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Identification of N_2-Fixing Plant- and Fungus-Associated *Azoarcus* Species by PCR-Based Genomic Fingerprints

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Most species of the diazotrophic *Proteobacteria Azoarcus* spp. occur in association with grass roots, while *A. tolulyticus* and *A. evansii* are soil bacteria not associated with a plant host. To facilitate species identification and strain comparison, we developed a protocol for PCR-generated genomic fingerprints, using an automated sequencer for fragment analysis. Commonly used primers targeted to REP (repetitive extragenic palindromic) and ERIC (enterobacterial repetitive intergenic consensus) sequence elements failed to amplify fragments from the two species tested. In contrast, the BOX-PCR assay (targeted to repetitive intergenic sequence elements of *Streptococcus*) yielded species-specific genomic fingerprints with some strain-specific differences. PCR profiles of an additional PCR assay using primers targeted to tRNA genes (tDNA-PCR, for $tRNA^{Ile}$) were more discriminative, allowing differentiation at species-specific (for two species) or infraspecies-specific level. Our protocol of several consecutive PCR assays consisted of 16S ribosomal DNA (rDNA)-targeted, genus-specific PCR followed by BOX- and tDNA-PCR; it enabled us to assign new diazotrophic isolates originating from fungal resting stages (sclerotia) to known species of *Azoarcus*. The assignment was confirmed by phylogenetic analysis of 16S rDNA sequences. Additionally, the phylogenetic distances and the lack of monophyly suggested emendment of the genus *Azoarcus*: the unnamed species *Azoarcus* groups C and D and a new group (E) of *Azoarcus*, which was detected in association with fungi, are likely to have the taxonomic rank of three different genera. According to its small subunit rRNA, the sclerotium-forming basidiomycete was related to the *Ustilagomycetes*, facultatively biotrophic parasites of plants. Since they occurred in a field which was under cultivation with rice and wheat, these fungi might serve as a niche for survival for *Azoarcus* in the soil and as a source for reinfection of plants.

Azoarcus spp. are strictly respiratory, nitrogen-fixing bacteria which belong to the beta subclass of the *Proteobacteria* (36). Most species have been isolated from roots or stems of Kallar grass (*Leptochloa fusca* (L.) Kunth) (34, 36) grown as a pioneer plant on salt-affected, low-fertility soils in the Punjab of Pakistan (38). These plant isolates show a surprising diversity, forming five groups distinct at the species level (36). After the initial description of the genus *Azoarcus* (36), the number of related strains and species isolated from different sources, such as the soil bacteria *A. tolulyticus* (56) and *A. evansii* (1), is increasing (26, 29). Therefore, studies on the diversity of members of *Azoarcus*, on the taxonomic structure of the genus, and on the ecological distribution are of importance.

Plant-associated strains of *Azoarcus* have interesting features with respect to plant-microbe interactions. Strain BH72 can live endophytically inside the original host plant, Kallar grass, but also inside rice without causing symptoms of plant disease (16). The bacteria invade the cortex region in the elongation zone above the root tip intra- and intercellularly and are capable of deep penetration into the stele of the roots. They can enter xylem vessels and spread systemically into shoots of young rice plants (16), most likely aided by bacterial cellulolytic enzymes (35). Although *Azoarcus* sp. strain BH72 is capable of colonizing rice plants in laboratory culture, bacteria of this genus have not yet been isolated from field-grown rice. However, molecular phylogenetic sequence analysis of structural genes of nitrogenase, which had been obtained from DNA of rice roots grown in Japanese fields (48), indicated that *Azoarcus* sp. might occur naturally in rice (13).

Azoarcus spp. appear to associate not only with plants but also with fungi. When grown in coculture with an ascomycete isolated from the roots of Kallar grass, the plant isolate *Azoarcus* sp. strain BH72 forms novel intracytoplasmic membrane stacks (17). These membrane stacks are formed in the course of hyperinduction, when bacterial cells show an enhanced respiratory efficiency and activity of nitrogen fixation (15, 17). Moreover, a strain of *A. communis* was isolated from a fungal resting stage originating from the rhizosphere of Kallar grass (14). To assess whether one or several species of *Azoarcus* naturally associate with resting stages of fungi, we attempted to isolate members of this genus from fungal sclerotia found in rhizosphere soil of rice.

In identification of new bacterial isolates, it is difficult to assign them to the genus *Azoarcus*, because most strains are phenotypically very similar and fail to give positive results in many classical tests for identification (36). Due to this problem and the lack of sufficient members per taxon, only two of five plant-associated species have been named as yet (36). DNA-based techniques may overcome some problems of classical biochemical tests for identification. PCR-based DNA typing methods allow simple and rapid identification of bacteria at the species or infraspecies level (7, 50, 54). Repetitive DNA sequences, which are dispersed in the genomes of bacteria in extragenic regions, have been used as targets for PCR primers to obtain genomic fingerprints of various bacteria. Different types of genetic elements have been used. ERIC (enterobacterial repetitive intergenic consensus) and REP (repetitive extragenic palindromic) elements were detected in the genomes of the enterobacteria *Salmonella* and *Escherichia* species (11,

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TABLE 1. Strains used

Taxon	Strain	Other designation(s)	Source of isolation (reference)
Azoarcus tolulyticus	Td-1		Petroleum-contaminated soil, western Washington (9)
A. evansii	KB740^T	DSM6898^T	Creek sediment, United States (1, 5)
A. indigens	VB32^T	LMG 9092^T	Surface-sterilized stem bases of Kallar grass, Pakistan (36)
	VW35a		Surface-sterilized roots of Kallar grass, Pakistan (36)
	VW34c		Surface-sterilized roots of Kallar grass, Pakistan (36)
	BS2-10		Fungal sclerotium from rice field, Pakistan (this study)
A. communis	SWub3^T	LMG 9095^T	Root piece of Kallar grass, Pakistan (36)
	S2	LMG 5514, BPD2	Refinery oily sludge, France (23)
	KGP1		Fungal ascocarp from Kallar grass soil, Pakistan (14)
Azoarcus sp. group C	S5b2		Surface-sterilized roots of Kallar grass, Pakistan (36)
	S5b1		Surface-sterilized roots of Kallar grass, Pakistan (36)
	SSa3		Surface-sterilized roots of Kallar grass, Pakistan (36)
	BS1-14		Fungal sclerotium from rice soil, Pakistan (this study)
	BS20-3		Fungal sclerotium from rice soil, Pakistan (this study)
Azoarcus sp. group D	6a3		Surface-sterilized roots from Kallar grass, Pakistan (36)
	6a2		Surface-sterilized roots from Kallar grass, Pakistan (36)
	BS2-3		Fungal sclerotium from rice soil, Pakistan (this study)
Azoarcus sp. group E		BS5-8	Fungal sclerotium from rice soil, Pakistan (this study)
	BS19-2		Fungal sclerotium from rice soil, Pakistan (this study)
	BS19-5		Fungal sclerotium from rice soil, Pakistan (this study)
	BS19-7		Fungal sclerotium from rice soil, Pakistan (this study)
	BS22-6		Fungal sclerotium from rice soil, Pakistan (this study)
	BS1-10		Fungal sclerotium from rice soil, Pakistan (this study)
Herbaspirillum seropedicae	Z67^T	LMG 6313^T, ATCC 35892^T	*Sorghum bicolor* roots, Brazil (3)

44); similar DNA motifs were found to occur in other gram-negative and gram-positive bacteria, such as *Rhizobium*, *Citrobacter*, *Arthrobacter*, *Bacteroides*, *Thermus* (50), *Azorhizobium*, *Bradyrhizobium*, and *Agrobacterium* (7, 19) species. The BOX element (highly conserved repeated sequences) was discovered in the gram-positive bacterium *Streptococcus pneumoniae* (27) but was also used successfully to analyze *Proteobacteria* such as members of the genera *Xanthomonas* (25), *Burkholderia* (41), and *Rahnella* (40). Only the BOX element was found to be efficient for typing of *Azoarcus* species in the present study. In addition, we used a PCR assay for DNA typing that uses primers targeted to tRNA genes. By using these methods, bacterial isolates from fungal sclerotia, which originated from a rice field, could be assigned to known species of *Azoarcus*; the assignment was confirmed by sequence analysis of 16S rRNA genes (rDNA) for several strains. However, phylogenetic analysis of almost complete 16S rDNA sequences suggests an amendment of the taxonomic structure of the genus *Azoarcus*. In summary, genotypic fingerprinting allowed a rapid identification of eukaryote-associated *Azoarcus* spp. from a new host and will be helpful for learning more about their ecological distribution.

MATERIALS AND METHODS

Microorganisms. All of the bacterial cultures used in this study are listed in Table 1. The strains or cell pellets either were isolated by us or were kind gifts of James M. Tiedje (strain Td-1, Center for Microbial Ecology, Michigan State University, East Lansing), Georg Fuchs (strain KB740, Lehrstuhl für Mikrobiologie, Universität Freiburg, Freiburg, Germany), Monique Gillis (strain S2, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium), and Anton Hartmann (strain Z67, GSF-Forschungszentrum für Umwelt und Gesundheit, Oberschleissheim, Germany). Fungal sclerotia were collected from rhizosphere soil of rice plants on a field near Shakot in the Punjab of Pakistan.

Media, growth conditions, and isolation of bacterial pure cultures. For DNA extraction, bacteria were grown at 37°C in VM medium (36) supplemented with ethanol (35) instead of potassium malate. Isolation of nitrogen-fixing bacteria was carried out as described previously (34) after enrichment on semisolid, nitrogen-free SSM medium supplemented with vitamins (17). Fungal sporangia were crushed in enrichment medium without previous surface sterilization. Nitrogenase activity of bacterial isolates was assayed gas chromatographically by the acetylene reduction test (34).

To analyze cell morphology, bacteria were grown at 37°C on SM medium containing combined nitrogen (33) and a mixture of vitamins (17), with N_2 as the sole nitrogen source (semisolid SM medium [34]). Colony morphology was determined on agar plates with VM ethanol medium after growth at 37°C. Tests for vitamin requirement were carried out on liquid or semisolid SM medium containing 0.5 g each of NH_4Cl and KNO_3 per liter as a nitrogen source. The medium was supplemented with a vitamin mixture (17) or single vitamins. In tests for carbon source utilization, potassium malate was replaced by fructose or glucose (1% [wt/vol]) in this medium.

DNA extraction. DNA was isolated from 1 to 2 ml of well-grown bacterial cultures as previously described (12).

Genomic fingerprints: primers, PCR conditions, and data analysis of BOX- and tDNA-PCR. For BOX-directed (BOX-PCR), a single primer corresponding to the BOX element (25), 5′-CATCGGCAAGGCGACGCTGACG-3′, labeled at its 5′ end with the fluorescent dye Cy-5 was used. PCR cycling profiles were described by Selenska-Pobell et al. (40) in buffer consisting of 20 mM Tris-HCl (pH 8.7), 10 mM KCl, 0.005% Tween 20, 100 μg of nuclease-free bovine serum albumin per ml, 7.5 mM $MgCl_2$, 1.25 mM each deoxynucleoside triphosphate, 50 pmol of each primer, and 2 U of *Taq* polymerase (Beckman Instruments, Munich, Germany) in a 25-μl reaction mixture, with 50 ng of chromosomal DNA as template. A Techne Progene thermocycler (Thermo-Dux, Wertheim, Germany) was used. For analysis of amplification products, 0.2 to 1 μl of reaction mixture was applied. For tDNA-directed PCR (tDNA-PCR), the Cy-5 end-labeled primers MS9 [5′-GGACC(CT)TTAGCTCAG-3′] and MS10 [5′-ACT(CT)GAACC (AT)(AG)(CT)GAC-3′] were used. The PCR buffer described above was modified as following: 1.5 mM $MgCl_2$, 50 μM each deoxynucleoside triphosphate, 10 pmol of each primer, 1.25 U of *Taq* polymerase, and 20 ng of template DNA in a 50-μl reaction mixture. Cycling conditions were as follows: initial denaturation at 95°C for 4 min, 30 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min, with a final extension at 72°C for 4 min. For analysis of amplification products, 1 to 4 μl of reaction mixture was used. Analysis was carried out on a denaturing polyacrylamide (5% [wt/vol]) gel with 0.5× Tris-borate-EDTA (TBE [2]) as a running buffer, using an ALF*express* automated sequencer (Pharmacia, Uppsala, Sweden). Fragment size was documented by using the Fragment Manager program of ALF*express* (Pharmacia). To determine the size of fragments obtained from tDNA-PCR, a Cy-5-labeled DNA ladder ranging from 50 to 500 bp (Pharmacia) was used. To analyze the BOX-PCR products, size standards were generated by using 16S rDNA-targeted PCR primers, one of which was labeled with Cy5 (see below), and DNA of *Azoarcus* sp. strain BH72 as a template. A fragment length of 527 bp (primers 35fC and 522r), 920 bp (primers 35fC and 926r), or 1,517 bp (primers 35fC and 1492r) was obtained.

PCR amplification and sequence analysis of small subunit (SSU) rDNA genes. To amplify and sequence 16S rRNA genes, general primers for eubacterial 16S rDNA (24) were slightly modified. The PCR primers 25f (5′-AACTKAAGAG TTTGATCCTGGCTC-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) were used with 1 to 2 ng of template DNA in PCR buffer described above for

TABLE 2. Characteristics of *Azoarcus* strains isolated from fungi[a] and other strains[b]

Characteristic	Result for strain(s):						
	A. indigens VB32^T, BS2-10	*A. communis* SWub3^T, KGP1	*Azoarcus* sp. group C S5b2, BS1-14, BS20-3	*Azoarcus* sp. group D 6a3, BS2-3	*Azoarcus* sp. group E BS5-8, BS19-2, BS19-5, BS19-7, BS22-6	*A. tolulyticus*	*A. evansii*
Cell width (μm)	0.5–0.7	0.8–1.0	0.6–0.8	0.4–0.6	0.6–0.8	ND[g]	0.6–0.8
Colony diameter (mm) after 4 days of growth	2–3	2–4	1.5–2[c]	1–2	1–2	ND	ND
Requirement for *p*-aminobenzoic acid	+[d]	−	−	−	−	−	−
Requirement for cobalamin	−	−	±[e]	+[f]	+	−	−
Growth on glucose	−	−	−	−	−	+	ND
Growth on fructose	−	−	−	−	−	ND	+
PCR amplification product							
With primers TH3 and TH5	+	+	+	−	−	+	+
With primers TH14 and TH2	+	+	+	+	+	+	+

[a] Strains with designation BS and strain KGP1 were isolated from fungal sclerotia.
[b] Data from this study and from references 1, 14, 36 and 56.
[c] Growth of strain BS20-3 on agar plates negligible.
[d] Characteristic valid for fungus-associated or all known strains of this taxon, if not stated otherwise.
[e] Only strain BS20-3 positive.
[f] Only strain BS2-3 positive.
[g] ND, not determined.

tDNA-PCR. DNA was amplified after initial denaturation at 95°C with 27 cycles of 1 min of denaturation at 95°C, 2 min of annealing at 59°C, and 2 min of extension at 72°C, with a final extension step for 4 min at 72°C. Prior to direct sequencing, 5 μl of the amplification product was treated with exonuclease I (10 U) and shrimp alkaline phosphatase (2 U; Amersham International, Little Chalfont, United Kingdom) for 15 min at 37°C and then for 15 min at 80°C without previous agarose-gel purification. This DNA template was dialyzed against distilled water for 15 min before being used for ThermoSequenase fluorescence-labeled primer cycle sequencing with 7-deaza-dGTP (catalog no. 2438; Amersham International) according to the manufacturer's instructions. A Techne Cyclogene thermocycler (Thermo-Dux) was used with 25 cycles of denaturation at 95°C for 30 s and annealing/extension at 60°C for 30 s, after an initial denaturation at 97°C for 1 min. Sequencing primers were labeled at the 5′ end with the fluorescent dye Cy-5 in order to allow automated sequencing with the ALF*express* (Pharmacia). Primers were 35fC (5′-CTKAAGAGTTTGATCMT GGCTCAGATTGAACG-3′), 342fC (5′-CTCCTACGGGAGGCAGCAG-3′), 530mfC (5′-CTACGTGCCAGCMGCCGCGG-3′), and 930fC (5′-GGTTAAA ACTYAAAKGAATTGACGGGGAC-3′).

Fungal SSU rRNA genes were amplified by using primers NS1 and NS4 as outlined by White et al. (55). PCR fragments of approximately 1,150-bp length were blunt-end cloned into pUC19 according to standard techniques (2). Sequencing was carried out from double-stranded plasmid DNA as described above without pretreatment.

The rDNA sequences were aligned by direct examination using the MUST package (31). For phylogenetic analysis, all base ambiguities and gaps were removed from the alignment. Aligned sequences are available from the authors upon request.

Phylogenetic analysis. Parsimony analysis of the aligned sequences was done with the DNAPARS program of the PHYLIP 3.5c package (8). Bootstrap values were calculated from 100 pseudoreplicates generated by the SEQBOOT program. Neighbor-joining analysis was also performed on the aligned DNA sequences, using the MEGA package (22). Bootstrap tests of the neighbor-joining trees inferred from Jukes-Cantor distances (20) were done with 500 replications. 16S rDNA trees were compared with a nonparametric and a parametric test under the parsimony (47) and maximum-likelihood criteria (21), respectively, using DNAPARS and DNAML programs (L option off) of the PHYLIP 3.5c package.

Nucleotide sequence accession numbers. Sequences were submitted to the GenBank database. Accession numbers are as follows: *A. indigens* VB32^T, AF011345; *A. communis* SWub3^T, AF011343; *Azoarcus* sp. strain BH72, AF011344; *Azoarcus* sp. group C strains S5b2, BS1-14, and BS20-3, AF011346, AF011348, and AF011349, respectively; *Azoarcus* sp. group D strains 6a3 and BS2-3, AF011347 and AF011351, respectively; *Azoarcus* sp. strain BS5-8, AF011350; black sclerotium, AF010303.

RESULTS

Isolation of fungus-associated *Azoarcus* spp. As a source for isolation, we selected the same field in the Punjab of Pakistan as was previously studied with respect to populations of *Azoarcus* spp. in roots of Kallar grass (34, 36). For 2 years, this field had been under alternating cultivation of rice and wheat. Black fungal sclerotia (ca. 0.5 mm in diameter) formed by a basidiomycete (see below) were collected from rhizosphere soil of rice and used as an inoculum for enrichment cultures. Success of enrichment of microaerobically nitrogen-fixing bacteria was judged by formation of a subsurface pellicle and reduction of acetylene to ethylene. Bacteria showing typical morphology of *Azoarcus* spp. (highly motile, slightly curved bacteria with a cell width of ≤1μm) were isolated from several sclerotia, the first number in the strain designation indicating the sclerotium of origin (Table 1). Approximately 30% of the sclerotia tested harbored diazotrophic bacteria of this type.

For a preliminary identification of *Azoarcus* spp., isolates were characterized genotypically. Total DNA of the pure cultures was subjected to PCR amplifications with genus-specific primers targeted to 16S rDNA (12). Highly specific primers TH3 and TH5 amplified fragments from isolates BS2-10, BS1-14, and BS20-3, showing that they belonged to the genus *Azoarcus* (Table 2). Primers of lesser specificity, TH14 and TH2, amplified fragments from strains BS2-3, BS5-8, BS19-2, BS19-5, BS19-7, BS22-6, and BS1-10, indicating that they also might belong to this genus.

Genomic fingerprints by tRNA- and BOX-PCR can identify *Azoarcus* species and group strains. To group and identify strains by genomic fingerprints, PCR amplification with primers directed to various targets were used. Primers MS9 and MS10 were developed to amplify bacterial tRNA^{Ile} genes with the anticodon CAU. The profile of this tDNA-PCR from different groups of bacteria consists of several fragments (45). From most *Azoarcus* strains, the tDNA-PCR yielded multiple amplification products, ranging in size from ca. 50 to 380 bp (Fig. 1A). Additionally, we tested whether PCR primers for genotypic analysis of other bacterial groups were applicable also for *Azoarcus* spp. When applied to *Azoarcus* sp. strain BH72 and *A. communis* SWub3^T DNA, primers targeted to ERIC or to REP sequences (7) did not yield any amplification products visible in ethidium-bromide-stained agarose gels. However, complex patterns were obtained with primers corresponding to the subunit A of the BOX element (25) (Fig. 1B). The PCR profiles were generally more complex and contained

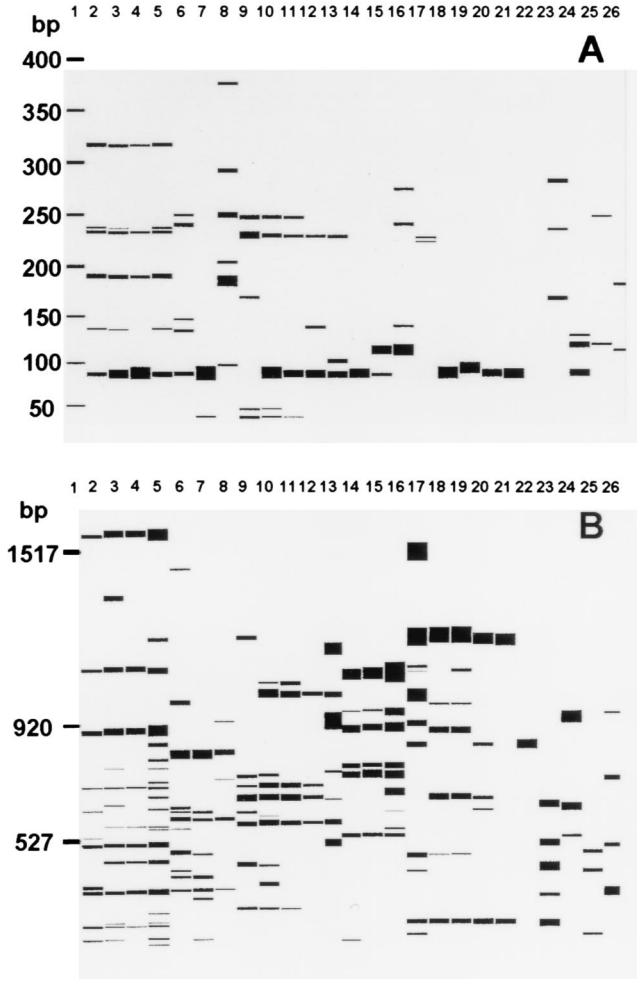

FIG. 1. Fingerprints generated by tRNA-PCR (A) and BOX-PCR (B) in various *Azoarcus* species and reference strains. Amplification products were analyzed on an ALF*express* automated sequencer, using the Fragment Manager program (Pharmacia). Lane 1, size marker (positions are indicated at the left); lanes 2 to 5, *A. indigens* VB32^T, VW34a, VW35c, and BS2-10, respectively; lanes 6 to 8, *A. communis* Swub3^T, S2, and KGP1, respectively; lanes 9 to 13, *Azoarcus* sp. group C strains S5b2, S5b1, SSa3, BS1-14, and BS20-3, respectively; lanes 14 to 16, *Azoarcus* sp. group D strains 6a3, 6a2, and BS2-3, respectively; lanes 17 to 21, *Azoarcus* sp. group E strains BS5-8, BS19-2, BS19-5, BS19-7, and BS22-6, respectively; lane 22, strain BS1-10; lane 23, *Azoarcus* sp. strain BH72; lane 24, *A. tolulyticus* Td-1; lane 25, *A. evansii* KB740; lane 26, *H. seropedicae* Z67^T.

larger fragments than those obtained with tRNA-PCR. Fragments of up to 1.6 kb in size were resolved by the automated sequencer on polyacrylamide gels. When the same thermocycler was used, PCR patterns were very reproducible, prominent bands that were consistently present (not shown).

Species-specific genotypic fingerprints were obtained for all species of *Azoarcus*; however, the resolution of the assay varied with the primer set used and the bacterial group analyzed. The intraspecies similarity of *A. indigens* and *Azoarcus* sp. group C was high in both PCR assays, whereas only the BOX-PCR assay revealed similarity within *A. communis* and *Azoarcus* sp. group D (Fig. 1). In contrast, the tRNA-targeted fingerprint detected major interstrain differences within the latter two groups (Fig. 1). Several new isolates from sclerotia were assigned to known species of *Azoarcus* according to their similarity in genotypic fingerprints: BS2-10 to *A. indigens* (Fig. 1, lane 5), BS1-14 and BS20-3 to *Azoarcus* sp. group C (Fig. 1, lanes 12 and 13), and BS2-3 to *Azoarcus* sp. group D (Fig. 1, lane 16). Other isolates which were tentatively assigned to *Azoarcus* according to 16S rDNA-targeted PCR reactions (see above) did not belong to a known taxon but were likely to form a new group (group E) (Fig. 1, lanes 17 to 21); since these strains had only two major bands in common (Fig. 1B), grouping is preliminary; however, they also showed very similar colony morphologies. Strain BS1-10, which was similar in colony morphology to strains of group E, showed neither a similar fingerprint nor nitrogenase activity, nor did it belong to *Azoarcus* spp. according to its 16S rDNA sequence (not shown). *Herbaspirillum seropedicae*, a diazotroph which also occurs in roots of subtropical gramineae (3), was different from *Azoarcus* spp. in genotypic analysis (Fig. 1) and did not react in genus-specific, 16S rDNA-targeted PCR assays (not shown); thus, it can be clearly distinguished from *Azoarcus* spp. by our PCR assays.

Phylogenetic analyses of 16S rDNA sequences of *Azoarcus* spp. To evaluate if the assignment of isolates to *Azoarcus* spp. by genomic fingerprints was correct, selected isolates were subjected to 16S rDNA sequencing and phylogenetic analysis. Additionally, we sequenced 16S rDNA genes of representatives of plant-associated species of *Azoarcus* for which complete sequences were not yet available. According to 16S rDNA sequence homologies (Table 3), several isolates were assigned to known species of *Azoarcus* in agreement with results of genotypic fingerprints. Strain BS2-3 had 100% sequence identity to strain 6a3 (*Azoarcus* group D). Strains BS1-14 and BS20-3 both were related to strain S5b2 (*Azoarcus* group C); according to sequence identities of 99.8 and 97.2%, respectively, they may belong to the same species as strain S5b2. Organisms sharing more than 97% sequence similarity often belong to a single species (43); however, this will have to be confirmed by DNA-DNA homology studies. As in genotypic analysis, strain BS5-8 representing group E was not closely related to any known species of *Azoarcus*. The highest sequence identities were obtained with *Azoarcus* sp. group C (strain S5b2, 93.3%) and *Azoarcus* sp. group D (strain 6a3, 93.4%) (Table 3).

The 16S rRNA gene sequences of *Azoarcus* spp. and related genera, comprising of 1183 positions, were subjected to phylogenetic analysis using different methods. Both neighbor-joining and maximum-parsimony (Fig. 2) analyses confirmed the assignment of our new isolates to known species of *Azoarcus*. Isolates BS1-14 and BS20-3 or isolate BS2-3 formed monophyletic units with *Azoarcus* sp. strain S5b2 or 6a3, respectively, a finding supported at a significant (≥95%) level by bootstrap analysis. Bootstrap values also indicated that the *Thauera* clade consists of two species and that one clade consists of soil isolates, including *A. tolulyticus* and *A. evansii*. In the neighbor-joining analysis, bootstrap confidence levels above 50 supported most other nodes (Fig. 2A), which was, however, not the case in the parsimony analysis (Fig. 2B). Topologies of the two trees were not identical and varied in the position of the *Thauera* clade and of *Azoarcus* sp. strains S5b2 and 6a3. In parsimony analysis, *Thauera* and soil isolates of *Azoarcus* formed a monophyletic unit suggesting that they belonged to the same genus, which was a sister lineage of *A. indigens*. In contrast, neighbor-joining analysis yielded a topology in which five species of *Azoarcus* including the soil isolates formed a monophyletic unit which was in one clade with *Thauera*. In both topologies, *Azoarcus* sp. group D was most closely related to *Rhodocyclus*. Neighbor joining analysis placed *Azoarcus* sp. group C in the *Rhodocyclus* clade, whereas it was at the bottom of the *Azoarcus* lineage according to parsimony topology. Isolate BS5-8 could not be clearly related to *Azoarcus* sp. group C or group D. When both trees were compared under maximum-

likelihood and parsimony criteria, scores obtained (Fig. 2) were not significantly different, indicating that none of the topologies is superior to the other, and some relationships within the *Azoarcus-Thauera-Rhodocyclus* group were uncertain.

Identification of the fungal source of *Azoarcus* isolates by phylogenetic analysis of SSU rDNA. Segments encompassing 933 positions were analyzed. The overall toplogy of the neighbor joining tree (Fig. 3) was in agreement with those of Bruns et al. (6) and Berbee and Taylor (4) and shows monophyletic fungi with *Chytridiomycota* and *Zygomycota* as basal and *Ascomycota* and *Basidiomycota* as sister groups. The SSU sequences from the black sclerotium grouped with the *Ustilagomycetes* within the *Basidiomycota*. The placement of the fungal isolate within the *Basidiomycota* was supported at a significant level (≥95%), while the confidence in the *Ustilagomycetes* branch was high but not significant.

Morphological and physiological characteristics of the fungus-associated isolates. A key characteristic of the plant-associated *Azoarcus* species is the restricted range of carbon sources: carbohydrates are not utilized (36). None of the new putative *Azoarcus* strains grew on D-glucose or fructose, but they all grew on malic acid, the best carbon source for the plant-associated species (Table 2). Colony morphology and cell size were similar in the new isolates and in the species to which they were assigned by genetical methods (Table 2). However, there were some phenotypic differences. For example, isolate BS20-3, which was related to *Azoarcus* group C, was obligately microaerobic. Since growth on agar plates and in well-aerated liquid media was poor, all phenotypic tests were carried out in semisolid media under microaerobic conditions. In contrast to the plant-associated strains of *Azoarcus*, most of the new isolates required vitamins for growth on combined nitrogen, such as vitamin B_{12} (cobalamin) (Table 2). All members of *Azoarcus* group E required this vitamin, a feature which they share with *Rhodocyclus purpureus* (18).

DISCUSSION

Bacteria of the genus *Azoarcus* have been recently identified in several habitats (1, 9, 14, 29) which are different from that originally described for the first isolates, plant roots (34). This study was undertaken to continue analysis of the ecological distribution of bacteria of this genus. Phenotypic similarity and lack of positive results in many classical tests hinder the rapid accurate assignment of new isolates. Therefore, we first tried to develop a rapid and accurate approach for identification of *Azoarcus* at the species level. PCR-generated genomic fingerprints appeared to be the method of choice for such studies. PCR protocols which yielded the best results in molecular characterization of the genus were then used for identification of new isolates from a new habitat, and the results were compared with those of molecular phylogenetic analysis.

Since REP-, ERIC-, and BOX-PCR amplifications (rep-PCR) often detect the same similarities in a given group of bacteria, it is likely that the distribution of these repetitive sequences is a true reflection of the genome structure (25, 40). It was anticipated that REP- and ERIC-like sequences are virtually ubiquitous in bacteria (7, 50), allowing a rapid molecular characterization by PCR-based fingerprints. However, REP- and ERIC-PCR did not yield any amplification products from the two species of *Azoarcus* tested. Apparently, these repetitive sequences are not present in their genomes at all or not in a distance suitable for PCR amplifications. Similarly, other members of *Proteobacteria*, *Neisseria* or *Proteus* and *Vibrio*, failed to show complex amplification patterns in ERIC-

TABLE 3. Percent similarity (1,296 positions) between 16S rDNA sequences of members of the beta subclass of the *Proteobacteria*

Sequence no.	Organism	% Similarity																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*Azoarcus tolulyticus* L33687																					
2	*A. evansii* X77679	99.2																				
3	Isolate X83531	96.1	96.1																			
4	Isolate X83532	96.1	96.3	98.1																		
5	*Azoarcus* sp. strain BH72 L15531	95.1	95.0	94.6	94.8																	
6	*A. indigens* L15532	94.4	94.7	94.6	94.8	97.0																
7	*A. communis* SWuB3	94.1	94.3	93.8	94.1	96.0	94.6															
8	Isolate U46748	92.9	92.7	92.1	93.0	93.5	93.9	91.9														
9	*Thauera selenatis* X68491	93.1	93.4	93.4	93.1	93.6	93.9	93.6	92.6													
10	*T. aromatica* X77118	93.2	93.2	93.2	92.6	93.4	93.7	93.6	93.1	97.3												
11	*Azoarcus* sp. S5B2	92.0	92.0	92.8	93.0	93.6	93.8	94.3	92.9	92.3	92.9											
12	Isolate BS1-14	92.8	92.8	92.6	92.8	93.5	93.6	94.2	91.7	92.0	92.7	99.8										
13	Isolate BS20-3	91.0	90.9	92.3	91.8	92.5	92.6	92.7	91.2	91.5	92.2	97.2	97.0									
14	Isolate BS5-8	92.4	92.6	92.6	92.2	92.3	91.3	92.0	90.6	90.4	90.8	93.3	93.2	92.9								
15	Isolate BS2-3	91.2	91.2	91.1	90.9	92.4	92.0	91.4	91.6	91.0	91.9	93.2	93.1	93.5	93.4	100.0						
16	*Azoarcus* sp. 6a3	91.2	91.2	91.1	90.9	92.4	92.0	91.4	91.6	91.0	91.9	93.2	93.1	93.5	93.4	92.7	92.7					
17	*Rhodocyclus purpureus* M34132	90.2	90.3	90.9	90.9	90.8	90.8	89.9	89.4	90.9	90.3	91.8	91.6	91.1	92.4	92.7	92.7	91.2				
18	*Zoogloea ramigera* X74913	92.8	92.2	91.8	91.7	92.5	91.8	91.8	90.3	91.7	91.9	90.5	90.3	91.4	91.1	92.7	92.7	91.2				
19	*Gallionella ferrugina* L07897	88.3	88.0	88.1	88.7	89.6	89.0	87.3	88.2	87.5	87.8	89.8	89.7	89.8	89.8	91.1	91.1	89.9	90.6			
20	*Rubrivivax gelatinosus* M60682	88.1	87.9	88.8	89.0	89.0	88.1	88.2	87.8	87.7	87.7	88.4	88.2	87.7	88.6	88.1	88.1	88.3	90.2	86.7		
21	*Herbaspirillum seropedicae* Z67	87.5	87.6	87.5	87.6	88.0	87.7	88.1	86.1	86.7	87.0	88.6	88.5	88.0	89.8	89.2	89.2	88.5	88.1	88.2	89.8	
22	*Chromatium vinosum* M26629	84.0	84.0	85.1	84.1	83.5	84.1	83.7	84.3	83.9	84.3	83.8	83.6	84.2	83.1	83.9	83.9	82.8	83.8	81.5	82.4	81.9

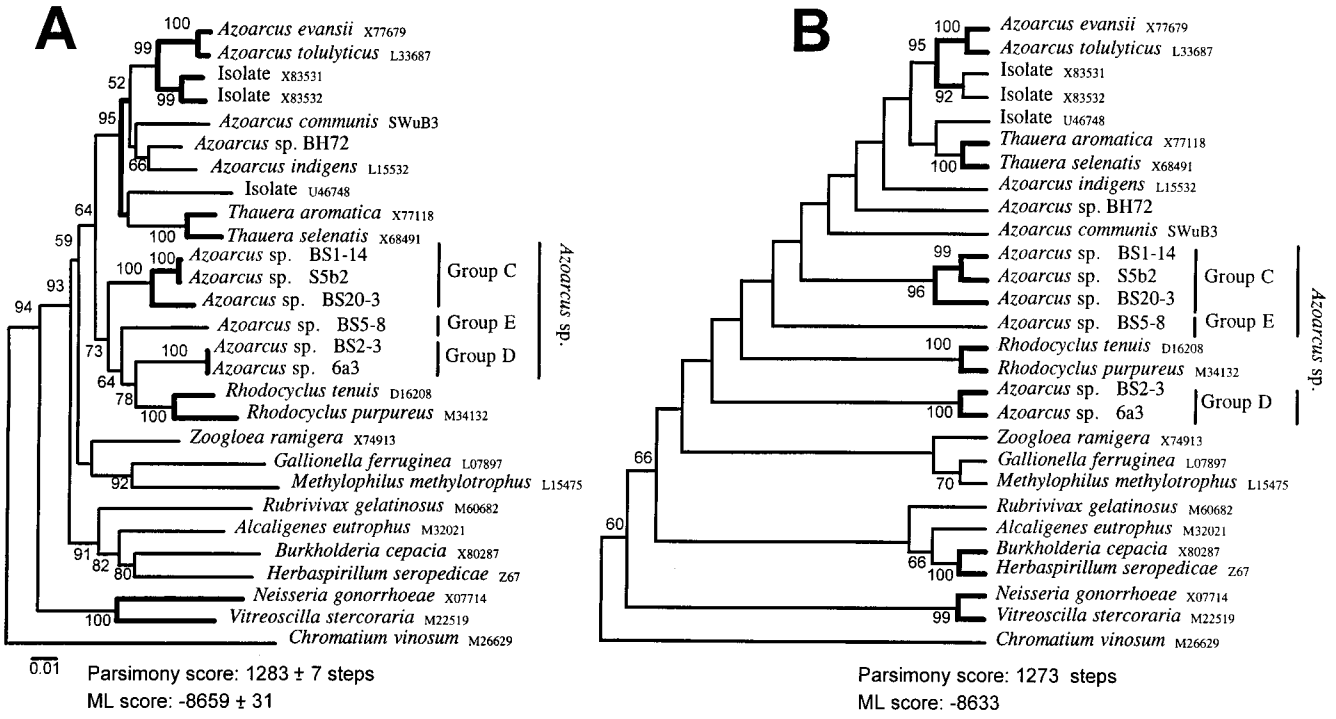

FIG. 2. Phylogenetic analysis of 16S rDNA sequences from beta-subclass *Proteobacteria* showing alternative hypothesis about the relationship of *Azoarcus* with other genera. The gamma-subclass proteobacterium *Chromatium vinosum* was used as an outgroup. (A) Neighbor-joining phenogram. Bootstrap confidence levels greater than 50% are indicated at internodes. (B) Bootstrap consensus cladogram resulting from parsimony analysis without branch lengths. Numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees out of hundred. Bootstrap values greater than 50% are indicated above internodes. Topologies that are supported by more than 95% of the bootstrap trees are depicted in bold. Accession numbers of sequences are given next to the species or strain name. ML, maximum likelihood.

or REP-PCR, respectively (50). However, we have demonstrated that BOX-like sequences are present in the genomes of *Azoarcus* spp. These repetitive sequences, which were originally observed in gram-positive bacteria (27), are also widely distributed in *Proteobacteria* (25, 40, 41). For the correct identification of unknown isolates, it is useful to rely on not only one test. Since REP- and ERIC-PCR protocols failed to give satisfying results for *Azoarcus*, we adopted a tRNA-targeted PCR protocol which may be used to confirm results of BOX-PCR in two species of *Azoarcus*; however, it has a higher resolution and might be more useful for strain typing than for species determination. A complete protocol which allows identification of *Azoarcus* species by several consecutive PCRs, consisting of genus-specific, 16S rDNA-targeted PCR (12) followed by BOX- and tDNA-targeted PCR amplifications, was developed.

Genomic fingerprints are usually visualized on ethidium bromide-stained agarose or polyacrylamide gels. Recently, a method using REP-PCR with fluorescently labeled primers followed by analysis with an automated sequencer has been introduced (51). In our experience, analysis with the Fragment Manager program of the ALF*express* automated sequencer has several advantages over ethidium bromide-stained gels. The sensitivity is higher, differences of the mobilities in different parts of the gel can be calibrated after the run, and lanes may be rearranged after the run to fit profiles similar to each other. For routine analysis, the possibility to store the data will also be of advantage.

tDNA-PCR has been used to differentiate species of the same genus (53, 54) or groups of strains of the same species (39) from each other. Bacterial tRNA genes are often organized in clusters (10, 28, 52), where they are separated from each other by spacer regions of different lengths (53). To increase the probability of length polymorphisms of amplification products, we used primers to a tRNA gene, Ile^{CAU}tRNA, which is related to other tRNA genes with the same anticodon (^{Met}tRNA and $^{F\text{-}Met}$tRNA [42]) and might amplify them at conditions of low stringency. This approach was successful for most species of *Azoarcus*: fingerprint patterns of at least three different bands were obtained. In contrast, a less complex pattern of only one to three bands was obtained with different primers for *Burkholderia solanacearum* (39), which divided biovars of the same species into several groups. Our tDNA-PCR allowed differentiation at the infraspecies-specific or species-specific level, depending on the *Azoarcus* species studied. This variation was not clearly correlated with the phylogenetic relatedness of the strains. For example, within *A. indigens* and *Azoarcus* groups C and D, strains were highly related to each other according to their protein patterns (36) or according to 99.8 to 100% identity of 16S rRNA genes (this study); however, *A. indigens* and group C had either species-specific fingerprints, whereas they were highly variable within group D. However, since up to now only a limited number of strains are known to exist per species, the infraspecific diversity might not have been fully described yet, and genomic fingerprints might not prove to be species-specific for new isolates.

Genomic fingerprints with REP, ERIC, and BOX primers have mostly been used for infraspecific comparisons of bacteria (6, 37, 40, 41). Infraspecific differences were also detected in *Azoarcus*, which in part correlated with phylogenetic relatedness. In group C, strain BS20-3 had only 97.2% 16S rDNA homology to strain S5b2; moreover, it was phenotypically dif-

ferent since it was the only obligately microaerobic strain in this group. This was reflected in BOX-PCRs, which detected different major bands (in addition to similar bands) in both strains, indicating some differences in the overall genomic structure. BOX-PCR allowed differentiation at the species level in *Azoarcus*, since the PCR profiles showed several prominent bands of equal mobility which were specific for each species. Even members of *A. communis* were effectively grouped, although they had only 70% DNA-DNA homology (36) and originated from different habitats such as French oily sludge and grass roots from Pakistan. They had entirely different patterns in tDNA-PCR, demonstrating that the tDNA-PCR assay may be more discriminative in *Azoarcus*. It has also been observed by others (19, 25) that the discriminative power of genomic fingerprints depends on the taxon under study and the assay used. Species-specific similarities in REP-PCR fingerprint profiles have been previously observed with highly related pathovars of *Xanthomonas oryzae* and *X. campestris* (25) and *B. solanacearum* (41). Since only highly related bacteria can be grouped by genomic fingerprints, this method is of limited use for bacterial taxonomy when new species are isolated. This was the case for strains related to BS5-8, which we isolated from fungi. They were assigned to *Azoarcus* (group E) by phylogenetic analysis of 16S rDNA sequences, while they could not be identified by PCR profiles.

Since the original description of this genus (36), new species have been described (1, 56). Almost complete sequences of 16S rDNA genes of all known *Azoarcus* species (references 1, 12, and 56 and this study), genetically related unidentified isolates from a hot spring (U46748 [29]), and the related genus *Thauera* (26) are now available, allowing the analysis of the taxonomic structure of these genera. Our phylogenetic analysis was impeded by the instability of the branching levels between *Azoarcus*, *Thauera*, and *Rhodocyclus*, according to neighbor-joining as well as parsimony criteria. Selection of taxa included in the analysis may affect the branching order, as has been observed for other groups of microorganisms (6, 49). When the conflict of topologies cannot be resolved by tests for comparing two trees (as in our case), additional data are needed to resolve the phylogenetic structure. It might be helpful to apply sequence analysis of other phylogenetic markers such as 23S rDNA genes, which bear a higher resolution of closely related taxa due to higher sequence variability (30).

Despite some unresolved branching pattern, our phylogenetic sequence analysis suggests an emendment of the genus *Azoarcus*. It should be divided into several new genera for the following reasons. (i) When founded on molecular phylogenetic data, a genus should be monophyletic. The genus *Azoarcus* is not. At least two different groups were consistently found, one related to *Thauera* (top groups) and one, comprising *Azoarcus* groups C, D, and E, related to *Rhodocyclus* (bottom groups). (ii) 16S rDNA sequence homology of several "species" of *Azoarcus* to each other is low. *Azoarcus* groups C, D, and E are only distantly related to species of the top groups and to each other; the same sequence identities (ca. 92 to 93%) are found between these *Azoarcus* species as well as between them and the genera *Thauera* and *Rhodocyclus*. Therefore, *Azoarcus* groups C, D, and E are likely to have the taxonomic rank of three genera that are different from *Azoarcus*. Based on the thermal stability of DNA-rRNA hybrids, these strains are located on the *Azoarcus* branch, albeit close to the bottom of this branch (36). Due to this relatively low hybrid stability, these groups could have been excluded from the genus *Azoarcus* sensu stricto; however, they were included in order to stress their phenotypic similarity (36). We are currently undertaking a polyphasic taxonomic approach to describe these genera.

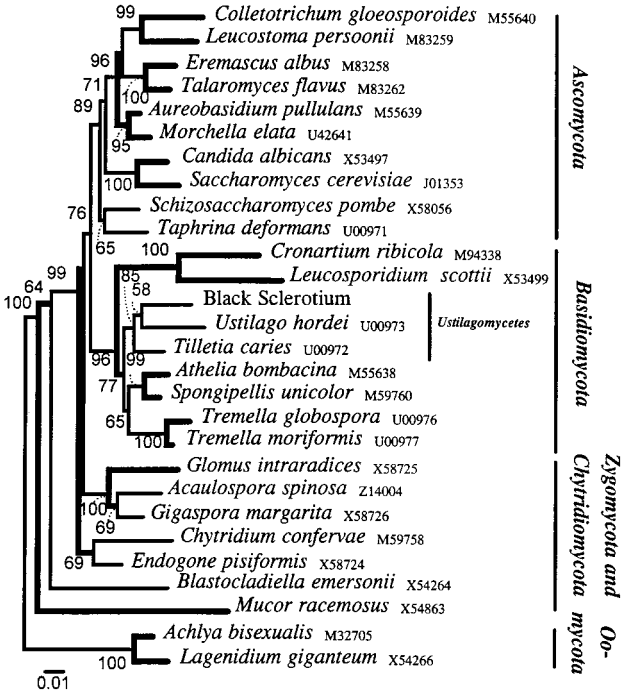

FIG. 3. Neighbor-joining phenogram resulting from analysis of SSU rRNA gene sequences of fungi. Oomycota were used as an outgroup. Statistics are as in Fig. 2.

Azoarcus spp. occur on fungal sclerotia in a remarkable diversity: they belong to three of five known plant-associated species and to one newly described group. This indicates that these fungal structures might represent an important microhabitat for *Azoarcus* spp. It might be an inherent feature of the plant-associated species to interact with eukaryotes, since *A. tolulyticus* and *A. evansii*, which are found in plant-free soil, were not isolated from fungal resting stages. What might be the role of this microhabitat for survival of *Azoarcus*? Plant-associated species other than *A. communis* (23) have never been isolated from root-free soil (12, 14, 34, 36) and can be regarded as closely associated with grasses. In a detailed population study on the same field from which the sclerotia were collected, we were not able to isolate *Azoarcus* spp. from sclerotium-free, sieved soil (34, 36). This indicates that these bacteria cannot survive freely in the soil, as has been proposed for other grass-associated diazotrophs as well (3). At first sight, it was therefore surprising that the root-associated *Azoarcus* species could be isolated from root-free soil in this study, albeit associated to the fungal resting stages present in this soil. Fungi, including their resting stages, might therefore serve as niches for survival of *Azoarcus* in soil. They might as well be an alternative host, which harbors strains of *Azoarcus* different from plant-associated strains. Indeed most of the strains found on sclerotia had some phenotypic differences (for instance, different vitamin requirements) in comparison to plant isolates. Moreover, group E of *Azoarcus* has never been isolated from any other source but the fungi. One of the strains, *A. indigens* BS2-10, was almost indistinguishable in PCR profiles from plant-associated members of this species, indicating that the fungi might be an intermediate host or a vector for plant-associated strains. Indeed, this view might be supported by the identity of the sclerotia. According to their SSU rDNA sequences, they are related to the *Ustilagomycetes*; hyphal members of this clade

are usually parasites of higher plants with a facultatively biotrophic life cycle (46). The sclerotia were similar to those of *Sclerotium rolfsii*, a deuteromycete which is a widespread plant pathogen in subtropical and temperate regions (32); a sexual state of this fungus, which resembled the basidiomycete *Athelia rolfsii* (32), could be induced; however, molecular data for *Sc. rolfsii* are not yet available. In any case, the fungi harboring *Azoarcus* spp. are likely to infect plants and may thus serve as a shuttle for endophytic *Azoarcus* spp.

Since the fungus harboring *Azoarcus* spp. occurs on a field cultivated with rice and wheat, it is tempting to speculate that these plants might be colonized by *Azoarcus* spp. in situ. In gnotobiotic culture, *Azoarcus* sp. strain BH72 is able to infect and grow endophytically in rice (16). Moreover, a sequence of a structural gene of nitrogenase which was highly homologous to *nifH* genes of *Azoarcus* spp. (13) was retrieved from rice roots grown in Japan (48), indicating that rice may indeed be a natural host plant for these bacteria.

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