDQHFGSSRSFAIYGVNPE
AvVnfX                    MIKVAFASNDRVNVNLHFGAADTLMVYDISPG

        51                                              100
AvNifX  RSQLLSVVEFGELEQDGN...............................E
AvVnfX  YAELLGVGEFVQANMKGENRFKSLSDGQTNIIDLQLSAEELERLAAKPPE

        101                                             150
AvNifX  DKLARKIDLLDGCVAVYCCACGASAVRQLMAIGVQPIKVSEGARIAELIE
AvVnfX  DKVIAKLKFLEGCSAIYAASIGTSSIKRLIMAGIQPIIVGTGQTIEDLLN

        151                                             200
AvNifX  ALQVELREGPSAWLAK.......AIQRTRGPDMRRFDAMAAEGWDE*
AvVnfX  EVSLALHCGGLSWVERAKAKAERAAAQNPAAPHTPSGNGLRLITSIEELE

        201
AvNifX
AvVnfX  *
```

FIG. 3. Comparison of the predicted amino acid sequences of the products of the *vnfENX* region with those of the *nifENX* region of *A. vinelandii* (Av). (A) NifE compared with VnfE; (B) NifN compared with VnfN; (C) NifX compared with VnfX.

TABLE 2. Predicted molecular weights and isoelectric points (pIs) of VnfE, VnfN, VnfX, NifE, NifN, and NifX

Gene	No. of amino acids	Calculated mol wt of predicted protein	Estimated pI
vnfE	469	51,089	6.58
nifE	474	52,162	6.16
vnfN	460	48,701	5.24
nifN	458	49,207	5.92
vnfX	182	19,447	5.14
nifX	158	17,289	4.47

Strain CA47, which contains Tn*5*-B21 inserted in *vnfN*, exhibited the same growth pattern as did CA48. In order to test the possibility that the *nifEN* gene products substitute for the missing *vnfEN* products in strains CA47 and CA48, we constructed a double mutant that is inactivated for both *nifEN* and *vnfEN*. This strain, DJ42.48 (Δ*nifEN vnfE750*::*kan*), was unable to grow under any of the nitrogen-fixing conditions tested. These results suggest that *A. vinelandii* probably does not have a third set of *nifEN*-like genes and that the *vnfEN* products function in the synthesis of cofactors for both dinitrogenase-2 and dinitrogenase-3. Since strain DJ42 (Δ*nifEN*) is Nif^- (but is Vnf^+ Anf^+), the products of *vnfEN* are apparently unable to substitute for those of *nifEN*. However, on the basis of results with strains CA47 and CA48, the *nifEN* gene products appear to function in place of the *vnfEN* products when these genes are mutationally inactivated. This suggests that NifE and NifN participate in the synthesis of FeVaco for dinitrogenase-2 and in the formation of the putative cofactor for dinitrogenase-3 when VnfE and VnfN are unavailable.

Expression of a *vnfN*::*lacZ* transcriptional fusion. Expression of the *vnfENX* transcriptional unit was monitored with a transcriptional *lac* fusion constructed by inserting Tn*5*-B21 (45) in *vnfN*. The results of two independent experiments for each strain are shown in Table 3. Strain CA47 (*vnfN47*::Tn*5*-B21) accumulated β-galactosidase in the presence of V as well as under Mo- and V-deficient conditions. These results indicate that the *vnfENX* genes are transcribed under conditions where either of the two alternative nitrogenases

TABLE 3. Expression of the *vnfN-lacZ* fusion (*vnfN47*::Tn*5*-B21)

Strain (phenotype)	Medium[a]	β-Galactosidase activity (%)[b]
CA47	−Mo, −V	3,950 ± 505 (88.0)
	+V	4,491 ± 1,006 (100.0)
	+Mo	439 ± 191 (9.8)
	$+NH_4^+$	2,149 ± 462 (47.9)
CA66.47 ($AnfA^-$)	−Mo, −V	3,701 ± 690 (82.4)
	+V	4,384 ± 547 (97.6)
	+Mo	353 ± 102 (7.9)
	$+NH_4^+$	1,794 ± 994 (40.0)

[a] Cells were cultured in N-free modified Burk medium without added Mo and V (−Mo, −V), with 1 μM V_2O_5 (+V), or with 1 μM Na_2MoO_4 (+Mo) or in modified Mo- and V-deficient Burk medium containing ammonium acetate (2.2 mg/ml) ($+NH_4^+$).

[b] β-Galactosidase activities are expressed in units as described by Miller (31). Results represent the mean ± standard deviation of activity values from two independent experiments, with percentages of the mean activity found in cells of strain CA47 derepressed for 12 h in modified N-free Burk medium containing 1 μM V_2O_5 given in parentheses.

are expressed. This strain also accumulated appreciable amounts of β-galactosidase when normally repressive amounts of NH_4^+ were present in the medium. Since NH_4^+ repression of *nifHDK* in *K. pneumoniae* is not as effective in $VnfX^-$ mutant strains as it is in wild-type strains (19), it is possible that polarity effects on *vnfX* lead to the lack of repression of the *vnfENX* genes by NH_4^+ observed in strain CA47. It is also possible that *vnfENX* transcripts are made in the presence of NH_4^+, as has been found for the *vnfHorfFd* and *vnfDGK* operons (21). In the case of these operons, repression by NH_4^+ appears to occur at the posttranscriptional level.

Walmsley et al. (51) used a transcriptional *lac* fusion similar to that carried by CA47 to show that VnfA is required for expression of the *vnfENX* operon. Since the *vnfN-lacZ* fusion in CA47 is also expressed under Mo-deficient conditions in the absence of V, we asked whether AnfA is also required for expression of this fusion. β-Galactosidase activities determined for the $AnfA^-$ mutant strain CA66.47 were approximately the same as those found for the $AnfA^+$ strain CA47 (Table 3); thus, AnfA does not appear to be necessary for expression of the *vnfENX* operon.

Conclusion. The complete DNA sequence for a second copy of *nifENX*-like genes (*vnfENX*) in *A. vinelandii* has been determined. There does not appear to be a third copy of *nifENX*-like genes in this organism, and the *vnfENX* genes appear to be expressed under Mo-deficient conditions in the presence or absence of V. This suggests that VnfE and VnfN are involved in the synthesis of cofactors for the alternative nitrogenases, nitrogenase-2 and nitrogenase-3. Furthermore, it appears that NifE and NifN can substitute for VnfE and VnfN in *vnfEN* mutants.

ACKNOWLEDGMENTS

We thank R. Simon for providing us with strains necessary for Tn*5*-B21 mutagenesis, C. Kennedy and J. Walmsley for help with the Tn*5*-B21 mutagenesis procedures, D. Dean for providing strain DJ42, and R. Pau for providing plasmid pAU48. We also thank R. Joerger and R. Pau for helpful discussions.

This study was supported by USDA competitive grant 88-37120-3872 and NATO travel grant 0532/88.

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