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Identification and Characterization of the *nifV-nifZ-nifT* Gene Region from the Filamentous Cyanobacterium *Anabaena* sp. Strain PCC 7120

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The *nifV* and *leuA* genes, which encode homocitrate synthase and α-isopropylmalate synthase, respectively, were cloned from the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 by a PCR-based strategy. Since the N-terminal parts of NifV and LeuA from other bacteria are highly similar to each other, a single pair of PCR primers was used to amplify internal fragments of both *Anabaena* strain 7120 genes. Sequence analysis of cloned PCR products confirmed the presence of two different *nifV*-like DNA fragments, which were subsequently used as *nifV*- and *leuA*-specific probes, respectively, to clone *Xba*I fragments of 2.1 kbp (pOST4) and 2.6 kbp (pOST2). Plasmid pOST4 carried the *Anabaena* strain 7120 *nifV-nifZ-nifT* genes, whereas pOST2 contained the *leuA* and *dapF* genes. The *nifVZT* genes were not located in close proximity to the main *nif* gene cluster in *Anabaena* strain 7120, and therefore *nifVZT* forms a second *nif* gene cluster in this strain. Overlaps between the *nifV* and *nifZ* genes and between the *nifZ* and *nifT* genes and the presence of a 1.8-kb transcript indicated that *nifVZT* might form one transcriptional unit. Transcripts of *nifV* were induced not only in a nitrogen-depleted culture but also by iron depletion irrespective of the nitrogen status. The *nifV* gene in *Anabaena* strain 7120 was interrupted by an interposon insertion (mutant strain BMB105) and by a plasmid integration via a single crossover with a *nifV* internal fragment as a site for recombination (mutant strain BMB106). Both mutant strains were capable of diazotrophic growth, and their growth rates were only slightly impaired compared to that of the wild type. Heterologous complementation of the *Rhodobacter capsulatus nifV* mutant R229I by the *Anabaena* strain 7120 *nifV* gene corroborated the assumption that *Anabaena* strain 7120 *nifV* also encodes a homocitrate synthase. In contrast, the *Anabaena* strain 7120 *leuA* gene did not complement the *nifV* mutation of R229I efficiently.

In the facultative anaerobic enterobacterium *Klebsiella pneumoniae*, 20 nitrogen fixation (*nif*) genes, which are clustered in one region of the chromosome, are involved in the process of N_2 reduction by the molybdenum-containing nitrogenase enzyme complex (2). The molybdenum nitrogenase is composed of the Fe protein, a dimer of identical subunits (NifH) containing a single [4Fe-4S] cluster, and the MoFe protein, a heterotetramer of two NifD and two NifK subunits including two iron-molybdenum cofactors (FeMo-co) and two P clusters (for reviews, see references 18 and 39). Biosynthesis of the FeMo-co requires the gene products of *nifH*, *nifQ*, *nifB*, *nifV*, *nifE*, and *nifN* (16, 35, 36). Hoover et al. (14, 15) have shown that homocitrate is an integral part of the FeMo-co and that *nifV* encodes a homocitrate synthase.

NifV exhibits similarity to LeuA from different organisms (reference 25 and references therein). In yeasts and fungi, homocitrate synthase carries out the condensation between acetyl coenzyme A and α-ketoglutarate, an analogous reaction to that catalyzed by LeuA (α-isopropylmalate synthase), which carries out the condensation between acetyl coenzyme A and α-ketoisovalerate.

Anabaena sp. strain PCC 7120 is a filamentous cyanobacterium capable of aerobic nitrogen fixation, which is carried out in specialized cells called heterocysts. Heterocysts provide the anaerobic environment necessary for the oxygen-sensitive process of N_2 fixation. Despite the conservation of many *nif* genes, some features of *nif* gene organization in *Anabaena* strain 7120 differ from those of *K. pneumoniae* and other diazotrophs. The main *nif* gene cluster in *Anabaena* strain 7120 heterocysts includes (in order) *nifB*, *fdxN*, *nifS*, *nifU*, *nifH*, *nifD*, *nifK*, *nifE*, *nifN*, *nifX*, ORF3, *nifW*, ORF1, ORF2, and *fdxH* (for reviews, see references 12 and 41). One of the *nif* genes essential in other diazotrophic organisms not previously found in cyanobacteria was *nifV*.

In this study, we identified the *Anabaena* strain 7120 *nifVZT* genes, which form a second *nif* gene cluster separated from the main *nif* gene cluster. The *nifVZT* gene region was characterized by transcriptional and mutational analysis. Furthermore, the *Anabaena* strain 7120 *nifV* gene was shown to complement a *nifV* mutation in *Rhodobacter capsulatus.*

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Anabaena* strain 7120 was grown at 30°C in BG11 medium (30) containing either 17.7 mM $NaNO_3$ or 5 mM NH_4NO_3 as the nitrogen source. Cultures were illuminated with white fluorescent light at 100 microeinsteins m^{-2} s^{-1} and bubbled with air enriched to 1% CO_2. Nitrogen-fixing cultures were grown in $BG11_0$ medium, in which combined nitrogen was omitted. Standard BG11 medium (iron replete) contained 30 μM ferric ammonium citrate; for iron-deficient cultures, 0.9 μM ferric ammonium citrate was added. For *Anabaena* strain 7120 BMB105 and BMB106 mutants, neomycin was added to a final concentration of 50 μg/ml. The growth conditions, media, and antibiotic concentrations used for *Escherichia coli* and *R. capsulatus* strains were described previously (19, 24, 26, 27).

DNA techniques. Preparation of genomic or plasmid DNA and cloning procedures were carried out by standard techniques (31, 33). PCR amplification was carried out with an Eppendorf MicroCycler E and *Taq* polymerase (Gibco). Oligonucleotides synthesized in a Pharmacia LKB Gene Assembler Plus were

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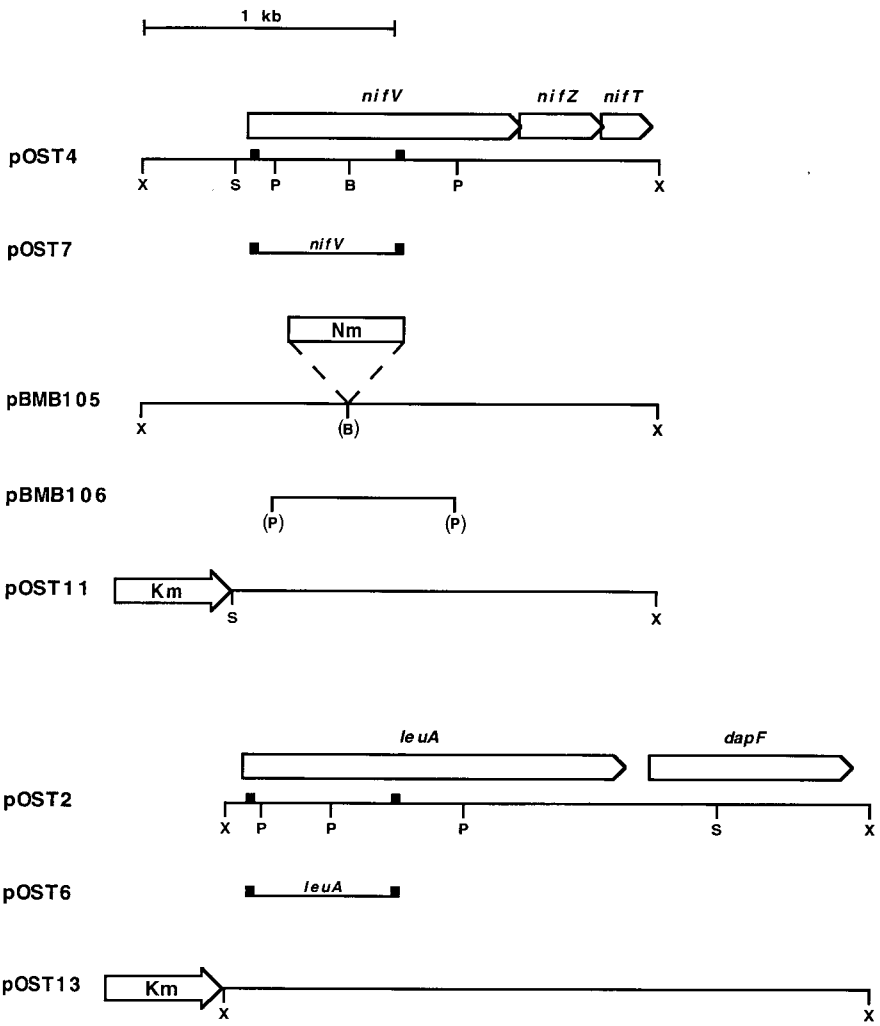

FIG. 1. Physical and genetic maps of the *Anabaena* strain 7120 *nifV* and *leuA* gene regions. The physical maps are given for the enzymes *Xba*I (X), *Spe*I (S), *Pvu*I (P), and *Bcl*I (B). The nucleotide sequence of the 2.1-kbp *Xba*I fragment carrying *nifV-nifZ-nifT* is shown in Fig. 2. Solid squares mark the approximate locations of the synthetic oligonucleotides used for PCR amplification of internal fragments of *nifV* (pOST7) and *leuA* (pOST6), respectively. The neomycin (Nm) and kanamycin cassettes (Km) in plasmids pBMB105, pOST11, and pOST13 are not drawn to scale. In pOST11 and pOST13, the kanamycin resistance gene was used to drive transcription of *nifV* and *leuA*, respectively, in *R. capsulatus.*

used as primers in PCRs. Two *nifV*-specific oligonucleotides were designed on the basis of sequences conserved between the NifV proteins from *K. pneumoniae* (2), *Azotobacter vinelandii* (17), and *R. capsulatus* (23). The degenerate oligonucleotides OST2 (5′-GA[C/T]AC[A/T/C]AC[A/T/C][C/T]T[A/G]CG[C/T]GA[C/T]GG[C/T]GAACA-3′) and OST1 (5′-GT[A/G]TT[A/T/C]GC[G/A/T]GT[A/T/C]GCCAT[G/A/T]CC[A/G]AA[A/G]TC-3′) correspond to positions 8 to 16 (DTTLRDGEQ) and 197 to 205 (DLGMATANT) of *K. pneumoniae* NifV, respectively. Nucleotide sequencing was performed for both DNA strands by the chain termination method (32). DNA sequence analyses were done with the BLAST programs (1) in the nonredundant database.

Construction of *Anabaena* strain 7120 BMB105 and BMB106 mutants. The *Anabaena* strain 7120 *nifV* gene was interrupted by interposon insertion. For this purpose, the 2,061-bp *Xba*I fragment carrying the entire *nifV* gene was cloned into pSUP202 (38) and then the neomycin resistance gene of pRL648 (C.K3 [8]) was inserted into the *Bcl*I site within *nifV*, resulting in hybrid plasmid pBMB105 (see Fig. 1). Plasmid pBMB105 was introduced into *Anabaena* strain 7120 by triparental mating (7), and exconjugants were selected for neomycin resistance. Loss of the vector-encoded chloramphenicol resistance indicated marker rescue via double crossover. One chloramphenicol-sensitive mutant chosen for further studies was designated BMB105. Marker rescue and complete segregation in BMB105 were confirmed by Southern blot hybridization (data not shown).

In addition, the *nifV* gene was inactivated by plasmid integration as described for *Anabaena* strain 7120 *xisF* and *nifJ* mutants (3, 5). For this purpose, the internal 728-bp *Pvu*I fragment of *nifV* (see Fig. 2) was cloned into a pSUP202 derivative carrying the neomycin resistance gene of pRL648 (C.K3), resulting in hybrid plasmid pBMB106 (8, 38). The *nifV* fragment lacks 85 bp of the 5′ sequence and 318 bp of the 3′ sequence of the *nifV* coding region. Plasmid pBMB106 was introduced into *Anabaena* strain 7120 by triparental mating (7), and exconjugants were selected for neomycin resistance. A single homologous recombination event between pBMB106, which does not replicate in *Anabaena* strain 7120, and the chromosomal copy of *Anabaena* strain 7120 *nifV* results in plasmid integration into the chromosome, creating two incomplete copies of the *nifV* gene. One neomycin-resistant exconjugant chosen for further studies was designated BMB106.

RNA techniques. RNA from *Anabaena* strain 7120 was extracted from 1 g (wet weight) of cells (10). A 30-μg sample of each RNA sample was glyoxalylated and separated in a 1.0% agarose gel. Transfer of RNA to Hybond-N membranes was carried out in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization of the probes and subsequent washing steps were performed at 65°C (4).

Nucleotide sequence accession number. The EMBL accession number for the DNA sequence reported in this paper is X99902.

RESULTS AND DISCUSSION

Identification and cloning of the *Anabaena* strain 7120 *nifV* and *leuA* genes. The N-terminal parts of NifV (homocitrate synthase) and LeuA (α-isopropylmalate synthase) from differ-

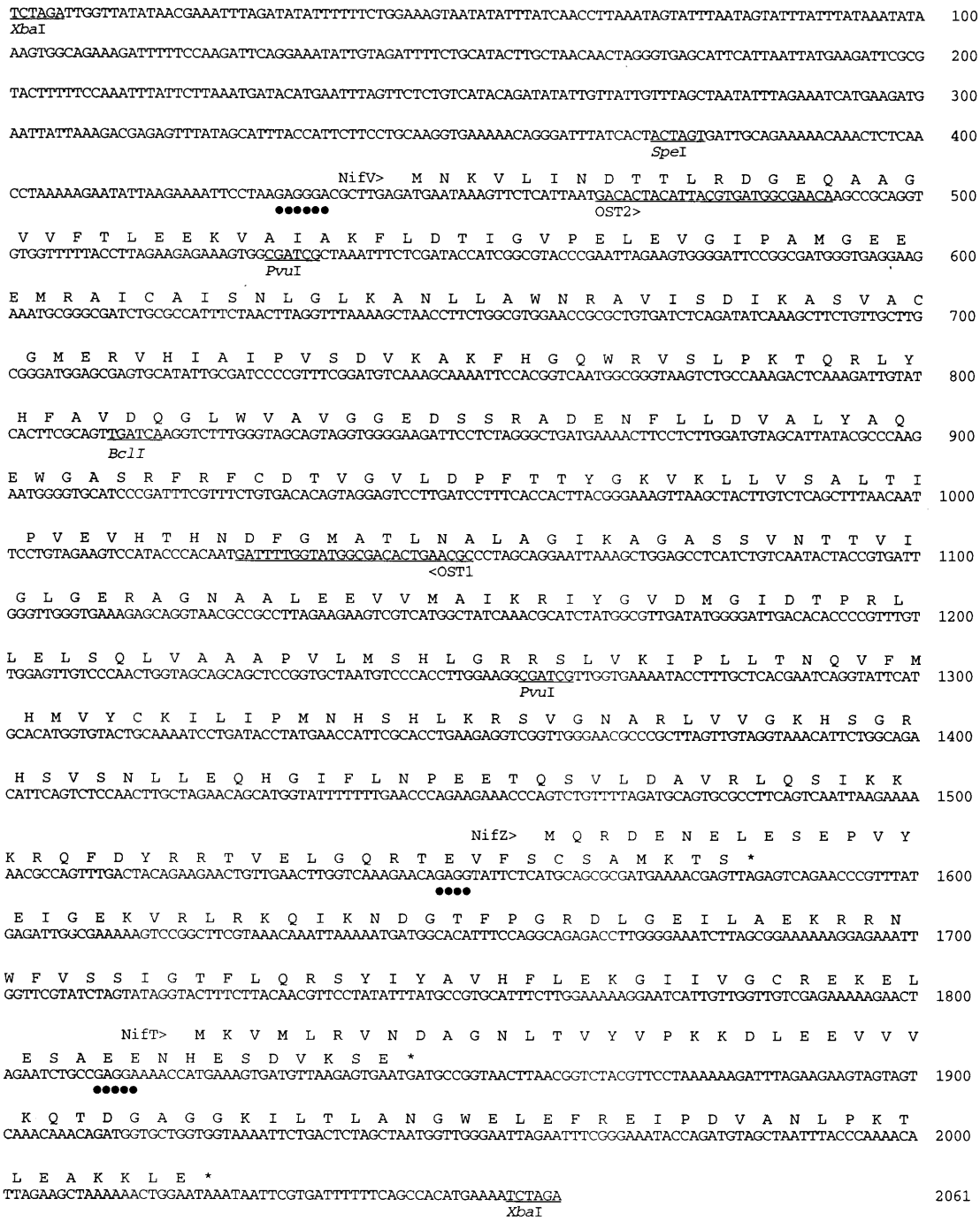

FIG. 2. Nucleotide sequence of the *Anabaena* strain 7120 *nifV-nifZ-nifT* gene region. Predicted amino acid sequences given in the single-letter code indicate overlapping of the *nifV* and *nifZ* genes and the *nifZ* and *nifT* genes. Putative ribosomal binding sites are marked by dots. Synthetic oligonucleotides OST2 and OST1 (see Materials and Methods) used for PCR amplification are underlined.

ent organisms show strong similarity (25). One pair of primers for PCR amplification was designed on the basis of sequences conserved between the NifV proteins from different diazotrophic bacteria (for details, see Materials and Methods), which were used to identify both the *nifV* and *leuA* genes from *Anabaena* strain 7120. PCR amplification resulted in DNA fragments of the expected size of approximately 0.6 kbp, which were cloned into the *Sma*I site of vector plasmid pUC19. Plasmid DNAs of four individual clones were subjected to DNA sequence analysis. Two hybrid plasmids contained the same internal fragment of the *nifV* gene, and one of these plasmids chosen for further studies was designated pOST7. The other two hybrid plasmids, one of which was called pOST6, contained the same internal fragment of *leuA*.

Southern blot analysis of *Anabaena* strain 7120 total DNA digested with different restriction enzymes, using pOST7 and pOST6 as probes, was first carried out under stringent hybridization conditions. We found a 2.1-kbp *Xba*I fragment hybrid-

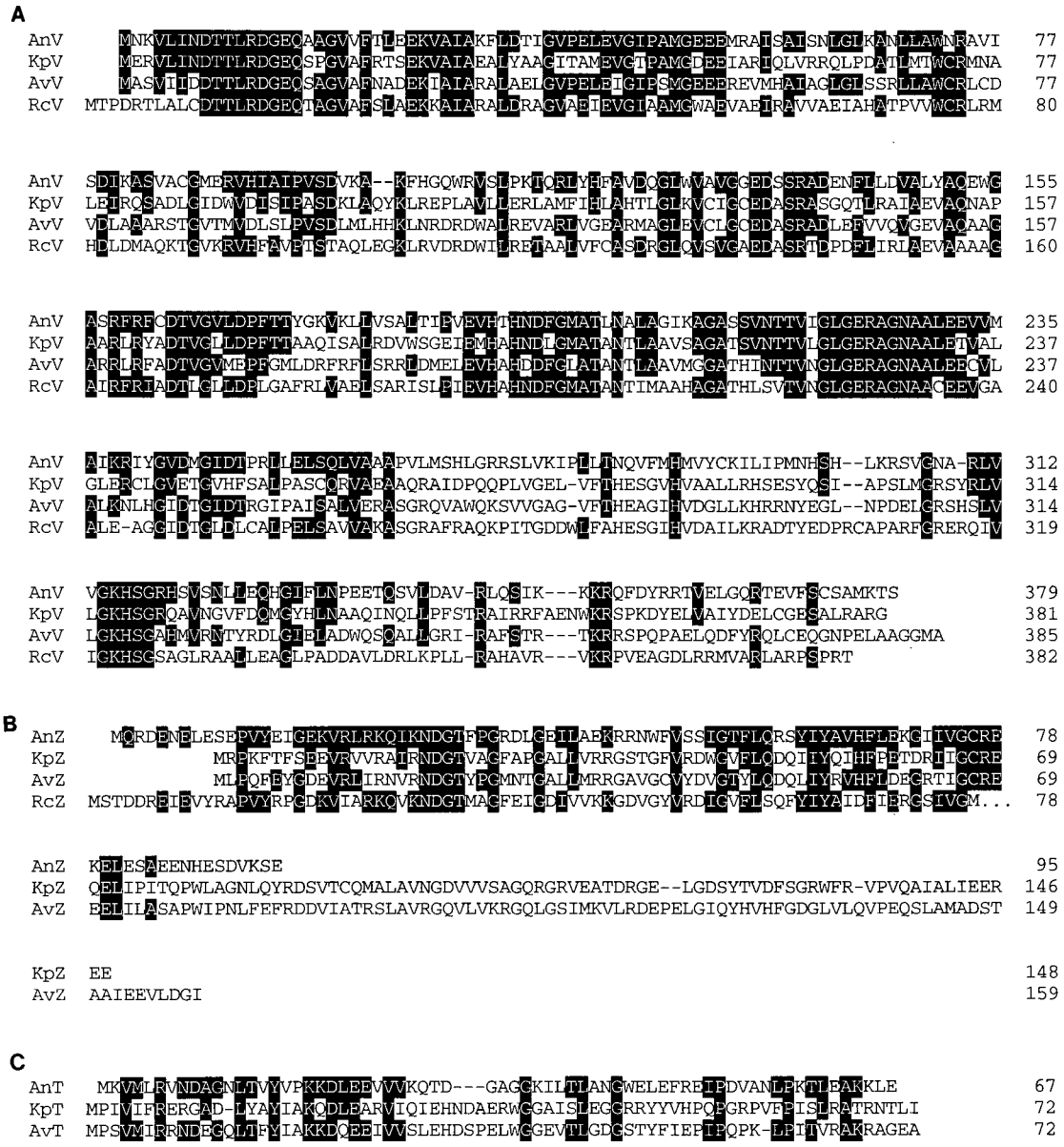

FIG. 3. Comparison of the deduced *nifV* (A), *nifZ* (B), and *nifT* (C) gene products of *Anabaena* strain 7120 (An) to corresponding proteins from *K. pneumoniae* (Kp), *A. vinelandii* (Av), and *R. capsulatus* (Rc). The amino acid sequences are aligned for maximum matching, and amino acid residues identical to those in *Anabaena* strain 7120 are emphasized by inverse lettering. The DNA sequence of the 3′ end of the *R. capsulatus nifZ* gene has not yet been determined; therefore, only the N-terminal part of NifZ is shown in panel B.

izing to the *nifV*-specific probe (pOST7) and a 2.6-kbp *Xba*I fragment showing homology to the *leuA*-specific probe (pOST6). Hybrid plasmids carrying the 2.1- or 2.6-kbp *Xba*I fragment were isolated from a size-fractionated *Xba*I gene bank in pUC18 and designated pOST4 (*nifV*) and pOST2 (*leuA*), respectively. The physical and genetic maps of pOST4 and pOST2 are shown in Fig. 1.

In addition to the strongly hybridizing 2.1-kbp *Xba*I fragment, weakly hybridizing *Xba*I fragments of 2.6 and >12 kbp were detected by the *nifV*-specific probe under nonstringent hybridization conditions (data not shown). Southern blot analysis of *Anabaena* strain 7120 total DNA digested with *Xba*I-*Eco*RI or *Hin*dIII indicated that the 2.6-kbp *Xba*I fragment might correspond to *leuA*. Altogether, these hybridization experiments demonstrated that the *nifV* gene described in this study does not exist in (almost) perfect duplication as do, e.g., *R. capsulatus nifA* and *nifB* (19, 24) but that a second (less highly conserved) copy of *nifV* might be present in *Anabaena* strain 7120.

DNA sequence analysis of the *Anabaena* strain 7120 *nifV* and *leuA* gene regions. Despite the strong similarity between the N-terminal parts of NifV and LeuA, the two proteins differ in their C-terminal parts. In addition, LeuA proteins contain a C-terminal extension of about 140 amino acids (aa) compared to NifV proteins. Therefore, nucleotide sequence analysis of the 2.1-kbp (pOST4) and the 2.6-kbp (pOST2) *Xba*I fragments was carried out, and comparison of the deduced amino acid sequences to NifV and LeuA from other organisms suggested

that pOST2 contained the complete *Anabaena* strain 7120 *leuA* gene (Fig. 1) (39a) and that pOST4 contained the complete *nifV* gene.

Downstream of *nifV*, we identified the *Anabaena* strain 7120 *nifZ* and *nifT* genes (Fig. 1 and 2). The *Anabaena* strain 7120 *nifV* and *nifZ* genes and the *nifZ* and *nifT* genes overlap by 25 and 26 bp, respectively (Fig. 2), indicating transcriptional coupling. Such long overlaps between adjacent genes are not very common but have already been described for other *nif* genes, including *R. capsulatus nifN* and *nifX* (26-bp overlap [27]) and *K. pneumoniae nifN* and *nifX* (14-bp overlap [2]). In contrast, many genes in *Anabaena* strain 7120 *nif* operons are separated by long intergenic regions containing tandemly repeated heptameric sequences (12). Cotranscription of *Anabaena* strain 7120 *nifV-nifZ-nifT* was corroborated by the presence of a 1.8-kb transcript in nitrogen-fixing cultures (see below), which was close to the expected size of a putative *nifVZT* transcript.

Comparison of restriction maps and partial-sequence analysis of the DNA regions upstream of *nifV* and downstream of *nifT* showed that the *Anabaena* strain 7120 *nifVZT* gene region was not located close to the main *nif* gene cluster (data not shown). However, high-resolution mapping with rarely cutting restriction endonucleases in combination with pulsed-field gel electrophoresis indicated that *nifV* might be located within the same *Sal*I fragment (fragment F; approximately 390 kbp) which contains the major *nif* gene cluster of *Anabaena* strain 7120 (reference 20 and data not shown).

The predicted *Anabaena* strain 7120 *nifV* gene product (379 aa) showed strong similarity to NifV proteins from *K. pneumoniae* (2) (381 aa; 39% identity), *A. vinelandii* (17) (385 aa; 40% identity), and *R. capsulatus* (23) (382 aa; 35% identity) (Fig. 3A). Despite the strong similarity between the N-terminal parts of NifV and LeuA, *Anabaena* strain 7120 NifV exhibited only 20% identity to LeuA from *Anabaena* strain 7120 and *E. coli* (reference 43 and data not shown) over the entire length of the proteins.

A comparison of the deduced *Anabaena* strain 7120 *nifZ* and *nifT* gene products to the corresponding proteins from *K. pneumoniae* (2), *A. vinelandii* (17), and *R. capsulatus* (23) is given in Fig. 3B and C, respectively. *Anabaena* strain 7120 NifZ is considerably smaller than its counterparts from the other diazotrophic bacteria. NifZ appears to be involved in the formation or accumulation of active MoFe protein, but it is not essential for nitrogen fixation in *K. pneumoniae* (28). Pertubation of *nifT* expression in *K. pneumoniae* also has only a limited effect on nitrogen fixation (37). Therefore, no clear function has been assigned yet for NifZ and NifT. However, based on the map position of *Anabaena* strain 7120 *nifZ* and *nifT* directly downstream of the *nifV* gene, one might speculate that NifZ and/or NifT are also involved in the biosynthesis of the FeMo cofactor of nitrogenase.

Transcriptional analysis of *Anabaena* strain 7120 *nifV*. To test whether *nifV* expression in *Anabaena* strain 7120 was regulated by combined nitrogen, Northern analysis was carried out. For this purpose, *Anabaena* strain 7120 was grown under nitrogen-replete (N+) or nitrogen-deficient (N−) conditions and RNA was prepared. Northern blot hybridization experiments with a *nifV*-specific probe (pOST7) led to the detection of a 1.8-kb transcript under nitrogen-fixing conditions (N−), which was completely missing in the presence of combined nitrogen (Fig. 4). Therefore, the *Anabaena* strain 7120 *nifV* gene was nitrogen regulated (at least under iron-replete conditions). The smaller RNAs in Fig. 4 might have resulted from degradation of the 1.8-kb transcript, indicating low stability of the *nifV* transcript, because the use of the same blot with a *petF* probe revealed a single transcript in all four lanes (29).

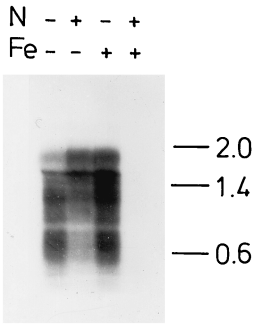

FIG. 4. Northern blot analysis of *Anabaena* strain 7120 *nifV* gene expression. RNA was isolated from cultures grown under nitrogen- and/or iron-replete or -deficient conditions as indicated by + and −. Cultures were grown in $BG11_0$ medium containing 5 mM NH_4NO_3 (N+) or no combined nitrogen (N−) and 30 μM (Fe+) or 0.9 μM (Fe−) ferric ammonium citrate. The insert of plasmid pOST7 (Fig. 1) was used to identify *nifV*-specific transcripts. The size of the marker (in kilobases) is indicated at the right.

In contrast to iron-replete conditions (Fe+), nitrogen regulation was overcome under iron-deficient conditions (Fe−). At low iron concentrations, the *nifV* gene was expressed not only under N− but also under N+ conditions (Fig. 4). A similar result has been observed for the expression of other *Anabaena* strain 7120 *nif* genes. Transcription of *nifH*, *fdxH*, and *nifJ* was induced by iron depletion irrespective of the nitrogen status (3, 29). Although transcription of *nifH* and *fdxH* was induced by iron starvation, neither the FdxH protein nor nitrogenase activity was detected. Unlike *nifH*, *fdxH*, and *nifV*, the *nifJ* gene was not expressed in cultures containing iron (3).

It has been suggested that the induction of *nif* genes is under developmental control, responding to one or more signals that also direct heterocyst differentiation (9, 34, 40). However, the *nifH*, *fdxH*, *nifV*, and *nifJ* genes are transcribed under Fe−N+ conditions, which prevent the formation of mature heterocysts.

Mutational analysis of the *Anabaena* strain 7120 *nifV* gene region. To analyze the role of *Anabaena* strain 7120 *nifV* in

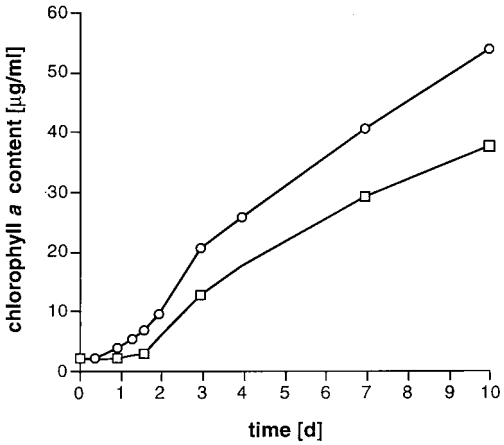

FIG. 5. Diazotrophic growth of *Anabaena* strain 7120 wild-type and *nifV* mutant BMB106. Precultures of the wild-type and mutant strains were grown in BG11 medium containing nitrate as the nitrogen source. After the filaments were washed with N-free medium ($BG11_0$), wild-type and mutant strains were diluted in $BG11_0$ and adjusted to the same cell density (measured by the chlorophyll *a* content). Diazotrophic growth was analyzed in three independent experiments, and one representative example of growth curves is shown in this figure. Open circles, *Anabaena* strain 7120 wild type; open squares, *nifV* mutant strain BMB106.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Bacterial strains		
E. coli		
MC1061	Host for pUC and pSUP plasmids	6
J53 (RP4)	Conjugal strain in triparental matings	7
HB101 (pRL528)	Cargo strain in triparental matings	7
S17-1	Host for mobilizable plasmids in biparental matings	38
Anabaena sp.		
PCC 7120	Wild type	11
BMB105	*Anabaena* strain 7120 *nifV*::[Nm]	This work
BMB106	*Anabaena* strain 7120:pBMB106 (*nifV* mutant)	This work
R. capsulatus		
B10S	Spontaneous Sm^r mutant of *R. capsulatus* B10	19
R229I	B10S *nifV*::[>Gm]	23
R229I-11	R229I:pOST11 ([Km>]-*nifV*)	This work
R229I-12	R229I:pOST12 ([<Km]-*nifV*)	This work
R229I-13	R229I:pOST13 ([Km>]-*leuA*)	This work
Plasmids		
pUC18, pUC19	Ap^r, *mcs*	42
pSUP202	Tc^r, Cm^r, Ap^r, *mob*	38
pRL648	Nm^r (C.K3)	8
pOST2	2.6-kbp *Xba*I fragment carrying *Anabaena* strain 7120 *leuA-dapF* cloned into pUC18	This work
pOST4	2.1-kbp *Xba*I fragment carrying *Anabaena* strain 7120 *nifV-nifZ-nifT* cloned into pUC18	This work
pOST6	0.6-kbp PCR fragment (*leuA*) cloned into pUC19	This work
pOST7	0.6-kbp PCR fragment (*nifV*) cloned into pUC19	This work
pOST11	pSUP202 carrying ([Km>]-*nifV*), [Gm]	This work
pOST12	pSUP202 carrying ([<Km]-*nifV*), [Gm]	This work
pOST13	pSUP202 carrying ([Km>]-*leuA*), [Gm]	This work
pBMB105	pSUP202 carrying *Anabaena* strain 7120 *nifV*::[Nm]	This work
pBMB106	728-bp *Pvu*I internal fragment of *Anabaena* strain 7120 *nifV* cloned into pSUP202, Nm^r	This work

[a] > and <, direction of transcription of the Gm or Km resistance gene relative to *nifV* or *leuA*.

nitrogen fixation, we constructed mutant strains BMB105 and BMB106. In mutant strain BMB105, the *nifV* gene was interrupted by an interposon inserted into the *Bcl*I site within *nifV* (Fig. 1). In mutant strain BMB106, the *nifV* gene was inactivated by chromosomal insertion of a plasmid carrying the internal 728-bp *Pvu*I fragment of *nifV* (Fig. 1), resulting in a tandem duplication in which neither copy of the *nifV* gene was complete. The mutations in BMB105 and BMB106 might be polar on *nifZ* and *nifT* expression, assuming that *nifVZT* are cotranscribed from a promoter upstream of *nifV*, and therefore both mutant strains were presumably defective not only for *nifV* but also for *nifZ* and *nifT*.

The Nif phenotype of mutant strains BMB105 and BMB106 was determined by analyzing growth under nitrogen-fixing conditions (Fe+N−). Both strains exhibited the same Nif phenotype, and a representative growth curve for BMB106 is shown in Fig. 5. Diazotrophic growth of BMB106 indicated that *nifV*, *nifZ*, and *nifT* were not essential for nitrogen fixation in *Anabaena* strain 7120. However, at least one of the three genes was required for maximum growth under N_2-fixing conditions (Fig. 5). Consideration of the effects of mutations in *nifV*, *nifZ*, and *nifT* genes in other organisms (17, 23, 28, 37) suggested that the *nifV* mutation was responsible for the observed Nif phenotype in *Anabaena* strain 7120. However, the NifV phenotype in BMB106 was less severe than in any other diazotrophic bacterium analyzed until now (reference 23 and references therein). It is worth noting that in contrast to an *Anabaena* strain 7120 *nifJ* mutant, which is not able to grow under Fe−N− conditions (3), the *nifV* mutant strain BMB106 was capable of diazotrophic growth under iron-depleted conditions (data not shown).

As mentioned above, hybridization data demonstrated that no perfectly duplicated copy of *nifV*, which might substitute for *nifV* in mutant strains BMB105 and BMB106, is present in the genome. However, as indicated by (additional) weak hybridization signals, a less highly conserved functional analog of *nifV* might exist in *Anabaena* strain 7120, which might be responsible for the observed Nif phenotype. Despite the similarity between NifV and LeuA, it seemed unlikely that the *leuA* gene substituted for *nifV*, since identity was restricted mainly to the N-terminal parts of the two proteins. Furthermore, only the *nifV*, not the *leuA*, gene was able to complement a *nifV* mutation in *R. capsulatus* (see below).

A variety of different organic acids other than homocitrate can be used for in vitro synthesis of the FeMo cofactor, resulting in dinitrogenases with altered substrate specificities (13, 21, 22). Since diazotrophic growth of BMB106 reached almost wild-type levels (Fig. 5), substrate specificity for N_2 was not changed dramatically in the *Anabaena* strain 7120 *nifV* mutant. However, it is unknown whether substitution of homocitrate by another organic acid might be responsible for the ability of BMB106 to grow with atmospheric dinitrogen as the sole nitrogen source.

***Anabaena* strain 7120 *nifV* complements a *nifV* mutation in *R. capsulatus*.** To analyze the function of the *Anabaena* strain 7120 *nifV* gene product in nitrogen fixation, complementation studies with an *R. capsulatus nifV* mutant were carried out. A nonpolar mutation in the *R. capsulatus nifV* gene (mutant strain R229I) resulted in a severe decrease in diazotrophic growth (doubling time of 55 h compared to 4 h for the wild type), and growth of R229I with N_2 as the sole source of

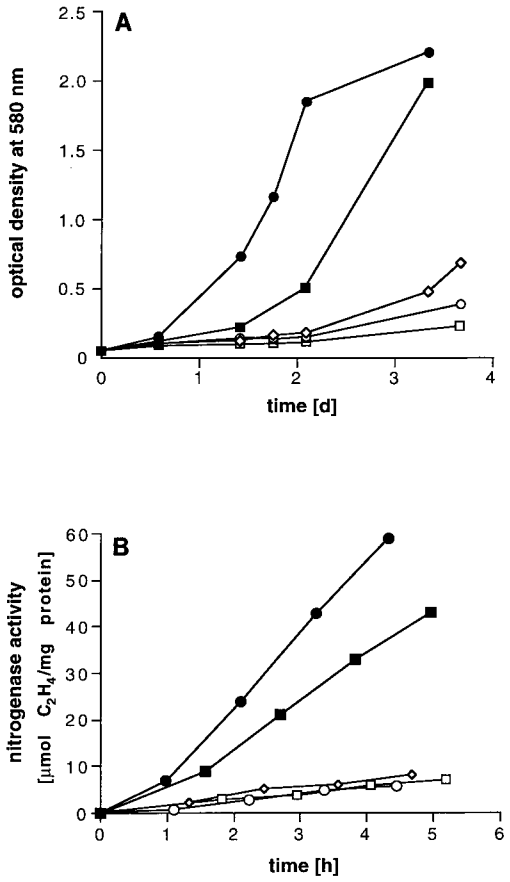

FIG. 6. Heterologous complementation of the *R. capsulatus nifV* mutant R229I by *Anabaena* strain 7120 *nifV*. Precultures of *R. capsulatus* wild-type and mutant strains were grown in RCV minimal medium with ammonium as the nitrogen source (23). Cells were washed with nitrogen-free medium, diluted in nitrogen-free RCV medium, and incubated under an N_2 atmosphere. (A) Diazotrophic growth was monitored by determining the optical density at 580 nm. Solid circles, *R. capsulatus* B10S (wild type); open circles, R229I (*R. capsulatus nifV* mutant [23]); solid squares, R229I-11 (R229I carrying the *Anabaena* strain 7120 *nifV* gene under control of the constitutive promoter of the kanamycin resistance gene [Km]); open squares, R229I-12 (R229I carrying the heterologous *nifV* gene in inverse orientation relative to the Km promoter); open diamonds, R229I-13 (R229I carrying the *Anabaena* strain 7120 *leuA* gene under control of the Km promoter). (B) The nitrogenase activity of the same strains was analyzed in the acetylene reduction assay by determining the accumulation of ethylene as described previously (23). Diazotrophic growth and nitrogenase activity were analyzed in two independent experiments, and one representative example of the curves is shown.

nitrogen was stimulated by the addition of homocitrate to the culture medium (23).

For complementation analysis of R229I, the 1,690-bp *Spe*I-*Xba*I fragment from pOST4 carrying the entire *Anabaena* strain 7120 *nifV* gene was cloned into a derivative of the mobilizable vector plasmid pSUP202, resulting in hybrid plasmid pOST11 (Fig. 1; Table 1). To ensure the expression of the *Anabaena* strain 7120 *nifV* gene in *R. capsulatus*, plasmid pOST11 contained a kanamycin cassette, which is known to constitutively transcribe genes located downstream (23), in front of the *Anabaena* strain 7120 *nifV* gene reading in the same direction. Plasmid pOST11 was mobilized from *E. coli* S17-1 into *R. capsulatus* R229I by biparental mating (24). Since pOST11 was not able to replicate in *R. capsulatus*, selection for kanamycin-resistant exconjugants yielded strains in which the entire plasmid was inserted into the chromosome via a single crossover between the gentamicin resistance genes in pOST11 (Table 1) and in R229I (*nifV*::[Gm] [23]). One exconjugant used for further studies was designated R229I-11.

In addition to R229I-11, two other mutant strains (R229I-12 and R229I-13) were constructed in a similar manner (Table 1). Strain R229I-12 contained the *Anabaena* strain 7120 *nifV* gene and the kanamycin resistance gene reading in opposite directions, whereas R229I-13 contained the *Anabaena* strain 7120 *leuA* gene under the control of the kanamycin cassette (Fig. 1).

The ability of *Anabaena* strain 7120 *nifV* and *leuA* to complement the *R. capsulatus nifV* mutant R229I was determined by monitoring diazotrophic growth (Fig. 6A) or estimating the nitrogenase activity by acetylene reduction (Fig. 6B). The results of these experiments can be summarized as follows. (i) Diazotrophic growth of R229I-11 demonstrated that *Anabaena* strain 7120 *nifV* can complement the *R. capsulatus nifV* mutation, corroborating that *Anabaena* strain 7120 *nifV* also encodes a homocitrate synthase. (ii) Comparison of mutant strains R229I-11 and R229I-12 showed that heterologous expression of *Anabaena* strain 7120 *nifV* was indeed driven by the kanamycin cassette. (iii) The *Anabaena* strain 7120 *leuA* gene complemented the *nifV* mutation in R229I to a much lower degree than did *nifV*. Therefore, it seems unlikely that *leuA*, which was constitutively transcribed in *Anabaena* strain 7120 (39a), might substitute for *nifV* in an *Anabaena* strain 7120 *nifV* mutant.

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