Identification and Characterization of the \textit{ni}f\textit{V-ni}f\textit{Z-ni}f\textit{T} Gene Region from the Filamentous Cyanobacterium \textit{Anabaena} sp. Strain PCC 7120

OLAF STRICKER,1 BERND MASEPOHL,1* WERNER KLIPP,2 AND HERBERT BÖHME1

Botanisches Institut der Universität Bonn, D-53115 Bonn, 1 and Lehrstuhl für Genetik, Fakultät für Biologie, Universität Bielefeld, D-33501 Bielefeld, 2 Germany

Received 24 September 1996/Accepted 18 February 1997

The \textit{ni}f\textit{V} and \textit{leu}A genes, which encode homocitrate synthase and \textit{α}-isopropylmalate synthase, respectively, were cloned from the filamentous cyanobacterium \textit{Anabaena} sp. strain PCC 7120 by a PCR-based strategy. Since the N-terminal parts of \textit{ni}f\textit{V} and \textit{Leu}A from other bacteria are highly similar to each other, a single pair of PCR primers was used to amplify internal fragments of both \textit{Anabaena} strain 7120 genes. Sequence analysis of cloned PCR products confirmed the presence of two different \textit{ni}f\textit{V}-like DNA fragments, which were subsequently used as \textit{ni}f\textit{V}- and \textit{leu}A-specific probes, respectively, to clone \textit{xhoI} fragments of 2.1 kbp (\textit{pO}ST4) and 2.6 kbp (\textit{pO}ST2). Plasmid \textit{pO}ST4 carried the \textit{Anabaena} strain 7120 \textit{ni}f\textit{V-ni}f\textit{Z-ni}f\textit{T} genes, whereas \textit{pO}ST2 contained the \textit{leu}A and \textit{dapF} genes. The \textit{ni}f\textit{V}ZT genes were not located in close proximity to the main \textit{ni}f gene cluster in \textit{Anabaena} strain 7120, and therefore \textit{ni}f\textit{VZT} forms a second \textit{ni}f gene cluster in this strain. Overlaps between the \textit{ni}f\textit{V} and \textit{ni}f\textit{Z} genes and between the \textit{ni}f\textit{Z} and \textit{ni}f\textit{T} genes and the presence of a 1.8-kb transcript indicated that \textit{ni}f\textit{VZT} might form one transcriptional unit. Transcripts of \textit{ni}f\textit{V} were induced not only in a nitrogen-depleted culture but also by iron depletion irrespective of the nitrogen status. The \textit{ni}f\textit{V} gene in \textit{Anabaena} strain 7120 was interrupted by an interposon insertion (mutant strain BMB105) and by a plasmid integration via a single crossover with a \textit{ni}f\textit{V} 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 \textit{Rhodobacter capsulatus} \textit{ni}f\textit{V} mutant R229I by the \textit{Anabaena} strain 7120 \textit{ni}f\textit{V} gene corroborated the assumption that \textit{Anabaena} strain 7120 \textit{ni}f\textit{V} also encodes a homocitrate synthase. In contrast, the \textit{Anabaena} strain 7120 \textit{leu}A gene did not complement the \textit{ni}f\textit{V} mutation of R229I efficiently.

In the facultative anaerobic enterobacterium \textit{Klebsiella pneumoniae}, 20 nitrogen fixation (\textit{ni}f) genes, which are clustered in one region of the chromosome, are involved in the process of \textit{N2} reduction by the molybdenum-containing nitrogenase enzyme complex (2). The molybdenum nitrogenase is composed of the Fe protein, a dimer of identical subunits (\textit{Ni}fH) containing a single [4Fe-4S] cluster, and the MoFe protein, a heterotetramer of two \textit{Ni}fD and two \textit{Ni}fK 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 \textit{ni}fH, \textit{ni}fQ, \textit{ni}fB, \textit{ni}fV, \textit{ni}fE, and \textit{ni}fN (16, 35, 36). Hoover et al. (14, 15) have shown that homocitrate is an integral part of the FeMo-co and that \textit{ni}f\textit{V} encodes a homocitrate synthase.

\textit{Ni}f\textit{V} exhibits similarity to \textit{Leu}A from different organisms (reference 25 and references therein). In yeasts and fungi, homocitrate synthase carries out the condensation between acetyl coenzyme A and \textit{α}-ketoglutarate, an analogous reaction to that catalyzed by \textit{Leu}A (\textit{α}-isopropylmalate synthase), which carries out the condensation between acetyl coenzyme A and \textit{α}-ketosiovalerate.

\textit{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 \textit{N2} fixation. Despite the conservation of many \textit{ni}f genes, some features of \textit{ni}f gene organization in \textit{Anabaena} strain 7120 differ from those of \textit{K. pneumoniae} and other diazotrophs. The main \textit{ni}f gene cluster in \textit{Anabaena} strain 7120 heterocysts includes (in order) \textit{ni}fB, \textit{fdxN}, \textit{ni}fS, \textit{ni}fU, \textit{ni}fH, \textit{ni}fD, \textit{ni}fK, \textit{ni}fE, \textit{ni}fN, \textit{ni}fX, \textit{ORF}3, \textit{ni}fW, \textit{ORF}1, \textit{ORF}2, and \textit{fdxH} (for reviews, see references 12 and 41). One of the \textit{ni}f genes essential in other diazotrophic organisms not previously found in cyanobacteria was \textit{ni}f\textit{V}.

In this study, we identified the \textit{Anabaena} strain 7120 \textit{ni}f\textit{VZT} genes, which form a second \textit{ni}f gene cluster separated from the main \textit{ni}f gene cluster. The \textit{ni}f\textit{VZT} gene region was characterized by transcriptional and mutational analysis. Furthermore, the \textit{Anabaena} strain 7120 \textit{ni}f\textit{V} gene was shown to complement a \textit{ni}f\textit{V} mutation in \textit{Rhodobacter capsulatus}.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. \textit{Anabaena} strain 7120 was grown at 30°C in BG11 medium (30) containing either 17.7 mM NaNO3 or 5 mM NH4NO3 as the nitrogen source. Cultures were illuminated with white fluorescent light at 100 mW/cm2 and bubbled with air enriched to 1% CO2. Nitrogen-fixing cultures were grown in BG11 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 \textit{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 \textit{Escherichia coli} and \textit{Rhodobacter 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 II and Taq polymerase (Gibco). Oligonucleotides synthesized in a Pharmacia LKB Gene Assembler Plus were...
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[G/A]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 *nifV* and *leuA* gene regions. The N-terminal parts of NifV (homocitrate synthase) and LeuA (α-isopropylmalate synthase) from differ-

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 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 NiF (homocitrate synthase) and LeuA (α-isopropylmalate synthase) from differ-
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 \textit{nifV} and \textit{leuA} genes from \textit{Anabaena} strain 7120. PCR amplification resulted in DNA fragments of the expected size of approximately 0.6 kbp, which were cloned into the SmaI 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 \textit{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 \textit{leuA}.

Southern blot analysis of \textit{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 XhoI fragment hybrid-
izing to the \textit{nifV}-specific probe (pOST7) and a 2.6-kbp \textit{XbaI} fragment showing homology to the \textit{leuA}-specific probe (pOST6). Hybrid plasmids carrying the 2.1- or 2.6-kbp \textit{XbaI} fragment were isolated from a size-fractionated \textit{XbaI} gene bank in \textit{pUC18} and designated pOST4 (\textit{nifV}) and pOST2 (\textit{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 \textit{XbaI} fragment, weakly hybridizing \textit{XbaI} fragments of 2.6 and >12 kbp were detected by the \textit{nifV}-specific probe under nonstringent hybridization conditions (data not shown). Southern blot analysis of \textit{Anabaena} strain 7120 total DNA digested with \textit{XbaI}-\textit{EcoRI} or \textit{HindIII} indicated that the 2.6-kbp \textit{XbaI} fragment might correspond to \textit{leuA}. Altogether, these hybridization experiments demonstrated that the \textit{nifV} gene described in this study does not exist in (almost) perfect duplication as do, e.g., \textit{R. capsulatus nifA} and \textit{nifB} (19, 24) but that a second (less highly conserved) copy of \textit{nifV} might be present in \textit{Anabaena} strain 7120.

\textbf{DNA sequence analysis of the \textit{Anabaena} strain 7120 \textit{nifV} and \textit{leuA} gene regions.} Despite the strong similarity between the N-terminal parts of NifZ 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) \textit{XbaI} 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 *SalI* 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 NiF 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 NiV and LeuA, *Anabaena* strain 7120 NiV 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). Perturbation 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).

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 *nifB* 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
nitrogen fixation, we constructed mutant strains BMB105 and BMB106. In mutant strain BMB105, the nifV gene was interrupted by an interposon inserted into the BeII 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 PvuI 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 nifZT 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 N2-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 nifH 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 N2 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.

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 N2 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 N2 as the sole source of
Diazotrophic growth was monitored by determining the optical density at 580 nm. Cells were washed with nitrogen-free medium, diluted in
"nitrogen-free RCV medium, and incubated under an N2 atmosphere. (A) Diazotrophic growth was stimulated by the addition of homocitrate to the
culture medium (23).

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 N2 atmosphere. (A) Diazotrophic growth was monitored by determining the optical density at 580 nm. Solid circles, R. capsulatus B105 (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 SpeI- XbaI fragment from pOST4 carrying the entire Anabaena strain 7120 nifV gene was cloned into a derivative of the mobi-
zilizable 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 biamplar 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 direc-
tions, 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 com-
plement 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 muta-
tion, corroborating that Anabaena strain 7120 nifV also en-
codes 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.

ACKNOWLEDGMENTS

We thank K. Görlitz for technical assistance and H. Geithmann for
photographic work.

This work was supported by Bundesministerium für Forschung und
Technologie (grant 9342A) and Deutsche Forschungsgemeinschaft
(grant Bo660/4-2).

REFERENCES

tinere fixation gene cluster of Klebsiella pneumoniae. J. Mol. Biol. 203:
715–738.
fixation of nitrogen. Chapman & Hall, New York, N.Y.
based on the nonviability of palindromic-containing plasmids that allows
8. Elhai, J., and C. P. Wolk. 1990. Developmental regulation and spatial pat-
tern of expression of the structural genes for nitrogenase in the cyanobac-
nitrogen fixation. Chapman & Hall, New York, N.Y.
trogenase with altered substrate specificity results from the use of ho-
mcitrate analogues for in vitro synthesis of the iron-molybdenum cofactor.
component of the iron-molybdenum cofactor of nitrogenase. Biochem-
istry 28:2768–2771.


