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Role of a Ferredoxin Gene Cotranscribed with the *nifHDK* Operon in N_2 Fixation and Nitrogenase "Switch-Off" of *Azoarcus* sp. Strain BH72

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The endophytic diazotroph *Azoarcus* sp. strain BH72 is capable of infecting rice roots and of expressing the nitrogenase (*nif*) genes there. In order to study the genetic background for nitrogen fixation in strain BH72, the structural genes of nitrogenase (*nifHDK*) were cloned and sequenced. The sequence analysis revealed an unusual gene organization: downstream of *nifHDK*, a ferredoxin gene (*fdxN*; 59% amino acid sequence identity to *R. capsulatus* FdxN) and open reading frames showing 52 and 36% amino acid sequence identity to *nifY* of *Pseudomonas stutzeri* A15 and ORF1 of *Azotobacter vinelandii* were located. Northern blot analysis, reverse transcriptase PCR and primer extension analysis revealed that these six genes are located on one transcript transcribed from a σ^{54}-type promoter. Shorter transcripts sequentially missing genes of the 3′ part of the full-length mRNA were more abundantly detected. Mutational analyses suggested that FdxN is an important but not the essential electron donor for dinitrogenase reductase. An in-frame deletion of *fdxN* resulted in reduced growth rates (59% ± 9%) and nitrogenase activities (81%) in nitrogen-fixing pure cultures in comparison to the wild type. Nitrogenase activity was fully complemented in an *fdxN* mutant which carried a *nifH* promoter-driven *fdxN* gene in *trans*. Also, in coculture with the ascomycete *Acremonium alternatum*, where strain BH72 develops intracytoplasmic membrane stacks, the nitrogenase activity in the *fdxN* deletion mutant was decreased to 56% of the wild-type level. Surprisingly, the *fdxN* deletion also had an effect on the rapid "switch-off" of nitrogenase activity in response to ammonium. Wild-type strain BH72 and the deletion mutant complemented with *fdxN* in *trans* showed a rapid reversible inactivation of acetylene reduction, while the deletion mutant did not cease to reduce acetylene. In concordance with the hypothesis that changes in the redox state of NifH or electron flux towards nitrogenase may be involved in the mechanism of physiological nitrogenase switch-off, our results suggest that the ferredoxin may be a component involved in this process.

In addition to the root surface, inner tissues of roots may also be colonized by bacteria. Endophytic diazotrophic bacteria invade roots and shoots of grasses without causing symptoms of plant disease (23, 46). Establishment in inner tissues of agriculturally important crops such as sugar cane or rice has been shown for several gram-negative bacteria, such as *Herbaspirillum seropedicae* (23, 24), *Acetobacter diazotrophicus* (25), and *Azoarcus* spp. (19).

Azoarcus sp. strain BH72, which was isolated from the endorhizosphere of Kallar grass in Punjab, Pakistan (45), is also capable of infecting roots of rice seedlings in the laboratory (19). Reporter gene studies have demonstrated that nitrogenase (*nif*) genes of *Azoarcus* spp. can be expressed endophytically in the aerenchyma of these seedlings, suggesting that the interior of rice roots provides a microenvironment suitable for nitrogen fixation (6). Expression of *nifHDK* genes (6) as well as nitrogenase activity (16) requires low concentrations of oxygen and ammonium (below 0.5 mM); however, anaerobic conditions do not permit nitrogen fixation in this strictly respiratory bacterium.

Azoarcus sp. strain BH72 is unusual in that it can shift into a state of "hyperinduction" under certain growth conditions that include extremely low oxygen concentrations (30 nM). Moreover, in contrast to other *Proteobacteria*, this strain harbors three instead of two copies of P_{II}-like proteins, the central signal transmitters of nitrogen metabolism (37). Hyperinduction of strain BH72 is characterized by increased activity and efficiency of nitrogen fixation (18), appearance of intracellular membrane stacks (diazosomes), and association of the iron protein of nitrogenase with diazosome membranes (20). Diazosome formation can be induced reproducibly in the laboratory by cocultivating *Azoarcus* sp. strain BH72 with the ascomycete *Acremonium alternatum*, which was isolated from the root interior of Kallar grass as well (17). The cells attach to the fungal mycelium, and the fungal respiration may provide sufficiently microaerobic niches for diazosome formation. The association of nitrogenase with these membranes suggests that they are involved in efficient nitrogen fixation, possibly by providing a more efficient electron flux to nitrogenase (20).

The electrons required to reduce N_2 are carried to nitrogenase by either flavodoxins or ferredoxins. Little is known about the generation of reductant for N_2 fixation in nonphototrophic bacteria. While the NifJF pathway via a pyruvate:flavodoxin oxidoreductase was characterized for *Klebsiella pneumoniae*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
Azoarcus sp. strain BH72	Wild type	45
BHΔfdxN	*fdxN* in-frame deletion in BH72	This study
BHΔfdxN(pfdxN)	BHΔfdxN complemented with pfdxN	This study
E. coli DH5αF′	F′ *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA relA1* ϕ*80lacZ*Δ*M15* Δ(*lacZYA-argF*)U169	13
Plasmids		
pUC19/18	Ap^r	59
pBSKII/pBKSII	Ap^r	Stratagene
pLAFR3	Tet^r, cosmid vector	56
pEN9	Ap^r, *Kpn*I fragment from GenBank clone pBGVN9 carrying *nif* gene cluster in pUC19	This study
pEN94	Ap^r, subclone from pEN9	This study
pEN9ΔF	Ap^r, *fdxN* deletion on pEN9	This study
pfdxN	Tet^r, *fdxN* gene fused to the *nifH* promoter region on pLAFR3	This study

(41), the generation of low-redox-potential electron carriers for nitrogenase reduction in many heterotrophic diazotrophs remains unclear. Nevertheless, the immediate molecule donating electrons to nitrogenase reductase had been identified for many diazotrophs. While in gram-positive bacteria (10) and in cyanobacteria (53) [2Fe-2S] ferredoxins have been shown to supply electrons to nitrogenase, the group of *Proteobacteria* favors more or less *nif*-specific 2[4Fe-4S] ferredoxins (30, 52). Some of the genes encoding these ferredoxins have been found to be localized in an operon with *nif* genes (other than nitrogenase genes) and are therefore regulated in a *nif*-dependent manner (26, 33).

In order to analyze nitrogen fixation in *Azoarcus* sp. strain BH72 genetically, nitrogenase structural genes were cloned and sequenced in the present study, revealing strong homologies with known *nif* genes of other *Proteobacteria*. In contrast to most other bacteria, strain BH72 was found to cotranscribe a ferredoxin gene with the structural *nifHDK* genes. Mutational analysis revealed that the ferredoxin is not obligatory for nitrogen fixation. However, it is essential for the rapid "switch-off" of nitrogenase activity in response to ammonium addition and thus a newly identified component involved in this process.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *Azoarcus* strain BH72 originated from roots of Kallar grass, *Leptochloa fusca* (L.) Kunth (45).

Culture media and growth conditions. If not stated otherwise, *Azoarcus* sp. strain BH72 was grown at 37°C in VM medium supplemented with ethanol (47, 48). For nitrogen fixation in pure culture, the cells were grown microaerobically in N-free SM medium (44) in a closed batch culture (32) or, for mixed-culture growth experiments, in a 2-liter fermentor (Biostat B; Braun Biotech, Melsungen, Germany) equipped with a regulated oxygen supply set at 0.1% O_2 in N_2, and stirred at 600 rpm. The cells were precultured overnight in SM medium supplemented with 0.01% yeast extract and 0.05% ammonium chloride, washed three times in N-free SM medium, and inoculated at an optical density at 578 nm (OD_{578}) of 0.02 in the fermentor. Cells were harvested at the late exponential growth phase (OD_{578} of 1.5) after six generations. Cocultures with the ascomycete *Acremonium alternatum* were carried out as described previously (20), with wild-type and mutant cells added to the cultures in the same amounts. The flasks were sealed with gas-tight rubber stoppers and incubated until the oxygen in the headspace had decreased from initially atmospheric concentrations to approximately 2%. This occurred 5 to 8 days after inoculation.

Analysis of bacterial growth and nitrogen fixation. To analyze the growth of the Δ*fdxN* mutant strain compared to that of the wild type under exactly the same growth conditions, the strains were coinoculated in the fermentor in equal amounts. Growth parameters were as described above. Culture samples of the mixed culture were taken at different time points during exponential growth, diluted in saline (0.9% NaCl), and plated on VM agar plates to give approximately 200 colonies per plate. These colonies were restreaked on Hybond N membranes in six replicates of 30 randomly picked colonies in the first experiment. Thus, 180 colonies total from each time point were used for colony hybridization, using a digoxigenin-labeled *fdxN* probe (fragment amplified with primers TE29 and TE30) to differentiate mutant colonies from the wild type and thereby determine the ratio of both cell types (standard deviations are given for the six replicates at 11 and 15 h in Fig. 4). In two more independent fermentor experiments, only the first and the last time points were evaluated in this way (standard deviations are given in Fig. 4 for all three independent experiments). Growth rates for individual strains were estimated from calculating cell densities of the mutant and the wild type according to the percentage of distribution obtained by colony hybridization at different time points.

To analyze the mutant phenotype in cocultures with the ascomycete, independent cultures of wild-type and mutant strains were grown. Nitrogen-fixing capacity was measured using the acetylene reduction assay (32) 5 to 8 days after inoculation as described below, and the amount of ethylene formed per vial was determined by gas chromatography. The oxygen concentration in the headspace was also determined by gas chromatography (32). Cultures had been inoculated with equal amounts of bacterial cells (8×10^8 per ml [5]), which do not grow significantly in coculture but adhere to the fungal mycelium (approximately 20 mg per culture), reduce acetylene, and form dumbbell-shaped, diazosome-containing cells which appear to be arrested in cell division (reference 20 and unpublished results). Acetylene reduction of cultures was estimated in three independent experiments with three to five replicates. To demonstrate an equal yield of bacterial protein from both wild-type and mutant cultures in coculture with the ascomycete, in which fungal mycelium and bacteria cannot efficiently be separated from each other, equal volumes of cultures were harvested and roughly disrupted in a kitchen blender (Braun) for 30 s. The bacteria and fungal debris were sedimented by centrifugation at 20,000 × *g* for 2 min and resuspended in sample treatment buffer to give 140 mg (fresh weight) per ml. After addition of 1% sodium dodecyl sulfate, the cells were incubated at 95°C for 5 min and centrifuged at 20,000 × *g* for 5 min to pellet DNA and cell debris. The clear protein-containing supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis, using antibodies against the iron protein of nitrogenase (kindly provided by R. Ludden, University of Wisconsin, Madison) to differentiate bacterial from fungal protein.

For analysis of switch-off of nitrogenase activity, nitrogen-fixing cells were grown in closed batch culture overnight as described above and then transferred to fresh N-free SM medium (5 to 15 ml of culture to give 30 ml total) in sealed Erlenmeyer flasks adjusted to 1% O_2 and 1% acetylene in the headspace. Acetylene reduction and optical density were monitored before and after addition of NH_4Cl (2 mM final concentration).

Statistical evaluations were carried out using the GraphPad Instat software, applying the Student *t* test or the Tukey-Kramer multiple comparison test.

Techniques for DNA and RNA manipulation. General techniques for DNA analysis were carried out according to standard protocols (3, 47). Homologous DNA gene probes for Southern and Northern blot analysis were digoxigenin labeled in a PCR using a digoxigenin labeling and detection kit (Boehringer,

TABLE 2. Primers used

Application	Primer and sequence (position)[a]
nifK probe	—[b]
ORF1 probe	TE27: 5′CCTGCGCTTCACCTATTGTT3′ (6240–6259)
	TE28: 5′AGCGAAATCCAACTCCGCCA3′ (110 nt downstream of ORF1)
fdxN probe	TE29: 5′ACCAAGTCGATCACCGACAA3′ (5200–5219)
	TE30: 5′CTACAAAAAACAGGGAGCC3′ (5384–5366)
Test for *fdxN* deletion	TE14: 5′CCGCCAGTGGTGGGCTTTCG3′ (4911–4930)
	TE19: 5′GACGACCTCGACACCGGGCA3′ (5499–5518)
Amplification of *nifHDK* promoter region	fdxpro2(*Pst*I): 5′AACTGCAGAGTCGAACAGCCAGCCGCGCA3′ (34–54)
	fdxprorev(*Bam*HI): 5′AAGGATCCAAGAAAAAAACTACCGGGTGTCCTGGG3′ (600–574)
fdxN gene	fdxvor(*Bam*HI): 5′CGGGATCCGGAGAAGGAATCATGGCTCTG3′ (5125–5145)
	TE36(*Eco*RI): 5′ACGAGCCTCATGGGAATTC3′ (5421–5403)
RT-PCR	nifKfw: 5′CGCCTTCGCGGCACCCGT3′ (4799–4816)
	Fdxrev: 5′CAGTCACCACAGGACGTGCAT3′ (5183–5163)
Primer extension	TH25rCy: 5′TGCCGATACCGCCCTTGCC (668–650)

[a] Nucleotide numbering according to GenBank accession number AF200742. Underlined sequences are restriction sites.
[b] See reference 20.

Mannheim, Germany). Primer sequences are given in Table 2. For Northern blot analysis, RNA was isolated from exponentially growing bacterial cells by the hot phenol method (1), and Northern blot analysis was carried out according to standard protocols (3, 47). Reverse transcriptase PCR (RT-PCR) was carried out with 1.5 μg of RNA using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech) according to manufacturer's instructions, using primers nifKfw and Fdxrev (Table 2) for the RT reaction at 42°C for 20 min and inactivation at 95°C for 5 min, followed by the PCR with 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min.

Primer extension was carried out with a CY5-labeled primer (TH25rCy) (Table 2) according to standard protocols (3) with 20 μg of RNA isolated from nitrogen-fixing cells, using an automated sequencer (ALF*express*; Amersham Pharmacia Biotech) for product analysis.

DNA sequencing and computational analysis. DNA sequencing was carried out as described by Sanger et al. (51). Primers were ^{35}S labeled and sequencing was carried out using the DNA sequencing kit Sequenase, version 2.0 (Amersham, Braunschweig, Germany). Products were separated on an 8% acrylamide gel (GENE-PAGE; Amresco, Solon, Ohio) and detected by autoradiography. Alternatively, CY5-labeled primers were used for sequencing with an automated sequencer as described by Hurek et al. (15).

Construction of *fdxN* mutants of *Azoarcus* sp. strain BH72. The construction of an *fdxN* in-frame deletion is depicted in Fig. 1. Plasmid pEN94, which carries the genomic region of the *fdxN* gene, was digested with *Bsm*I and *Eco*RI. The sticky ends of the restriction fragments were blunted using Klenow large fragment, which removed the 3′ overhang of the *Bsm*I site and filled the 5′ overhang at the *Eco*RI site. The blunt ends were religated, yielding a stop codon that interrupted the translation of the *fdxN* gene after the seventh amino acid. The mutation was confirmed by sequence analysis and reintroduced into pEN9 by cloning the *Kpn*I/BamHI insert of pEN94Δ*fdxN* into pEN9 digested with the same restriction endonucleases, yielding pEN9ΔF. The mutation was introduced into the *Azoarcus* sp. strain BH72 chromosome by allelic exchange mutagenesis. Specifically, the plasmid pEN9ΔF, which does not replicate in *Azoarcus* spp., was integrated into the chromosome by a single homologous recombination event after electroporation. Recombinants carrying the vector-borne Ap^r gene were used for a second recombination event: colonies were replica plated on medium with and without antibiotic and screened for cells that had lost the vector-encoded resistance. Six in 5,000 colonies tested which had lost the vector were tested for the *fdxN* deletion by PCR with primers TE14 and TE18. These primers amplified a 609-bp fragment in the wild type, while the mutant showed a 373-bp product. Two of six double recombinants had exchanged the wild-type for the mutagenized gene (strain BHΔfdxN).

To complement the mutation, the *fdxN* gene was genetically fused to the original *nifH* promoter and provided in *trans*. The coding sequence and the promoter region were both amplified by PCR using *Pfu* polymerase (Stratagene) and the primers (Table 2) fdxpro2(*Pst*I) and fdxprorev(*Bam*HI) at 64°C with 1.5 mM $MgCl_2$ for the promoter region or fdxvor(*Bam*HI) and TE36(*Eco*RI) at 53°C with 1.5 mM $MgCl_2$ for the coding sequence. The two amplified fragments were restriction digested with *Pst*I/*Bam*HI or *Eco*RI/*Bam*HI, respectively, and cloned into *Pst*I/*Eco*RI-digested vector pUC19, the two PCR fragments being fused at the *Bam*HI site. A clone of the correct sequence was subcloned into the broad-host-range plasmid pLAFR3 (56) after *Eco*RI/*Pst*I digestion, yielding plasmid pfdxN, which was conjugated into strain BH72 by triparental mating. Since the fragments provided in *trans* were very short (400 to 600 bp), a double recombination event of *fdx* into the chromosome was unlikely.

Nucleotide sequence accession number. The sequences of the *nifHDK* operon were submitted to GenBank (accession no. AF200742).

RESULTS

Characterization of the *nifHDK-fdxN* region of *Azoarcus* sp. strain BH72. The nitrogenase genes of *Azoarcus* sp. strain BH72 were obtained by screening a genomic library in pUC19 (*Sau*3A1-digested DNA cloned into the *Bam*HI site [47]) with a heterologous *nifH* gene probe of *Azorhizobium caulinodans*. The resulting plasmids carrying the *nifHDK* region and results of the sequence analysis of 6.5 kb are shown in Fig. 1. Southern hybridization assays at low stringency with either a homologous *nifH* or *nifK* probe indicated that these *nif* genes are present in a single copy on the chromosome of *Azoarcus* sp. strain BH72 (data not shown). Nitrogenase structural gene products showed the highest homology to the FeMo nitrogenase from *Azotobacter* spp., with 88% identity to NifH of *Azotobacter chroococcum* (27) and 82% identity to NifD and 77% identity to NifK of *Azotobacter vinelandii* (21). Downstream of the *nif* genes, a sequence coding for a 2[4Fe-4S] ferredoxin was detected which was most closely related to the ferredoxin FdxN from *Rhodobacter capsulatus*, with 59% amino acid identity (11, 29). Downstream of this *fdxN* gene, a *nifY* homologue (52 and 42% amino acid identity to NifY of *Pseudomonas stutzeri* A15 and *A. vinelandii*, respectively) and an open reading frame (ORF1) with weak amino acid identity (36%) to an open reading frame (named ORF1) from the *A. vinelandii nif* region (21) were found.

The upstream untranslated region of the *nifHDK* region

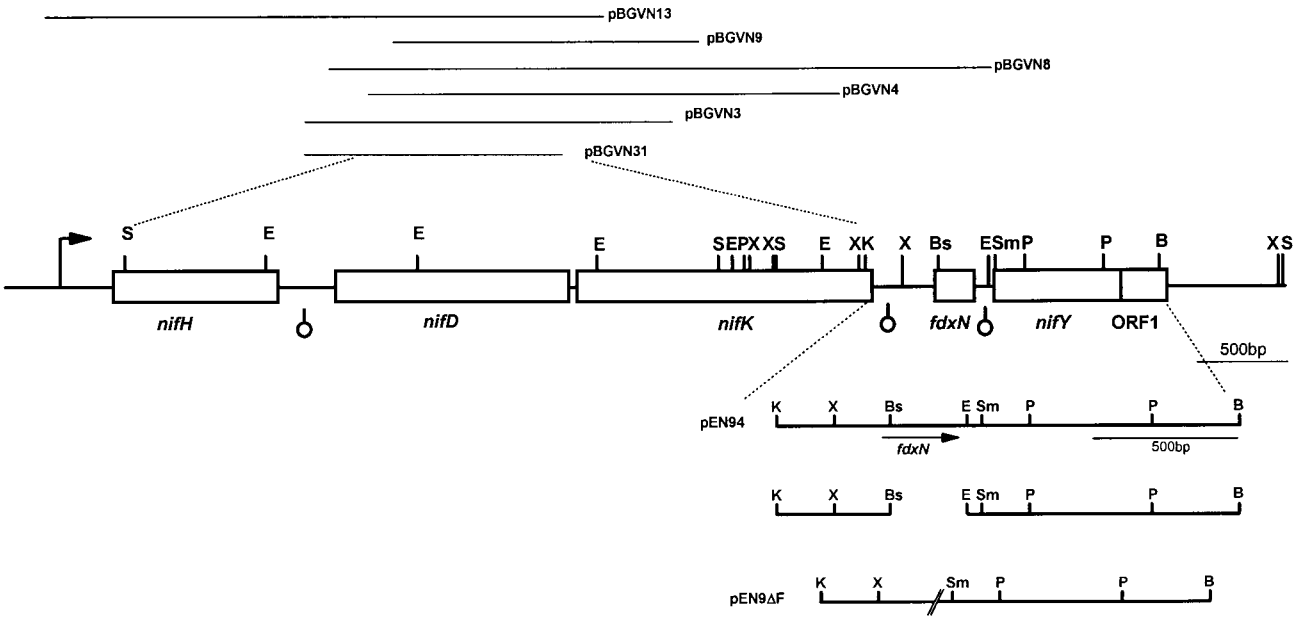

FIG. 1. Gene organization of the *nifHDK* locus. The restriction map of the *nifHDK* operon in *Azoarcus* sp. strain BH72 shows *nif* gene-containing clones from a genomic library above and the construction of the *fdxN* deletion (pEN9ΔF) below the map. The bold arrow indicates the promoter region, and the light arrow shows the position of *fdxN* in plasmid pEN94. Sequences with inverted repeats for stem-loop formation are indicated by hairpins below the map. S, *Sal*I; E, *Eco*RI; P, *Pst*I; X, *Xho*I; K, *Kpn*I; Bs, *Bsm*I; Sm, *Sma*I; B, *Bam*HI.

harbored sequence homologies to the consensus of σ^{54}-dependent promoters (Fig. 2A). Sequences for putative NifA binding sites (or putative upstream activating sequences) were detected approximately 120 and 140 bp upstream of the σ^{54} promoter. Putative ribosome binding sites (RBS) in *Azoarcus* sp. strain BH72 contained a minimal consensus of four or five bases (aGGAG) at a 6- or 7-base distance from the possible start codon (Fig. 2A), which is ATG. The transcriptional start site was verified by primer extension studies (Fig. 2B).

Analysis of the *nifHDK* mRNA. Nitrogenase genes in *Azoarcus* sp. strain BH72 are clustered in a region of approximately 6.3 kb (Fig. 1) covering six open reading frames. Since putative promoter sequences were detected only upstream of the *nifH* gene, these genes are likely to be part of the same operon.

In order to analyze whether the six genes formed one transcriptional unit, Northern blot analysis of RNA extracted from *Azoarcus* sp. strain BH72 was carried out with gene probes targeted to different genes of the *nifHDK* region (Fig. 3). All four probes hybridized only to RNA extracted from N_2-fixing cells, where they detected several apparently overlapping transcripts. A fragment of approximately 6 kb which is the same size as the entire *nifH-orf1* region was detected with an *orf1* probe; however, the signal was weak, requiring long exposure times. An RNA fragment of the same length hybridized with the *fdxN* probe; however, with this probe a stronger hybridization signal appeared at approximately 5 kb, corresponding in size to the *nifH-fdxN* region (Fig. 3). A *nifK* probe hybridized with three fragments of 6.5, 5, and 4.3 kb, the latter corresponding in size to the *nifH-nifK* region, which was most intensely stained. Lowest exposure times were required for the *nifH* probe, which hybridized most strongly with a 1.15-kb band (corresponding to *nifH* alone) and less intensely with the 4.3-kb fragment (Fig. 3). This mRNA hybridization pattern indicated that all genes are localized on one large transcript, which occurs, however, at low abundance. Shorter transcripts sequentially missing genes of the 3′ part of the region were more abundant, which might be due to multiple transcriptional termination sites or sequential degradation of the original transcript from its 3′ end in defined steps.

To prove that the *nifHDK* operon was transcriptionally linked with downstream genes, a PCR involving an RT step was carried out with RNA of the nitrogen-fixing strain BH72. The first primer for the RT reaction and the PCR was designed to anneal to the 5′ end of the ferredoxin gene, while the second primer was targeted to *nifK*. An RT-PCR product of the expected size was detected only in the presence of RNA and active RT (Fig. 3B, lane 4) and not after heat inactivation of RT (control for DNA contamination) (lane 3) or without addition of RNA (lane 2).

Role of the ferredoxin FdxN in nitrogen fixation. In order to investigate the role of the *nif* operon-encoded ferredoxin in nitrogen fixation, we constructed an in-frame deletion mutant of *fdxN* in *Azoarcus* sp. strain BH72. The mutant (BHΔfdxN) was still able to fix nitrogen in semisolid (0.2% agar) N-free medium. Whether nitrogen fixation reached wild-type levels was elucidated by quantitation of growth rates under nitrogen-fixing conditions.

To subject the wild type and the Δ*fdxN* mutant of *Azoarcus* to exactly the same growth conditions, the strains were cultivated in a mixed culture on N_2 in an oxygen-controlled bioreactor at initially identical cell numbers. The culture was grown to an OD_{578} of ~1.5, corresponding to six generations of bacterial growth (Fig. 4A). In three independent experiments, cultures were tested for the distribution of the different geno-

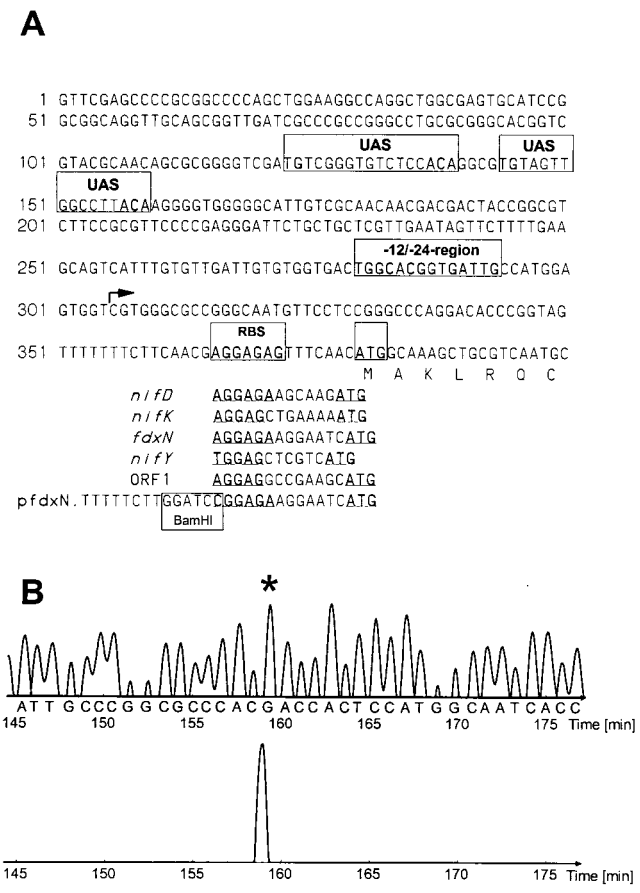

FIG. 2. Promoter region of *nifHDK*. (A) Sequence of the genomic region upstream of *nifH* and of the fusion of the promoter region of *nifH* fused to *fdxN* in plasmid pfdxN. The arrow indicates the putative transcription start site, and the unlabeled box indicates the possible start codon. Regulatory sequences are boxed and labeled: UAS, putative upstream activating sequence; −12/−24 region, putative σ^{54}-dependent promoter region; RBS, putative Shine-Dalgarno sequence of *nifH*. The putative RBS of the other genes of the *nifHDK* region are given at the bottom, as well as the sequence of the fusion region in plasmid pfdxN. (B) Primer extension analysis localizing the transcriptional start at minute 158.6, corresponding to nucleotides 541/542 (159 and 158.2 min). Top, sequencing reaction; bottom, primer extension.

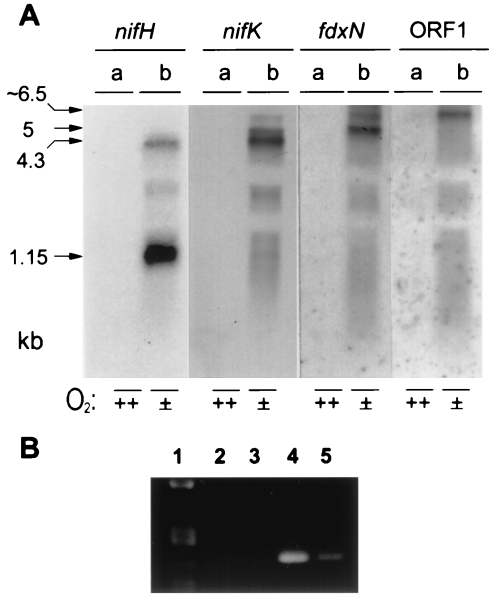

FIG. 3. Analysis of the *nifHDK* mRNA. (A) Northern blot analysis with RNA from *Azoarcus* sp. strain BH72 cells grown aerobically (lanes a) in VM medium containing combined nitrogen and microaerobically (lanes b) on N_2. Hybridization was carried out with probes directed against *nifH*, *nifK*, *fdxN*, and ORF1 as indicated above the lanes. Blank areas in lanes are of the sizes of rRNA. (B) RT-PCR using RNA of N_2-fixing cells of strain BH72 and primers annealing to *nifK* and *fdxN*, spanning 384 bp. Products were separated on a 1.5% agarose gel. Lane 1, size marker (lambda DNA digested with *Pst*I); lane 2, negative control (no RNA added); lane 3, RT inactivated by incubation at 95°C for 5 min prior to addition of 1.5 μg of RNA; lane 4, 1.5 μg of RNA added without heat inactivation; lane 5, 60 ng of chromosomal DNA of strain BH72 added to the RT-PCR mixture.

types at the first and the last time points by colony hybridization with a probe directed against the *fdxN* gene of strain BH72; in one experiment this was tested at several time points (Fig. 4B). While the proportions of wild-type and mutant strains were approximately 50% in the beginning of the experiment, the relative amount of the Δ*fdxN* mutant decreased during the course of the experiment (Fig. 4B). After 20 h or six generations, the wild type dominated the culture by a ratio of 4:1. Calculations of individual growth rates from these data revealed that the mutant BHΔfdxN had only 59% ± 9% of the growth rate of the wild type.

To investigate whether this growth deficiency was due to a decreased electron flux to nitrogenase or to other cellular processes, quantitative measurements of nitrogenase activity were carried out. To verify that the deficiency was due solely to the lack of FdxN and not to the destabilization of the *nifHDK* transcript, we complemented the *fdxN* gene in *trans* under the control of the *nifH* promoter of strain BH72, which was cloned into the mobilizable broad-host-range plasmid pLAFR3 to create pfdxN. Wild-type and mutant cells were precultured on N_2 in separate closed batch cultures and then transferred to fresh medium in microaerobic flasks (1% O_2) at 37°C (two experiments with three replicates each); after 3 h of incubation, acetylene (5%) was added, and the ethylene formation was quantified by gas chromatography after 1 h of incubation. While the wild type reduced 29.4 ± 2.4 μmol of acetylene/h/mg of protein, the *fdxN* deletion mutant reached significantly lower values ($P < 1\%$) of 23.9 ± 1.5 μmol of acetylene/h/mg of protein. This corresponded to approximately 81% of the wild-type nitrogenase activity rate. The complemented strain BHΔfdxN(pfdxN) restored the rates to 32.3 ± 1.7 μmol/h/mg of protein (110%, not significantly different from the wild-type value). Thus, a decrease in nitrogenase activity was observed which could be fully complemented by adding the *fdxN* gene in *trans*, suggesting that nitrogenase activity and thus also growth on N_2 were specifically affected by an impaired electron flow due to the lack of *fdxN* in strain BH72.

Because the diazotrophic growth was not completely abolished by the deletion of *fdxN*, we speculated that other electron donors, either other ferredoxins or flavodoxins, may be present in *Azoarcus* sp. strain BH72. In search of other, related ferredoxin genes chromosomal DNA was hybridized with a *fdxN*

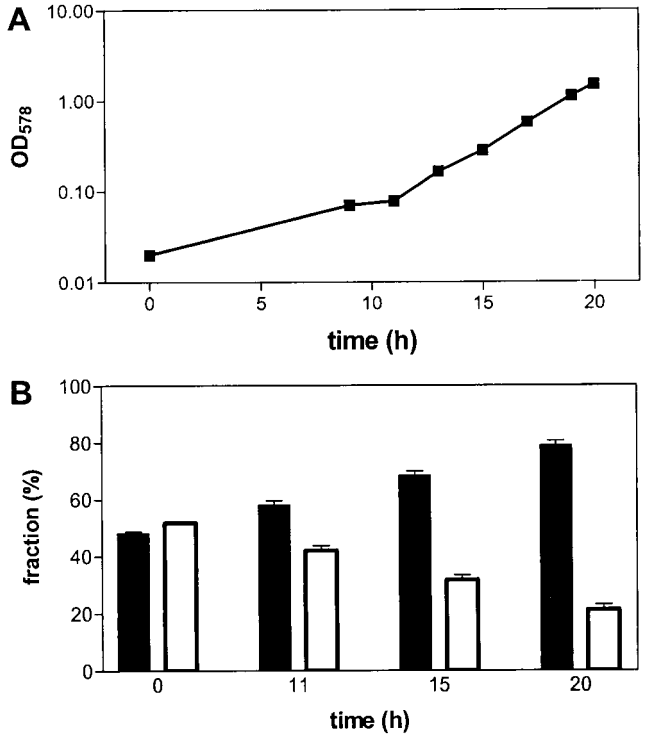

FIG. 4. Growth comparison of the wild type and the *fdxN* deletion mutant in mixed culture. The wild type and the *fdxN* deletion mutant were grown in an oxygen-controlled bioreactor on N_2 in mixed culture. (A) Growth measured as turbidity (OD_{578}). (B) Relative amount of each of the two strains at different time points during exponential growth of the mixed culture, determined by colony hybridization. Black bar, wild type; white bar, *fdxN* deletion strain. Values are means with standard deviations. For details of the calculations, see Materials and Methods.

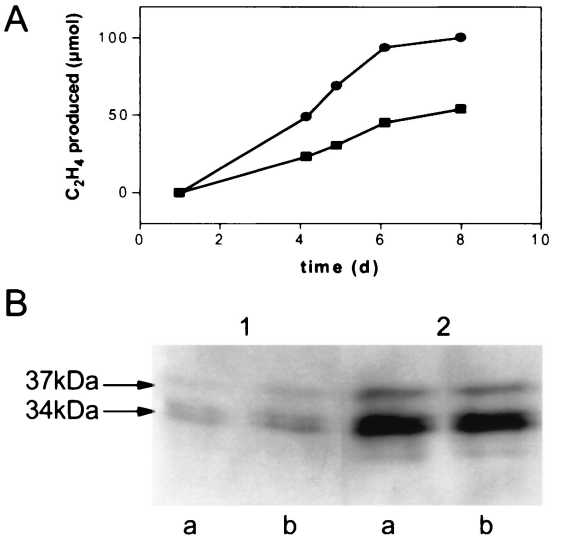

FIG. 5. Nitrogen fixation of the wild type and the *fdxN* deletion mutant in coculture. (A) Nitrogen fixation was measured as the amount of acetylene reduction per flask of *Acremonium alternatum* 2003 cocultured with *Azoarcus* sp. strain BH72 (circles) or with the mutant BHΔfdxN (squares). (B) Western blot analysis of cocultures with BH72 (lanes a) or BHΔfdxN (lanes b). Total protein extract of the coculture was diluted 2-fold (lanes 1) and 10-fold (lanes 2) and used for Western blot analysis with antibodies directed against the Fe protein of nitrogenase (34 and 37 kDa).

gene probe at low stringency. Specific hybridization signals were not observed at a hybridization temperature of 40°C with subsequent washing in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (data not shown). Thus, highly related ferredoxins were not detectable under these conditions. Whether flavodoxins may be alternatively used remains to be tested in the future.

Role of the ferredoxin FdxN for N_2 fixation of diazosome-containing cells. Coculture of *Azoarcus* sp. strain BH72 with the ascomycete *Acremonium alternatum* 2003 leads to formation of diazosomes, which occur in strain BH72 in a state of augmented activity and efficiency of nitrogen fixation (18, 20). Therefore, the effect of the *fdxN* mutation on nitrogen fixation was also analyzed in cocultures. The total ethylene formed per flask, measured when oxygen in the headspace had decreased to 2% at days 5 to 7, was determined in three independent experiments with five flasks each of strain BH72 and BHΔfdxN. Acetylene reduction of the mutant (6.9 ± 2.0 μmol of ethylene formed per flask) was significantly ($P < 0.0001$) different from that of the wild type (12.2 ± 2.6 μmol), corresponding to 56.6% of the wild-type fixation rate. A decreased acetylene reduction activity of the mutant was observed throughout the incubation period (Fig. 5A), when ethylene accumulates while the oxygen concentration decreases from 21 to 2% due to fungal and bacterial respiration (20, 32). Western blot analyses showed that comparable amounts of nitrogenase Fe protein were present in both cultures at the end of the experiment (Fig. 5B), indicating that the difference was caused not by decreased bacterial growth but by decreased nitrogenase activity. In both mutant and wild-type cells, diazosomes were detected (data not shown). Accordingly, an iron protein of nitrogenase of higher apparent molecular weight was observed (Fig. 5B), which is a covalently modified protein formed in diazosome-containing cells (20).

Effect of FdxN on rapid switch-off of nitrogenase in response to ammonium addition. Addition of 2 mM NH_4Cl to a nitrogen-fixing culture of *Azoarcus* sp. strain BH72 led to fast and complete (100%) inhibition of acetylene reduction, while cultures in N-free medium continued reducing acetylene. This nitrogenase switch-off was reversible, because nitrogenase activity was recovered within 30 to 50 min when only 0.2 mM ammonium was added (Fig. 6A), which was rapidly consumed to values below the detection limit (approximately 1 μM) by the bacteria within this time (data not shown). To assess whether the ferredoxin FdxN is involved in the process of nitrogenase inhibition, the deletion mutant BHΔfdxN with and without complementation of *fdxN* in *trans* was tested in switch-off experiments using 2 mM NH_4Cl. The complemented *fdxN* mutant showed almost wild-type-level inhibition of nitrogenase activity, while the *fdxN* mutant continued reducing acetylene (Fig. 6B). A rapid, complete switch-off was thus not observed in the *fdxN* mutant, but a slow retardation of nitrogenase activity which might be due to the repression of transcription of *nifHDK* genes by ammonium was observed.

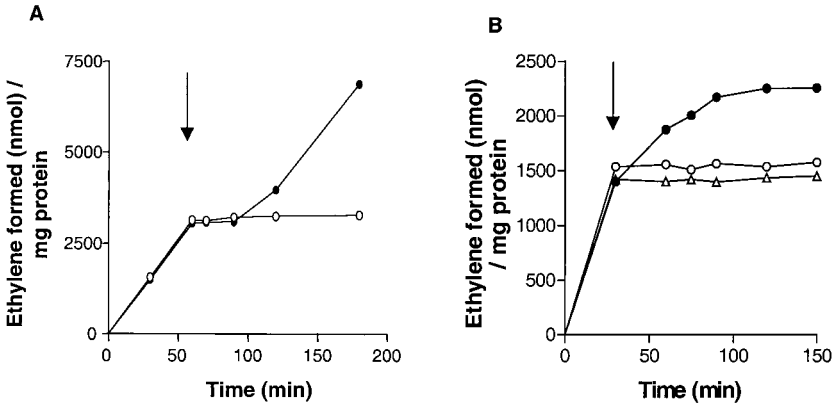

FIG. 6. Effects of ammonium addition on nitrogenase activity (acetylene reduction) of N_2-fixing cultures. (A) Reversible, fast, and complete inhibition (switch-off) of nitrogenase activity in *Azoarcus* sp. strain BH72 upon addition of 0.2 (closed circles) or 2 mM (open circles) NH_4Cl (final concentration). (B) Influence of ammonium addition (2 mM) on nitrogenase activity of BH72 (wild type; open circles), BHΔfdxN(pfdxN) (complemented mutant; triangles), and BHΔfdxN (deletion mutant; closed circles). Results are from one representative of three independent experiments where similar kinetics were observed.

DISCUSSION

Structural genes for the nitrogenase enzyme complex are often cotranscribed in one operon in bacteria. The structural nitrogenase genes *nifHDK* in *Azoarcus* sp. strain BH72 occur in a single copy on a large transcript that includes three more putative open reading frames, as shown by Northern hybridization and RT-PCR. While in *A. vinelandii* and *K. pneumoniae* the *nifHDK* genes are followed by *nifT*, a gene of unknown and nonessential function for nitrogen fixation (21, 55), the downstream gene in strain BH72 showed high sequence identities to bacterial 2[4Fe-4S] ferredoxins. Downstream of the *fdxN* gene, the operon structure resembles the situation in *A. vinelandii* with a *nifY* homologue and an open reading frame with weak homology to ORF1 (21). NifY is known to be involved in the maturation of nitrogenase (14) or may have a role in sensing and signaling the activity status of nitrogenase with respect to regulating *nifHDK* mRNA stability in *K. pneumoniae* (54), while a mutation in ORF1 had no obvious phenotype in *A. vinelandii* (21).

The *nifHDK* operon in *Azoarcus* sp. strain BH72 is transcribed only under N-limiting and microaerobic conditions (6). This is a common feature of free-living nitrogen-fixing bacteria, mediated by σ^{54}-dependent promoters (39). Also in this *Azoarcus* strain, the transcriptional start of the *nifHDK* mRNA corresponded in distance to $-12/-24$ promoter regions typical for σ^{54}-dependent promoters. Initiation of transcription from a σ^{54}-bound RNA polymerase needs to be facilitated by additional transcriptional activators (e.g., NifA or NtrC). Putative binding sequences for NifA were detected 120 and 140 bp upstream of the promoter region, suggesting a NifA-mediated regulation of nitrogen fixation in *Azoarcus* sp. strain BH72, as is commonly found in diazotrophic *Proteobacteria* (39).

In Northern blot analysis of the *nifHDK fdxN nifY* ORF1 operon of strain BH72, several different transcripts were observed, the full-length transcript appearing to be least abundant. As observed for *Azospirillum brasilense* (4), the *nifH* transcript appeared to be most abundant. Multiple transcripts of *nifHDK* mRNA were also observed for *A. vinelandii* (22) and *R. capsulatus* (57). Inverted repeat sequences potentially capable of forming stable stem-loop structures were detected in the intergenic regions of the latter two bacteria and also in strain BH72 between *nifHD*, *nifK fdxN*, and *fdxN nifY*. They might lead to differential termination of the transcript, or, as speculated for *R. capsulatus* (57), they may be a target for intramolecular processing of the *nifHDK* mRNA. Subsequent degradation of the full-length transcript from the 3′ end, giving stable intermediates that resisted RNA degradation, would be an alternative explanation for the transcript pattern observed in strain BH72. Formation of stem-loop structures may assist to protect RNA from 3′-end degradation (2). Differential stability of mRNA as a form of regulation was also demonstrated for other bacterial gene clusters, such as the *malEFG* operon in *Escherichia coli* (40) and the *puf* operon in *R. capsulatus* (34).

The close transcriptional linkage of the ferredoxin gene *fdxN* with the structural *nif* genes may imply a role for electron transport to nitrogenase in *Azoarcus* sp. strain BH72. 2[4Fe-4S] ferredoxins as electron carriers with a strong negative redox potential of −400 mV are known to play various roles in cellular electron transport. Ferredoxins and flavodoxins are proposed to be electron donors for nitrogenase in bacteria. In some organisms, such as *A. vinelandii* (26), *R. capsulatus* (52), or *Sinorhizobium meliloti* (33), *nif*-specific ferredoxins that are encoded in *nif* regions other than *nifHDK* have been identified. The *S. meliloti* ferredoxin was shown to be essential for symbiotic N_2 fixation with legumes. Localization of a ferredoxin gene in an operon of structural nitrogenase genes, which we describe here for an *Azoarcus* sp., has been reported only for *A. vinelandii*, where a ferredoxin-like gene is localized downstream of *vnfH*, which encodes the iron protein of vanadium nitrogenase (50).

Mutational and genetic complementation experiments in *Azoarcus* sp. strain BH72 showed that FdxN plays an important but not essential role in nitrogen fixation. The in-frame deletion of the *fdxN* gene reduced nitrogenase activity and diazotrophic growth in pure culture as well as nitrogen fixation of diazosome-containing cells in coculture with the ascomycete

Acremonium alternatum 2003 to a comparable degree (81, 59, and 56% of the wild-type rate, respectively). This defect is most likely due to a less efficient electron transport to dinitrogenase reductase in the absence of FdxN and not to destabilization of the nitrogenase gene mRNA, since nitrogenase activity could be fully restored by complementation of *fdxN* in *trans*. A similar nonessential role of a ferredoxin as an electron donor to nitrogenase was found, e.g., in *Anabaena* sp. (38), while in *S. meliloti* (33) and *R. capsulatus* (30) one ferredoxin was essential for nitrogen fixation. As no other ferredoxin gene with high sequence identity could be detected in *Azoarcus* sp., it is not clear whether the residual electron transport to nitrogenase is due to alternative ferredoxins or flavodoxins. As for *Azoarcus* sp., for most heterotrophic bacteria it is not yet known how ferredoxins are reduced. For *R. capsulatus*, the set of membrane-bound and Fe-S cluster-containing proteins of the *rnf* operon has been discussed as a candidate for electron donation to ferredoxins involved in N_2 fixation (28). The proteins show homology to NADH:ubiquinone oxidoreductase from *Vibrio alginolyticus*. (35). Analogous operons are also present in the fully sequenced genomes of *E. coli* and *Haemophilus influenzae* and suggest a general occurrence in bacteria (28).

That a ferredoxin can be essential for the physiological nitrogenase inactivation as detected in the *Azoarcus* sp. is a novel observation. Certain bacteria fixing N_2 react to a supply of ammonium rapidly by inactivation of nitrogenase activity (43, 49). The so-called nitrogenase switch-off by ammonium depends on two different mechanisms. In some diazotrophs, such as *Rhodospirillum rubrum* (43), *R. capsulatus* (31), and *Azospirillum brasilense* (9), the iron protein of nitrogenase (NifH) is subject to posttranslational modification, a reversible mono-ADP-ribosylation at a specific arginine residue. Additionally, a physiological switch-off mechanism which does not involve this covalent modification of nitrogenase exists in some bacteria (42). The mechanism is still unknown. Here we report that such a rapid reversible physiological switch-off mechanism also occurs in *Azoarcus* sp. strain BH72, similar to those in *R. capsulatus* and *Azospirillum brasilense* in terms of speed and extent of nitrogenase inhibition. Nitrogenase activity was almost completely abolished within a few minutes upon addition of ammonium. In contrast, nitrogenase switch-off in *Rhodospirillum rubrum* occurs more slowly and is incomplete (60). In *Rhodospirillum rubrum*, ammonium-induced switch-off was shown to be absolutely dependent on ADP ribosylation of NifH (36, 61), whereas in *R. capsulatus* and *Azospirillum brasilense*, modification of NifH is not absolutely required, indicating a second mechanism of regulation (7, 8, 42, 58, 61). Surprisingly, the rapid complete switch-off in response to ammonium was abolished in the *fdxN* deletion mutant of *Azoarcus* sp. strain BH72; only a slow decrease of nitrogenase activity was still observed. This suggested that the ferredoxin may be part of the mechanism of the process of rapid switch-off. This would be in concordance with the hypothesis that changes in the redox state of NifH or electron flux towards nitrogenase may be factors involved (12, 42), but no sensor or signal transduction proteins have been identified up to now. The essential role of FdxN for nitrogenase switch-off also suggests that it is the major electron donor for nitrogenase in wild-type BH72: putative alternative electron donors are apparently not able to compensate for the switch-off function in the deletion mutant, although they still allow approximately 50% of the nitrogen fixation activity. Therefore, they are unlikely to operate in the wild type but might be more abundant in the deletion mutant, as speculated for *R. capsulatus* (30).

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