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Azoarcus Grass Endophytes Contribute Fixed Nitrogen to the Plant in an Unculturable State

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The extent to which the N_2-fixing bacterial endophyte *Azoarcus* sp. strain BH72 in the rhizosphere of Kallar grass can provide fixed nitrogen to the plant was assessed by evaluating inoculated plants grown in the greenhouse and uninoculated plants taken from the natural environment. The inoculum consisted of either wild-type bacteria or *nifK*$^-$ mutant strain BHNKD4. In N_2-deficient conditions, plants inoculated with strain BH72 (N_2-fixing test plants) grew better and accumulated more nitrogen with a lower $\delta^{15}N$ signature after 8 months than did plants inoculated with the mutant strain (non-N_2-fixing control plants). Polyadenylated or polymerase chain reaction-amplified BH72 *nifH* transcripts were retrieved from test but not from control plants. BH72 *nifH* transcripts were abundant. The inocula could not be reisolated. These results indicate that *Azoarcus* sp. BH72 can contribute combined N_2 to the plant in an unculturable state. Abundant BH72 *nifH* transcripts were detected also in uninoculated plants taken from the natural environment, from which *Azoarcus* sp. BH72 also could not be isolated. Quantification of nitrogenase gene transcription indicated a high potential of strain BH72 for biological N_2 fixation in association with roots. Phylogenetic analysis of nitrogenase sequences predicted that uncultured grass endophytes including *Azoarcus* spp. are ecologically dominant and play an important role in N_2-fixation in natural grass ecosystems.

The extent to which potentially nitrogen-fixing bacteria in the rhizosphere of gramineous plants are functional in providing combined N_2 to the plant is a long-standing question (Boddey et al. 1995; Bormann et al. 1980; van Berkum and Bohlool 1980). For several uninoculated, soilgrown *Gramineae* spp., ^{15}N-enriched tracer studies have shown that microbial communities can fix substantial amounts of atmospheric N_2 from which the plants profit significantly (Boddey et al. 1995; Giller and Day 1985). It was estimated that up to 70% of plant nitrogen originates from biological nitrogen fixation for certain Brazilian sugar cane cultivars (Boddey 1995; Urquiaga et al. 1992) or 20% for certain Asian rice varieties (Boddey et al. 1995; Shrestha and Ladha 1996). Identifying the diazotrophic bacteria responsible for the N-gain is important for agricultural applications as well as for understanding ecosystem processes (Bormann et al. 1980; Reinhold-Hurek and Hurek 1998) because nitrogen is one of the most important limiting nutrients for ecosystem production. However, most of the studies attempting to identify the functionally active diazotrophs are based on classical isolation techniques; although for most ecosystems, it is known that the majority of the bacteria cannot be cultivated yet (Pace 1997). Because of a tight association with the plant, it has been suggested that nitrogen-fixing grass endophytes contribute nitrogen to the plant (Boddey et al. 1995; James and Olivares 1998; Reinhold-Hurek and Hurek 1998).

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Grass endophytes are non-nodule-forming, nonleguminous microorganisms that spend most of their life cycle inside plant tissues without causing symptoms of plant damage. Diazotrophic grass endophytes, such as *Azoarcus*, *Herbaspirillum,* and *Acetobacter* spp., differ markedly from other plant-colonizing bacteria such as *Rhizobium* or *Azospirillum* in that they do not survive well in and often cannot be isolated from root-free soil (James and Olivares 1998; Reinhold-Hurek and Hurek 1998). However, it is not known which bacteria are responsible for that fixation in all cases investigated so far, except for one recent study on *Acetobacter diazotrophicus* associated with sugar cane (Sevilla et al. 2001).

In addition, *Azoarcus* sp. strain BH72 is likely to be a functionally active grass endophyte. In Kallar grass (*Leptochloa fusca* (L.) Kunth), which yields two to four hay harvests per year without application of nitrogen fertilizer on low-fertility, often flooded soil in Pakistan (Reinhold-Hurek et al. 1993b), it was found to be the predominant endophytic isolate from roots (Reinhold et al. 1986). In situ hybridization experiments demonstrated that *Azoarcus* sp. nitrogenase genes are expressed in the aerenchyma of field-grown Kallar grass roots (Hurek et al. 1997a). Light and electron microscopic studies revealed that strain BH72 is also able to infect rice seedlings and promote their growth, although there was no evidence for a contribution of fixed nitrogen in this gnotobiotic system (Hurek et al. 1994). In a different gnotobiotic culture system, *Azoarcus* sp. strain BH72 shows high levels of nitrogenase gene expression inside roots of rice seedlings as demonstrated by reporter gene studies using transcriptional fusions between the genes encoding the iron protein of nitrogenase (*nifH*) and the green fluorescent protein (*gfp*) or β-glucuronidase (*gus*) (Egener et al. 1998, 1999). However, these experiments did not prove whether *Azoarcus* sp. strain BH72 is capable of contributing significant amounts of fixed nitrogen to a host plant and is the predominantly active N_2-fixing endophyte in its host plant in situ.

Azoarcus sp. BH72 (Hurek et al. 1994) and other diazotrophs such as *Azospirillum* spp. (Okon and Labandera-Gonzalez 1994) can stimulate plant growth by mechanisms other than nitrogen fixation; therefore, we used a *nif* mutant as a control inoculum to evaluate the amount of fixed nitrogen contributed to the plant by the wild type bacterium. To identify the most active diazotrophic bacterium (in inoculated or uninoculated, in situ-grown plants), we developed molecular, culture-independent methods. For this purpose, culturing techniques have been widely used, but these techniques may lead to misinterpretations of the composition of the active population of diazotrophs and cause biased results due to cultivation techniques. Furthermore, widely used molecular techniques of *nif* DNA retrieval from the natural environment (Lovell et al. 2000; Ohkuma and Kudo 1996; Ueda et al. 1995) only show the mere presence of nitrogenase genes or the diversity of diazotrophs. However, only studies on *nif*-mRNA expression can evaluate the actual activity of a particular microbe within a pool of other diazotrophs in an uninoculated natural system. This applies also for inoculation experiments where plants might be contaminated by other diazotrophs, raising the question of who is responsible for the observed nitrogen contribution.

Expression of nitrogenase genes at the time of sampling can be detected by reverse transcription–polymerase chain reaction (RT-PCR). In *Azoarcus* sp. BH72 (Egener et al. 1998, 1999), as also in many other diazotrophs (Merrick and Edwards 1995), there is a tight relationship between nitrogenase activity and *nifH* transcription. In the presence of combined nitrogen or unfavorable oxygen concentrations for nitrogen fixation, nitrogenase expression also is repressed. One possible discrepancy is the rapid inactivation of nitrogenase activity upon addition of ammonium, leading to an immediate cessation in nitrogen fixation while mRNA is still present (Egener et al. 2001). However, in the equilibrium situations in the natural environment, rates of transcription will have been adjusted accordingly. Moreover, in bacteria, mRNAs underlie a rapid turnover. Therefore, RT-PCR followed by comparative sequence analysis of *nifH* mRNAs are methods of choice for quantification of nitrogen fixation as well as identification and evaluation of nitrogen-fixing bacteria in situ. These techniques have been used recently to detect mRNA of several genes, including *nifH,* in the natural environment (Kowalchuk et al. 1999; Noda et al. 1999; Zani et al. 2000) and have been adopted by us to study root-associated nitrogen fixation.

Here we show by three independent lines of evidence (two molecular and one isotopic) that inoculated plants benefit from N_2-fixation by *Azoarcus* sp. BH72, and provide evidence that other bacteria, including *Azoarcus* spp., may be functionally similar. We also show that *nifH* (encoding the iron protein of nitrogenase) of *Azoarcus* sp. strain BH72 is abundantly expressed in the rhizosphere of naturally grown and inoculated Kallar grass, although this bacterium could not be cultured anymore. Unprecedentedly so far in nonleguminous plant ecosystems, one defined bacterium proved to provide fixed nitrogen to the plant in an unculturable state.

Table 1. Growth parameters of Kallar grass plants inoculated with *Azoarcus* sp. BH72

Parameter	*nifk*⁻ Mutant[a]	Wild type[a]
Dry weight (g)		
Plant	0.61 (0.08)[b]	1.02 (0.13)
Shoot	0.37 (0.03)[b]	0.62 (0.08)
Root	0.24 (0.08)[c]	0.40 (0.08)
Total nitrogen (mg)		
Plant	2.8 (0.4)[b]	4.2 (0.5)
Shoot	1.5 (0.4)[c]	2.4 (0.04)
Root	1.2 (0.2)	1.9 (0.5)
Soil	1.7 (0.2)[d]	1.6 (0.2)[d]
$\delta^{15}N$ (‰)		
Plant	+0.2 (0.5)[b]	–3.2 (0.9)
Shoot	–3.1 (0.3)	–4.2 (0.9)
Root	+4.1 (0.8)[b]	–2.5 (1.3)
Soil	n. d.[e]	–0.68 (0.1)

[a] Values are means of n = 4 with 4 to 6 plants each, standard deviations given in parenthesis.
[b] Treatments were different at $P < 0.005$.
[c] Treatments were different at $P < 0.05$.
[d] Per compartment. Before planting, soil contained 2 (0.3) mg total nitrogen per compartment and had a $\delta^{15}N$ of – 0.63 (0.02); n = 3.
[e] n.d. = not determined.

RESULTS

Plant response to inoculation.

Kallar grass seedlings pregrown on a substrate containing combined nitrogen were transferred to an inoculated, nitrogen-poor substrate without further N fertilization. The inoculum consisted of either wild-type *Azoarcus* sp. BH72 (for N_2-fixing test plants) or of the isogenic *nifK*⁻ mutant strain BHNKD4 (non-N_2-fixing control plants). Final dry weight, total nitrogen content, and $\delta^{15}N$ of plants were determined in four separate experiments. Plants inoculated with wild-type bacteria had significantly higher dry weights, lower $\delta^{15}N$, and 1.4 mg more nitrogen than did plants inoculated with strain BHNKD4 (Table 1). Our N-balance indicated that this difference in plant nitrogen was not originating from N-uptake from the potting medium. Over the 8 months, plants were not fertilized with combined nitrogen. The total soil N in the compartment decreased equally in both treatments; however, by only 0.4 to 0.3 mg of N (Table 1).

In addition, our isotopic data indicated that the soil was not a major source of the observed nitrogen gain (Table 1). The ratio between the natural isotopes ^{15}N and ^{14}N ($\delta^{15}N$ signature), which can be determined by a high-resolution mass spectrometer, is characteristic of the nitrogen source used in a system. Generally, nitrogenase discriminates the heavier isotope, leading to a decreased abundance of ^{15}N when N_2 is the source of nitrogen (Hoering and Ford 1960). The $\delta^{15}N$ signature of the whole plant thus reflects the $\delta^{15}N$ signature of the immediate nitrogen source. The $\delta^{15}N$ signature of the plants which showed the nitrogen gain (plants inoculated with the wild type) was significantly different from the $\delta^{15}N$ signature of the potting medium (Table 1); therefore, these plants must have had access to nitrogen sources other than potting medium. Moreover, the difference in the $\delta^{15}N$ signature of these plants compared with the control plants indicated that the plants in both treatments had access to different nitrogen sources. A strong decrease in natural ^{15}N abundance (wild-type inoculum, –3.2‰; *nifK*⁻ inoculum, +0.2‰) indicated a contribution of fixed nitrogen in the plant inoculated with the wild-type *Azoarcus* sp. We also determined the ^{15}N-discrimination in pure cultures of strain BH72 when grown on N_2. A similarly negative signature ($\delta^{15}N$, –3.9‰) as in free-living cultures of *Anabaena* (–3.8‰) or *Azotobacter* (–4.4‰) (Rowell et al. 1998) was detected, corroborating the fact that the plant nitrogen gain had to be attributed to N_2 fixation. The slight deviation of the $\delta^{15}N$ signature of the control plants compared with the N-poor potting medium originates from the pregrowth of the seedlings in a different substrate.

Reisolation of the inoculum.

Koch's postulates provide criteria to determine whether a certain bacterium is the agent causing a certain disease. One of these criteria is to reisolate a bacterium from a patient showing

the symptoms after he had been infected with this bacterium. In analogy, the bacterium causing the nitrogen gain of a plant after inoculation should be reisolated from these plants. Therefore, we attempted to reisolate a *Azoarcus* sp. 3 months after inoculation and at harvest, using established protocols which had led to enrichment and isolation of *Azoarcus* spp. originally (Reinhold et al. 1986; Reinhold-Hurek et al. 1993b). All attempts to do so failed; cells with morphology or 16S rDNA sequences (Hurek et al. 1993) typical of strain BH72 could not be enriched from dilutions of macerated roots or root pieces or the shoot. The presence of strain BH72 in roots and shoots also was investigated by 16S rDNA-directed PCR. Total DNA extracted from roots at the time of harvesting was subjected to PCR with primers TH14/TH2 followed by oligonucleotide hybridization which specifically detects *Azoarcus* spp. (Hurek et al. 1993). Hybridization of amplification products was obtained from plants inoculated with both wild-type or *nifK*$^-$ *Azoarcus* (not shown), showing that both *Azoarcus* strains were present in roots, albeit the signal obtained from the *nifK* mutant was weaker. Thus, both strains were present but probably not culturable. No amplification product was obtained from shoot preparations, indicating that either the *Azoarcus* sp. did not colonize the shoot or colonization was below the detection level (data not shown).

Analysis of root-associated *nifH* mRNA by polyadenylation- and *nifH*-specific RT-PCR from inoculated plants.

Molecular ecological methods were developed in order to retrieve and analyze bacterial nitrogenase gene (*nifH*) mRNA from plant roots. We used (i) screening of a cDNA library and (ii) *nifH*-specific RT-PCR to show that *Azoarcus* sp. BH72 was expressing nitrogenase genes. Surprisingly, in *Azoarcus* sp. BH72, mRNA was polyadenylated as shown with a digoxigenin-labeled poly (dT) oligonucleotide as a probe in Northern blot hybridization experiments (Fig. 1A). The polyadenylated mRNA appeared as a smear from 2 kb down with its greatest intensity <1 kb, suggesting that polyadenylation in this bacterium occurs after posttranscriptional processing or degradation of polycistronic mRNA with a high fraction of polyadenylated degraded mRNA molecules. To detect mRNAs nonselectively, we screened a library which was synthesized on poly(A)$^+$ RNA isolated from roots of inoculated Kallar grass at harvest. Among 20,000 colonies, one *nifH* cDNA clone was detected. It carried a truncated *nifH* cDNA identical to the *Azoarcus* sp. BH72 *nifH* gene that was polyadenylated within the coding region (Fig. 1B). The 85-nt-long poly(A) tract was interspersed with other nucleotides as in polyadenylation of T7 mRNAs (Johnson et al. 1998) or chloroplast *pbsA* mRNAs (Lisitsky et al. 1996). The retrieval of this clone, albeit truncated, showed that *Azoarcus* sp. BH72 was metabolically active and expressed nitrogenase genes in roots of inoculated Kallar grass.

This conclusion was supported by RT-PCR with universal primers for the *nifH* gene, which was carried out on total RNA extracted from roots 3 and 8 months after inoculation. RT-PCR amplification resulted in DNA fragments which hybridized with a *nifH* probe from strain BH72 (Fig 2A), whereas a non-template control in which reverse transcriptase was inactivated was negative (Fig. 2A). Sequencing of three representative clones (362-bp inserts) revealed 99.7% nucleotide identity with the respective *nifH* sequence of *Azoarcus* sp. BH72. This confirmed that *nifH* expression was from the inoculum. No product was obtained with RNA preparations from non-N_2-fixing control plants inoculated with the *nifK*$^-$ mutant, in contrast to DNA preparations where a band amplified which did not hybridize (not shown). This suggested that *nifH* mRNA levels in control plants were not sufficiently high to allow detection in our assay, although diazotrophic bacteria were present in the rhizosphere. Apparently, during incubation in the greenhouse, the non-N_2-fixing control plants had become contaminated by bacteria other than the wild-type inoculum that did not fix N_2 at the time of sampling, although they were probably able to do so. The expression of BH72 *nifH* in test plants, but not in control plants, confirmed that the source of plant nitrogen for

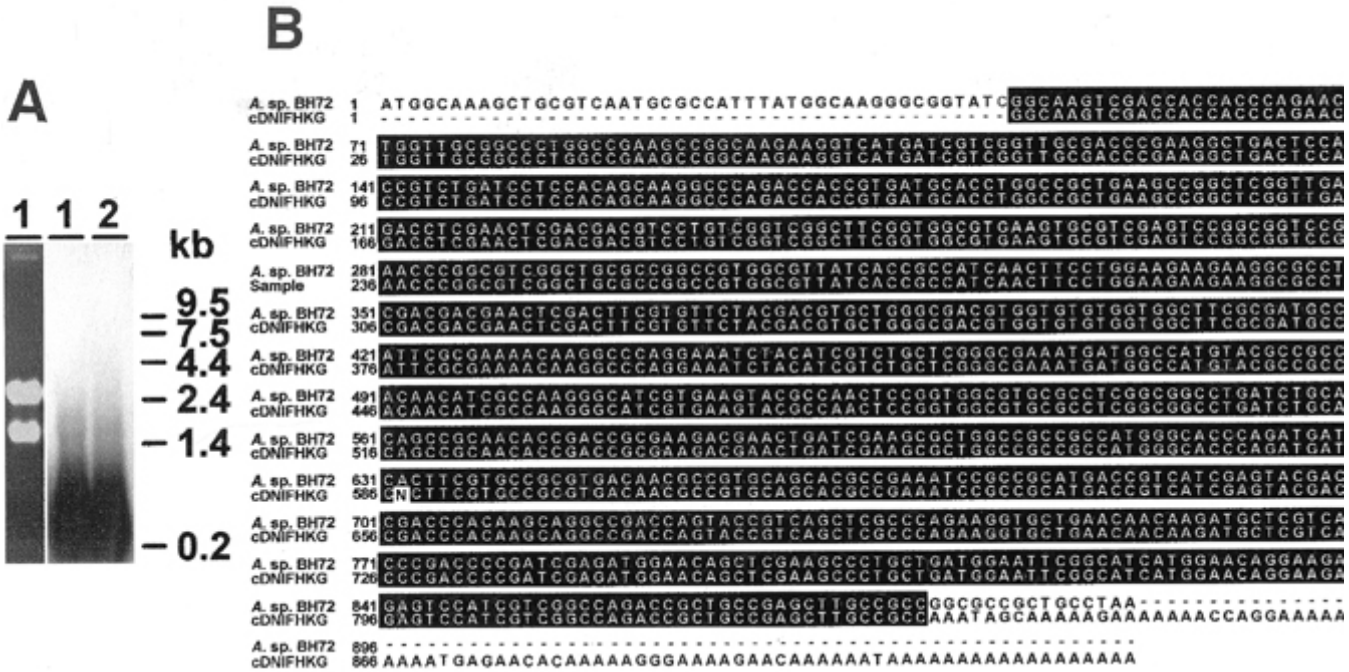

Fig. 1. Polyadenylation of *Azoarcus* mRNA. **A,** Agarose gel (left) and corresponding northern blot (right) of mRNA from *Azoarcus* sp. strain BH72 hybridized with a 27-base poly(dT) oligo at 54°C. Lane 1, total mRNA; lane 2, RNA purified with oligo(dT) cellulose. **B,** Alignment of the *nifH* DNA sequence of *Azoarcus* sp. strain BH72 with the sequence of a cDNA clone retrieved from a library of polyadenylated RNA from roots of inoculated Kallar grass; identical nucleotides are boxed in black.

the test plants was N_2-fixation by *Azoarcus* sp. BH72. No *nifH* PCR product was obtained from shoot DNA preparations, confirming our data from 16S rDNA directed PCR amplifications (data not shown).

Analysis of *nifH* mRNA in roots of noninoculated field-grown plants.

Studies in the natural environment are always required to evaluate laboratory experiments. In order to evaluate the role of *Azoarcus* sp. BH72 as a diazotroph in the environment, we collected soil cores containing Kallar grass from a waterlogged, salt-affected field in the Punjab of Pakistan which was surveyed for N_2-fixing bacteria in 1984 and 1988 (Reinhold-Hurek et al. 1993b). Additionally, soil cores containing *Oryza minuta* from a lowland area at Dayap, Laguna, Philippines (121°E, 15°N) were collected. Plants were grown in the original, permanently wet soil without application of nitrogenous fertilizer for several months in the phytotron, in order to deplete the nitrogen from the soil. To demonstrate that *Azoarcus* sp. BH72 was initially present in the rhizospheres of the samples and was not introduced later, roots were analyzed immediately after arrival using 16S rDNA-targeted, *Azoarcus* spp.-specific PCR protocols (see above), and by classical isolation procedures. Again, attempts to isolate *Azoarcus* sp. BH72 failed. In 16S rDNA clone libraries of Kallar grass, but not of *Oryza minuta,* 385-bp inserts with the identical sequence of this organism were found (not shown). This indicates that *Azoarcus*. sp. BH72 is a constant member of the natural microbial community associated with Kallar grass at the site of study in Pakistan.

After 3 months, Kallar grass roots were sampled and analyzed by RT-PCR for the occurrence of *nifH* transcripts. Amplification products were obtained from RNA (RT-PCR) as well as from DNA (PCR) preparations of Kallar grass roots (Fig. 2B). Southern analyses, using *nifH* probes from *Azoarcus* sp. BH72 and *Azospira oryzae* 6a3, indicated that the diversity among *nifH* amplicons was nonrandom (Fig. 2B). In Kallar grass, a majority of *nifH* transcripts and DNA amplicons were highly related to the gene from *Azoarcus* sp. BH72 and not to *nifH* genes typical for α-*Proteobacteria*, to which the *nifH* probe from *Azospira oryzae* 6a3 is highly homologous (Hurek et al. 1997a).

As a control for the efficiency of nucleic acid extraction, cells of the Gram-positive bacterium *Bacillus subtilis* were added to the root samples prior to the lysis. Nucleic acids could be detected by 16S rDNA-targeted PCR using specific primers (Fig. 3); thus, our method appeared to be effective for disruption of cells with different cell wall types. Therefore, our amplifications probably reflected the quantitative abundance of templates in the environmental samples.

To avoid interpreting misincorporations in early PCR cycles as template difference (Handt et al. 1996), sequences were determined from two independent RT-PCR amplifications. Six random clones were analyzed. All sequences showed 99.7% nucleotide identity to the respective *nifH* fragment of strain BH72. *NifH* and 16S rDNA sequence comparisons between closely related *Azoarcus* spp. (Hurek et al. 1997a; Reinhold-Hurek et all. 1993b) show strain level divergence or even organismal identity (Fig. 4). This shows that the environmental cDNA sequences are clearly derived from *Azoarcus* sp. BH72-type bacteria.

Estimation of *nifH*-mRNA abundance in roots.

In order to estimate how large the nitrogen-fixing populations were, we quantified *nifH* transcription by calibrating the RT-PCR. Pure cultures of *Azoarcus* sp. BH72 were grown under conditions optimal for N_2-fixation in an oxygen-controlled bioreactor (Hurek et al. 1987), total RNA was extracted, and serial dilutions were subjected to a most-probable-number (MPN) (n = 3) RT-PCR analysis. RT-PCR reactions were run in triplicates from three RNA preparations using the template concentrations mentioned in Figure 5. The numbers of positive and negative tubes that produced amplification products were scored, constituting the complete eight dilutions MPN RT-PCR data matrix. In all amplifications, products were obtained from 100 to 2,000 ng of RNA template. No bands were obtained from 1, 10, or 50 ng of RNA. The MPN estimate was determined to be 20.17. From dividing the undiluted RNA template by the MPN estimate, it was calculated that the RT-PCR detection limit was 99.2 ng of RNA, corresponding to 3.3×10^7 cells of strain BH72. Also, the RNA extracted from roots was subjected to a MPN RT-PCR (n = 4, five dilutions), starting with 5 of the 25 µl of RNA dissolved in diethyl pyrocarbonate-(DEPC) treated water. Using the same procedures detailed

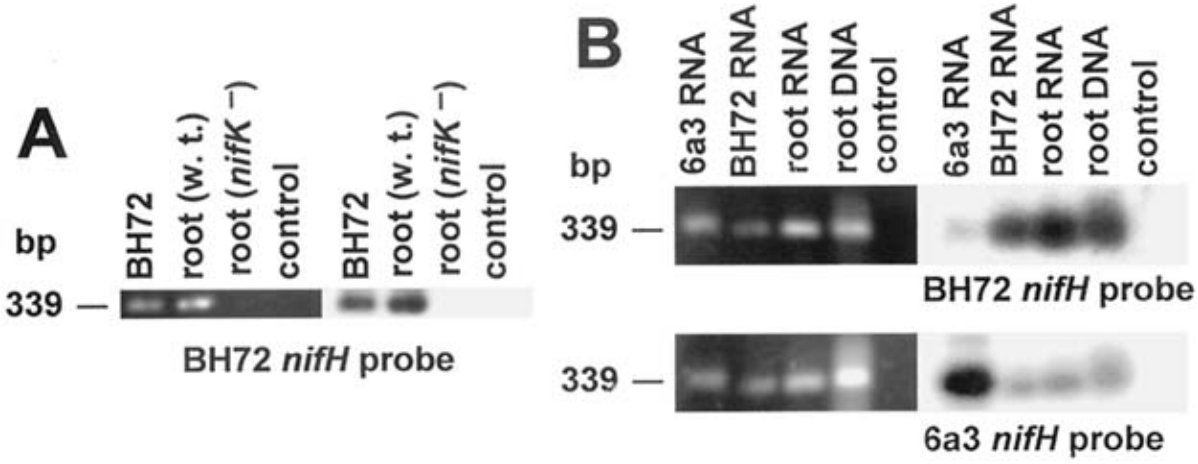

Fig. 2. Expression of *nifH* genes associated with roots of Kallar grass. **A** and **B,** Representative agarose gel (left) and corresponding Southern Blot (right) of polymerase chain reaction (PCR) products amplified with universal primers for *nifH.* **A** and **B,** Reverse transcription (RT)-PCR from RNA or **B,** PCR from DNA. Typical data from four RNA and DNA extractions are shown. **A,** Root samples taken from plants inoculated with *Azoarcus* sp. (2-µl templates) or **B,** from a natural site in the Punjab of Pakistan (0.5-µl templates). Controls, **A,** PCR amplification of RNA extracted from roots inoculated with strain BH72 or **B,** field-grown plants after heat inactivation of reverse transcriptase. Positive controls: RNA from *Azoarcus* sp. strain BH72 or *Azospira oryzae* 6a3. **B,** Root DNA: sample taken after RNA extraction prior to DNase treatment. Hybridization of Southern blots was carried out at high stringency with digoxygenin-labeled DNA probes of *nifH* genes of strains BH72 or 6a3, respectively.

above, the detection limit for *nifH* transcripts was estimated to be 1 µl of template from RNA preparations of plants inoculated with the wild type, corresponding to 1×10^{10} nitrogen fixing bacteria per gram of root dry weight. In the environmental samples (Kallar grass roots) the detection limit was two times lower and, accordingly, the level of *Azoarcus*. sp. BH72 *nifH* mRNA twice as high.

Distribution of *nifH*-mRNA in roots of rice.

In order to determine whether unculturability of plant-associated bacteria highly active in nitrogen fixation is a widespread phenomenon which is not only restricted to *Azoarcus* sp. BH72 and Kallar grass, we screened *nifH* cDNA and DNA clone libraries from the wild rice *Oryza minuta*. This plant had been surveyed for the presence of culturable nitrogen fixing bacteria before (Engelhard et al. 2000). Our screen revealed a higher diversity of N_2-fixing bacteria than found with Kallar grass. MPN RT-PCR revealed that the level of *nifH* mRNA corresponded to 1×10^{10} nitrogen fixing cells per gram of root dry weight. Clone libraries were constructed from the RT-PCR product and from *nifH* amplicons obtained from rice rot DNA. None of the eight clones picked at random harbored *nifH* sequences related to *Azoarcus* sp. BH72.

To determine the evolutionary relationships between the NifH sequences from diazotrophs associated with wild rice and those associated with Kallar grass, we performed a phylogenetic tree analysis as outlined by Hurek and associates (1997a) on NifH protein sequences from cultivated and uncultivated microorganisms (Fig. 6). This analysis identified two cDNA clones from roots of wild rice (Om13B and Om212A) as members of the Kg-2 and Kg-3 *nifH* clusters with bootstrap values of 70 and 98%, respectively. Two other clones from *Oryza minuta* (Om16D and OmA45) are highly related to *nifH* sequences 15(38) and 15(101) retrieved from roots of modern rice in Nepal (Engelhard et al. 2000) and to H-RIC19 and H-RIC14 from roots of *O. sativa* L. cv. nihonbare in Japan (Ueda et al. 1995). Numerous other *nifH* cDNA clones were recently retrieved from roots of other rice species which cluster with these sequences at high bootstrap values (T. Hurek, *unpublished data*). Since these sequences frequently occur and have been first detected in *Oryza* spp., we propose to call this group of entirely uncultivated microorganisms Ω-cluster. The other DNA or cDNA sequences from rice clustered with *nifH* genes from α-*Proteobacteria*, from Gram-positives, or, as in the case of OmD25, could not be assigned to other described bacteria.

DISCUSSION

There are many reports of plant growth promotion of gramineae by diazotrophic bacteria, such as *Azospirillum* spp. (Okon and Labandera-Gonzalez 1994), or even rhizobia (Biswas et al. 2000; Yanni et al. 1997). However, in all cases except for *Acetobacter diazotrophicus* and sugarcane (Sevilla et al. 2001), this growth response was not a result of the supply of biologically fixed nitrogen (Bashan and Holguin 1997; Yanni et al. 1997). Due to the following line of evidence, we have demonstrated that *Azoarcus* sp. BH72 can contribute significant amounts of fixed nitrogen to its host plant Kallar grass. First, after 8 months, plants inoculated with wild-type bacteria had higher dry weights, accumulated more nitrogen ($P < 0.05$), and had lower $\delta^{15}N$ signatures ($P < 0.005$) than plants inoculated with a *nifK*$^-$ mutant strain. The relatively small change of initial and final nitrogen content of the potting medium and its higher $\delta^{15}N$ signature with respect to wild type-treated plants in these experiments demonstrated that wild type-treated plants must have had access to combined nitrogen from nitrogen fixation. Second, sequence identity of retrieved partial *nifH* transcripts with the published *nifH* sequence of *Azoarcus* sp. BH72 (Egener et al. 2001) indicated that nitrogen fixation by *Azoarcus* sp. BH72 and not by other adventitious diazotrophic bacteria had provided combined nitrogen to the plant. Third, the low $\delta^{15}N$ signature of shoots indicated that combined nitrogen had been transferred from bacteria to plant material, because the inoculum could be not detected in the shoot. However, it is not clear yet whether this nitrogen had been transferred directly or after decay of the bacteria.

If our data are extrapolated to an annual hectare basis, approximately 34 kg of N ha^{-1} $year^{-1}$ were derived from N_2-fixation, an agronomically useful value, which would amount to approximately 50% of the nitrogen required for sustaining traditional wetland rice culture (Bennet and Ladha 1992). This indicates that biological nitrogen fixation by a particular endophyte can contribute substantially to plant growth. Our estimate is more than twice the contribution of nitrogen fixation calculated for hardwood species in the northeastern United States (Bormann et al. 1980). However, it is considerably lower than the estimated input of 150 kg of N ha^{-1} $year^{-1}$ by biological N_2 fixation in outdoor-grown sugar cane plants (Boddey et al. 1995). Probably, low light levels at certain periods of the year in the glasshouse prevented a full development of the N_2-fixation potential in the *Azoarcus* plant association.

In contrast to *Acetobacter diazotrophicus* and sugar cane, where the N_2-fixing bacteria contributing nitrogen to the plant remain culturable (Sevilla et al. 2001), in *Azoarcus* sp. BH72 and Kallar grass they became unculturable. As had been the case in 1988 (Reinhold-Hurek et al. 1993b), an attempt to isolate *Azoarcus* sp. BH72 failed, due to the low abundance of culturable diazotrophic endophytes. Wild-type and mutant *Azoarcus* sp. BH72 could not be reisolated using established protocols (Reinhold et al. 1986; Reinhold-Hurek et al. 1993b); therefore, the only means by which Koch's postulates could be fulfilled and the N_2-fixing agent be identified was by molecular taxonomy. We also intended to show that the identified N_2-fixing agent and not an adventitious microbe was highly active in nitrogen fixation; therefore, we had to make extensive use of molecular methods in microbial ecology.

Methods to obtain sequence data from microbial communities or pure cultures either include or omit a PCR amplification step. Although a routine method, PCR amplification can cause several problems, such as the retrieval of no, contaminating, or artefactual sequences (von Wintzingerode et al. 1997). The identification of polyadenylated *nifH* mRNA from unculturable *Azoarcus* sp. BH72 in roots of inoculated plants without a PCR amplification step indicated unambiguously that the inoculum was metabolically active and fixed nitrogen. Although it is established that RNA may be polyadenylated in prokaryotes as it is in eukaryotes (Cohen 1995), it is not known how widespread polyadenylation among bacteria is. Therefore, the retrieval of polyadenylated *nifH* mRNA is currently not a good

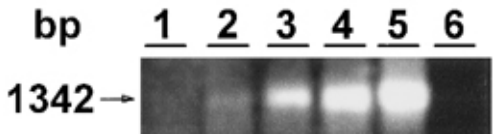

Fig 3. Control polymerase chain reaction (PCR) for lysing efficiency of RNA extraction protocol. Agarose gel of PCR products amplified using a combination of universal and taxon specific 16S rDNA-targeted primers for *Bacillus subtilis*. Prior to RNA extraction, no (lane 1), 10^4 (lane 2), 10^6 (lane 3), and 10^8 (lane 4) washed cells of *B. subtilis* were added to 400 mg root fresh weight of Kallar grass from experiment A; the DNase step was omitted from the purification protocol, and the sample used as template for PCR. Pure control DNA of *Bacillus subtilis* (lane 5), no-template control (lane 6).

method to evaluate N_2 fixation of diverse microbial populations as they occur in natural environments.

For the quantitative evaluation of *nifH* messengers in microbiologically undefined environments, PCR amplification with the universal primers of Zehr and McReynolds (1989) proved to be the method of choice. High abundance of partial *nifH* transcripts from *Azoarcus* sp. BH72, also in environmental samples from naturally grown Kallar grass in the Punjab of Pakistan, confirmed the important role of this bacterium in biological N_2 fixation with this plant in the natural environment. The phylogenetic analysis of the retrieved NifH sequences which we obtained agreed in all major clades with a previous study on the same coding region (Zehr et al. 1998). None of these sequences are represented by cultivated bacteria. Except for one sequence which clusters with *Azoarcus* NifH phylotypes (Hurek et al. 1997a) and for sequences belonging to the Ω-cluster, none of the sequences detected in a previous study on a modern line of rice (Ueda et al. 1995) were found in Kallar grass or wild rice. This result contrasts with those of a previous study in which N_2-fixing bacteria from most major groups were found associated with a modern line of rice (Ueda et al. 1995). The *nifH* primer set was shown to be unbiased to different types of templates (Zehr and Capone 1996); therefore, it is unlikely that these results are artifacts of amplification efficiencies for DNA or cDNA templates caused by the universal *nifH*-targeted primers (von Wintzingerode et al. 1997). This also is supported by retrieval of *nifH* sequences from all major clades in a previous study (Engelhard et al. 2000). Therefore, our amplifications probably reflected the quantitative abundance of templates in the environmental samples, a prerequisite for interpreting the community structure or function of microbiota in their natural environment (von Wintzingerode et al. 1997).

Quantitative evaluation of *nifH* transcription in the inoculation experiment and in environmental samples indicated a high potential for plant-associated N_2 fixation in *Azoarcus* sp. BH72. The amount of *nifH* transcript per gram of root dry weight was found to be equivalent to that produced by 1 to 2×10^{10} N_2-fixing cells of strain BH72 in pure culture. However, the actual numbers of BH72 cells per gram of root dry weight were probably much lower than 1 to 2×10^{10} because there is evidence from *nifH::gusA* expression in roots of rice by strain BH72 that the *nifH* expression level of bacteria inside the root can be more than a magnitude higher than in pure cultures(Egener et al. 1999; S. Wiese and B. Reinhold-Hurek, *unpublished data*). Nonetheless, considering that a root of Kallar grass with a length of 1 cm has approximately a dry weight of 0.3 mg and assuming that 30 bacteria would be present in the cortex per millimeter, Kallar grass could accommodate 1×10^9 bacteria per gram of root dry weight. Apparently no specialized, visible plant structures are required to accommodate the N_2-fixing agents, as in the rhizobium–legume symbiosis. A complex template mixture as used here might reduce the efficiency of specific amplification; therefore, these values are under- rather than overestimates. Environmental conditions that allow high nitrogenase activity also favor high levels of *nifH* expression in *Azoarcus* sp. BH72 (Egener et al. 1998, 1999). Abundant expression and function of *nifH* in the rhizosphere of inoculated and field-grown plants are consistent with an important role of *Azoarcus* sp. BH72 in biological N_2 fixation in the natural environment. Previous data on *nifH* expression of *Azoarcus* sp. BH72 in roots of field-grown Kallar grass plants were confirmed, which showed in situ hybridization with antisense *nifH* probes (Hurek et al. 1997a).

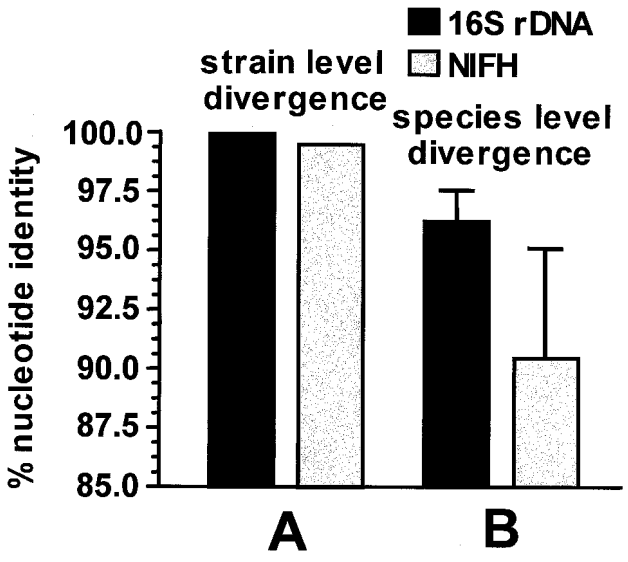

Fig. 4. 16S rDNA and *nifH* gene divergence in *Azoarcus* spp. **A,** Sequence comparisons of *Azoarcus communis* strains SWuB3 and S2. **B,** Sequence comparisons of the different species *Azoarcus*. sp. strain BH72, *A. indigens* VB32, and *A. communis* SWuB3. *NifH* sequences were **A,** 330 or **B,** 327 bases long. For 16S rDNA, almost full-length sequences were compared.

Our findings require a fundamental reconsideration of the ecology of N_2-fixing grass endophytes. The predominance and abundance of culturable diazotrophic endophytes in this habitat is believed to reflect the extent to which they contribute nitrogen to the plant. In contrast, we have shown here that a grass-endophytic–bacterium association functions in N_2-fixation with its host plant through the establishment of a nonculturable state, at least to our current knowledge of cultivation techniques. By maintaining an active metabolism, nonculturable *Azoarcus* sp. BH72 provided fixed nitrogen to the plant and contributed to plant growth, a characteristic, that this bacterium shares with many endosymbiotic microorganisms which also are not culturable, although metabolically active (Douglas 1995). Our data on the pattern of *nifH* expression and on the wide occurrence of culturable *Azoarcus* spp. in soils and in wild and modern lines of rice (Engelhard et al. 2000) suggests that only BH72 and the unculturable forms play a major role in N_2-fixation: known cultured N_2-fixing bacteria, in contrast to uncultured diazotrophs, were neither abundant nor very active, confirming observations from Ueda and coworkers (Ueda et al. 1995). Therefore, the overwhelming majority of N_2-fixing bacteria, which have been cultivated from associations with members of the *Gramineae* family, probably do not provide a large proportion of vascular plant nitrogen, confirming results obtained from inoculation experiments with various N_2-fixing bacteria (Bashan and Holguin 1997; Giller and Day 1985). Also, the small contribution of combined nitrogen to sugar cane by *Acetobacter diazotrophicus* estimated from inoculation experiments (Sevilla et al. 2001) cannot explain the huge contribution of N_2 fixation (Boddey et al. 1995; Urquiaga et al. 1992) observed in certain uninoculated, field-grown sugar cane cultivars. Furthermore, plant endophytes which are known to be taxonomically highly related can be very diverse in their genetic and biochemical capacities (Stierle et al. 1993; Young and Haukka 1996). It is therefore likely that endophytic bacteria, which respond to enumeration through classical culture procedures, are functionally different from microorganisms which usually maintain no free-living populations and only occasionally become culturable (e.g., *Azoarcus* sp. BH72) or are obligate endophytes. For *Azoarcus* sp. BH72, the interaction with rhizosphere fungi may be especially important for the maintenance of free-living populations of this bacterial species in the natural environ-

ment. N_2-fixing cells respond to coculture with an ascomycete from roots of Kallar grass with induction of complex intracytoplasmatic membranes (diazosomes) (Hurek et al. 1995) as a result of differential gene expression (Hurek et al. 1995; Karg and Reinhold-Hurek 1996). Microscopical appearance of morphologically changed strain BH72 suggested that complete binary fission might be inhibited and bacteria thus be arrested in growth (Hurek et al. 1995). Probably in nature, the formation of diazosomes would restrict differentiated bacteria in their ability to compete with other organisms, leading to physiologically active but unculturable *Azoarcus* cells. Therefore, the survival of the population may critically depend on the strict control of the differentiation process, still maintaining a sufficiently high number of viable, culturable cells. Otherwise, the ecological advantage of such an event for these bacteria would be hard to understand, because differentiations of populations would threaten this genotype by extinction. Thus, either this morphological change is a "dead end" in a fairly exclusive environment, analogous to the differentiation of rhizobia to bacteroids in nodule tissue of the Rhizobium–legume symbiosis, or it is an intermediate stage of an as yet undiscovered phenomenon. Future research should focus on unculturable N_2-fixing grass endophytes because they apparently play an important role in plant ecology that has been neglected.

MATERIALS AND METHODS

Plant growth and analysis.

Seedlings from surface-sterilized seeds of Kallar grass, which are generally too small to survive immediate growth under N-free conditions, were grown in a glasshouse in garden soil fertilized twice per week with Long Ashton nutrient solution (Hewitt 1996). Two-month-old plants (2.0 ± 0.3 g fresh weight) were transplanted into free draining compartments (Wyss et al. 1991), each containing 50 g of a mixture comprising sterile vermiculite (20% wt/wt), silica 70/25 (20% wt/wt), montmorillonite (20% wt/wt), and quartz sand (40% wt/wt). The potting medium of each compartment was inoculated with either 10^8 cells of *Azoarcus* sp. BH72 or the isogenic *nifK*$^-$ mutant strain BHNKD4 (Hurek et al. 1995). Plants were grown for up to 8 months and fertilized once a week with 20 ml of a quarter-strength combined nitrogen-free Long Ashton nutrient solution containing orthophosphate P at 10 mg liter^{-1}. $Ca(NO_3)_2{\cdot}4\ H_2O$ was replaced by $CaCl_2$. Dry weights were measured on freeze-dried plants.

Total nitrogen contents of the potting medium were measured using an elemental analyzer (NC 2100 Soil Analyzer, CE Instruments). $\delta^{15}N$ values of all samples were measured using a Europa Scientific Roboprep elemental analyzer coupled to a Europa Tracermass (Handley et al. 1993) and were calculated as $\delta^{15}N = [(R_{(sample)} - R_{(standard)})]/R_{(standard)})] \times 10^3$, where R is the ratio $^{15}N/^{14}N$ and the universally accepted standard is atmospheric N_2.

$\delta^{15}N$ of bacterial strain BH72.

In order to estimate the $\delta^{15}N$ of strain BH72, bacteria were grown in nitrogen-free medium in 1-liter rubber stoppered Erlenmeyer flasks with an initial oxygen partial pressure of 1.5 to 2.0% or in a fermentor at 2 μM dissolved O_2 according to standard conditions. Cultures were harvested at an optical density >100 times initial. No significant amounts of combined nitrogen were detected in the culture filtrate. The $\delta^{15}N$ of nitrogen (tank) gas introduced to the systems was 1.43 ‰.

RNA and DNA extraction.

Crude total RNA was isolated using a modification of the pine tree method (Chang et al. 1993) which increased the efficiency of nucleic acids recovery. Briefly, ground, tapwater–washed tissue (400 mg of root fresh weight equivalent to 80 mg dry weight) was homogenized with a tube pestle in extraction buffer. Debris was spun down, washed three times in TE buffer (10 mM TrisHCl, pH 8.0, 1 mM NaEDTA), incubated for 20 min at 37°C in 25% polyethylene glycol with lysozyme at 1 mg/ml, and spun down again. The supernatant from the first centrifugation was recovered. The pellet was incubated for 10 min at 37°C in 1 ml of lysing solution (0.5% sodium dodecyl sulfate and Proteinase K [Merck, Darmstadt, Germany] at 0.1 mg/ml in TE buffer). Extraction buffer from the first centrifugation was added and samples were incubated at 65°C for 15 min. For extraction of RNA from *Azoarcus* sp. BH72, cells were incubated in lysing solution before extraction buffer was added. For RT-PCR amplification, aliquots of RNA preparations were treated at 37°C for 20 min with 30 U RNAse-free DNAse I (Boehringer Mannheim, Mannheim, Germany) in 50 μl of DNAse I buffer (20 mM MES pH 7.2, 5 mM $MgCl_2$, 5 mM $CaCl_2$) and resuspended in 25 μl of DEPC-treated water. The quantity of total RNA was too small to be determined accurately by UV absorption. DNA samples were taken from the same samples previous to DNAse treatment.

DNA and RNA analyses.

DNA and RNA techniques such as Southern and Northern blotting (Reinhold-Hurek et al. 1993a), hybridization, cloning, and sequence analysis followed standard protocols (Ausubel et al. 1987). Sequence analysis using an automated sequencer (*ALFexpress,* AmershamPharmacia Biotech) and general 16S rDNA-targeted PCR (Hurek et al. 1997b) and *Azoarcus*-specific 16S rDNA-targeted PCR (Hurek and Reinhold-Hurek 1995) were carried out as previously described. PCR products were cloned into the vector pUC19 (Yanisch-Perron et al. 1985).

RT-PCR was done with Ready-to-Go RT-PCR beads following the manufacturer's specifications (Amersham Pharmacia Biotech, Freiburg, Germany). Reverse transcription was carried out with NIFHR-580 (5′-GTT(AG)(CT)A(AGT)AT-(AGC)A(GT)(AGC)CC(AGC)CC(AGC)AG-3′), a universal reverse primer for the nitrogenase Fe protein gene. For amplification of cDNA products, universal primers (Zehr and McReynolds 1989) were used. Amplification consisted of 40 cycles at 94°C for 1 min, 57°C for 2 min, and 72°C for 2 min in 0.2-ml thin-walled tubes and a Techne Progene cycler (Techne, Duxford, UK). For amplification of DNA templates, primers and PCR conditions were identical and the reverse transcriptase was heat-inactivated by incubation of the reaction mixture for 10 min at 95°C before the template was added.

Polyadenylated RNA was purified from total RNA preparations by chromatography on oligo(dT) cellulose. cDNA synthesis and cloning of double stranded cDNA into expression vector lambda ZAP was done with a ZAP-cDNA synthesis kit (Stratagene, San Diego, CA), following standard procedures.

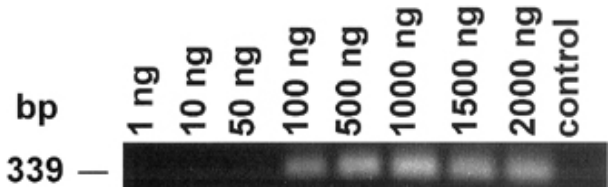

Fig. 5. Calibration of *nifH* mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR). RNA preparations from nitrogen-fixing cells of *Azoarcus* sp. BH72 grown in a fermentor at 2 μM dissolved O_2 were diluted and used as templates. Control: reverse transcriptase was heat inactivated prior to PCR. Agarose gel of PCR products. Typical data from three RNA extractions are shown.

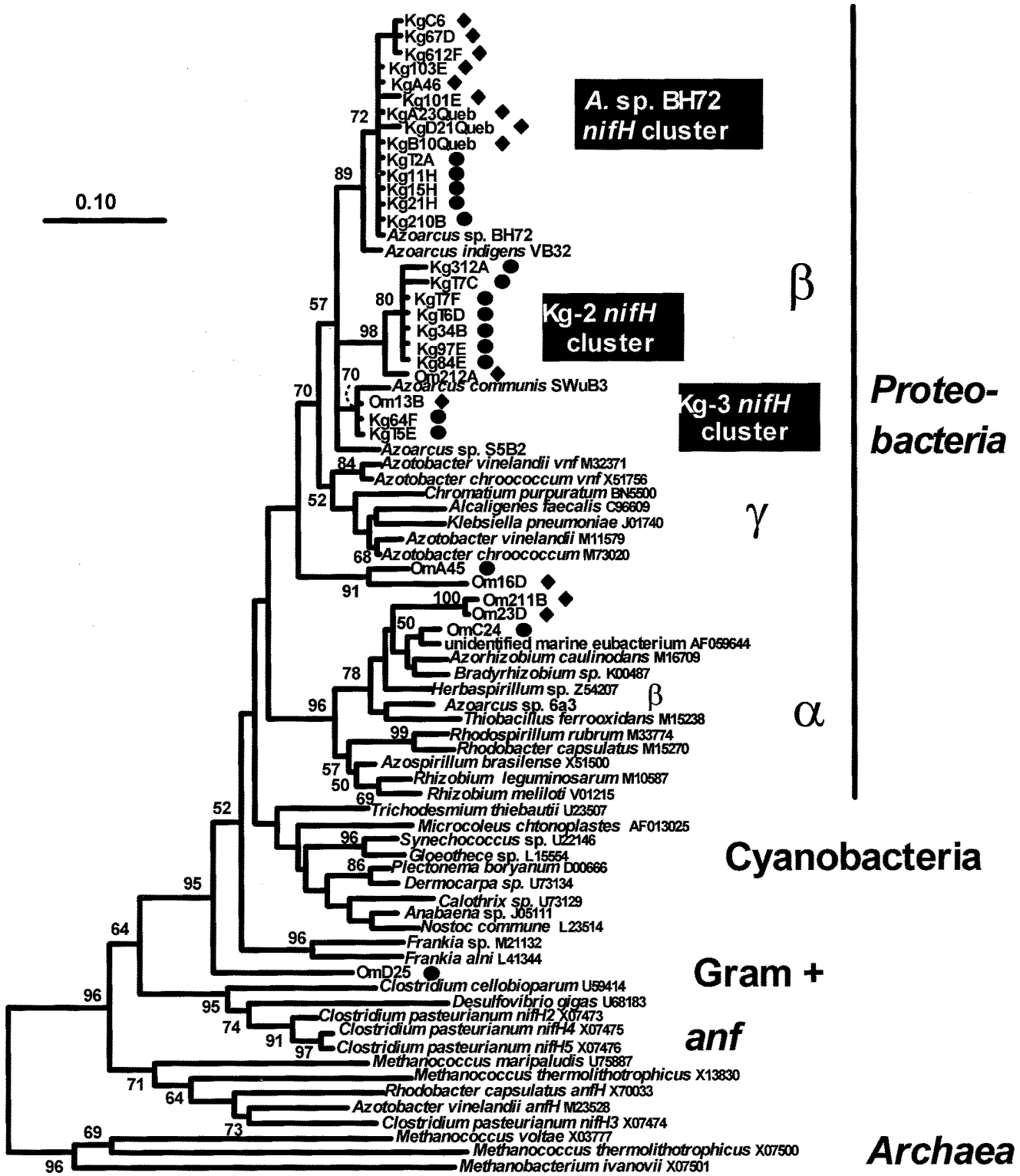

Fig. 6. Phylogenetic analysis of *nifH* DNAs and *nifH* mRNAs detected in the rhizospheres of Kallar grass and *Oryzae minuta*. Identical sequences were omitted in the phylogenetic analysis, but were included in the figure. Tree inference on comparisons of amino acids (122 sites) was done using a neighbor-joining algorithm and a Poisson correction distance matrix as described elsewhere (Hurek et al. 1997a). Horizontal branch lengths are drawn to scale with the bar indicating 0.1 replacements per site. Bootstrap confidence levels are shown as the percentage of 100 replications at the nodes, when greater than 50%. Accession numbers are given for reference sequences retrieved from databases. α, β, and γ represent the positions of different subclasses of the *Proteobacteria*; ● = DNA clones and ◆ = cDNA clones. Clone designation: retrieved from Kallar grass, field grown (Kg); Kallar grass, inoculated with *Azoarcus* sp. BH72 (KgQueb); or *O. minuta* (Om).

For analysis of lysing efficiency for nucleic acid extraction, *Bacillus subtilis* cells were added to the sample. 16S rDNA from crude RNA preparations was first amplified with PCR primers 25f (5′-AACTGTAAGAGTTTGATCCTGGCTC-3′) and 1492r (5′-TACGGCTTACCTTGTTACGACTT-3′), followed by a second amplification where the primer 25f was replaced by BS185 (5′-TGGTTGTTTGAACCGCATGG-3′), and 40 cycles. PCR primer BS185 is specific for *Bacillus subtilis*, and targets *Escherichia coli* position 185 in the 16S rDNA corresponding to the 3′ end of the primer.

MPN calculations were made using the MPNSolve program from Stephan Schneider, Department of Anthropology and Ecology at the University of Geneva, Switzerland.

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