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Identification of Grass-Associated and Toluene-Degrading Diazotrophs, *Azoarcus* spp., by Analyses of Partial 16S Ribosomal DNA Sequences

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The genus *Azoarcus* includes nitrogen-fixing, grass-associated strains as well as denitrifying toluene degraders. In order to identify and group members of the genus *Azoarcus*, phylogenetic analysis based on partial sequences of 16S rRNA genes (16S rDNAs) is proposed. 16S rRNA-targeted PCR using specific primers to exclude amplification in the majority of other members of the beta subclass of the class *Proteobacteria* was combined with direct sequencing of the PCR products. Tree inference from comparisons of 446-bp rDNA fragments yielded similar results for the three known *Azoarcus* spp. sequences and for analysis of the complete 16S rDNA sequence. These three species formed a phylogenetically coherent group with representatives of two other *Azoarcus* species which were subjected to 16S rRNA sequencing in this study. This group was related to *Rhodocyclus purpureus* and *Thauera selenatis*. New isolates and also sequences of so far uncultured bacteria from roots of Kallar grass were assigned to the genus *Azoarcus* as well. Also, strains degrading monoaromatic hydrocarbons anaerobically in the presence of nitrate clustered within this genus, albeit not with grass-associated isolates. All representative members of the five species harboring rhizospheric bacteria were able to form N_2O from nitrate and showed anaerobic growth on malic acid with nitrate but not on toluene. In order to visualize different *Azoarcus* spp. by whole-cell in situ hybridizations, we generated 16S rRNA-targeted, fluorescent probes by in vitro transcription directly from PCR products which spanned the variable region V2. Hybridization was species specific for *Azoarcus communis* and *Azoarcus indigens*. The proposed scheme of phylogenetic analysis of PCR-generated 16S rDNA segments will facilitate studies on ecological distribution, host range, and diversity of *Azoarcus* spp. and may even allow rapid identification of uncultured strains from environmental DNAs.

Recently, a new genus of grass-associated diazotrophs, *Azoarcus*, was described (22). It belongs to the beta subdivision of the class *Proteobacteria*, in which it constitutes a separate rRNA branch (22). All strains of this original description were isolated from one field located in the Punjab of Pakistan, from roots of the salt- and flood-tolerant grass *Leptochloa fusca* (L.) Kunth, commonly called Kallar grass (20, 22), except isolate BPD2, which originates from refinery oily sludge (14) and was assigned to the species *Azoarcus communis* (22). Regarding this restricted origin, strains show a surprising diversity; they cluster in five groups differing at the species level (22). Members of this genus are of particular interest with respect to plant-microbe interactions. Strain BH72 is capable of invading roots of its original host, Kallar grass, as well as of rice (11): it not only infects the cortex region but also is able to penetrate into the stele of gnotobiotically grown plants, spreading vertically into the plant shoot probably via xylem vessels. Enzymes which might contribute to the process of infection are cellulases, of which we detected two types in *Azoarcus* sp. strain BH72, an exoglycanase and an endoglucanase (21). Moreover, strain BH72 is capable of developing high rates of respiration and nitrogen fixation at close to anoxic conditions (around 30 nM dissolved O_2) (10), which may prevail in the rhizosphere of a flooded plant.

In order to better understand the ecological distribution and host range of *Azoarcus* spp., it will be necessary to screen for the presence of these bacteria in various habitats. The facts that members of the genus *Azoarcus* are, though genotypically diverse, phenotypically very similar and that they lack clearly differentiating characteristics impede such studies. Therefore, we developed molecular genetic tools to facilitate assignment to the genus *Azoarcus* (9). We proposed 16S rRNA gene (16S rDNA)-targeted PCR and oligonucleotide hybridization to screen for members of this genus. On the basis of sequence comparisons, new isolates from soil and industrial waste which are capable of degrading monoaromatic hydrocarbons such as toluene (7) could be assigned to the genus *Azoarcus*. These isolates can grow anaerobically and produce N_2 from denitrification, features which have not yet been described for members of the genus *Azoarcus* and are reevaluated in this study. Bacteria with similar phenotypic characteristics have recently been isolated by other groups, too (8). In order to facilitate studies on the intrageneric diversity of the genus *Azoarcus*, we propose here a scheme of 16S rDNA-targeted PCR combined with sequencing of PCR products to estimate phylogenetic relationships based on partial 16S rDNA sequences comprising only 446 bp. Since we use *Azoarcus*-specific primers, this method is applicable not only to pure cultures but also to DNA preparations of environmental samples. Using this method, we analyzed the two *Azoarcus* species which had not previously been subjected to 16S rDNA sequencing and assigned cultured

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and uncultured strains from Kallar grass as well as toluene-degrading isolates. Additionally, we propose a group-specific method for microscopic detection of *Azoarcus* spp. by in situ hybridization of whole cells, using fluorescent probes generated by in vitro transcription from PCR products.

MATERIALS AND METHODS

Bacteria and growth conditions. *Azoarcus indigens* VB32^T, *A. communis* SWub3^T, and *Azoarcus* sp. strains BH72, S5b2, and 6a3 originated from roots of Kallar grass grown in the Punjab of Pakistan (20, 22). Strain KGP1 was isolated after enrichment on SSM medium supplemented with vitamins as described by Reinhold et al. (20). The source of isolation was an ascoma of an ascomycete taken from Kallar grass rhizosphere soil; plants were grown for 4 years in pot culture in a soil core originating from Pakistan. *Azoarcus* sp. strain Td-1 was kindly provided by J. M. Tiedje.

Bacteria for DNA isolations were grown at 37°C on VM medium (22) with 6 g of ethanol per liter instead of potassium malate, either in liquid culture or on agar plates.

To test for denitrification, nitrogen-free SM medium (20) was used with one-fifth potassium malate content and supplemented with 0.1% (wt/vol) sodium succinate, 5 mM KNO_3, 50 mg each of Bacto Peptone and yeast extract per liter, and trace elements (per liter) $Na_2MoO_4 \cdot 2H_2O$ (2 mg), H_3BO_3 (3 mg), $MnSO_4 \cdot H_2O$ (2 mg), $ZnSO_4 \cdot 7H_2O$ (0.2 mg), and $CuSO_4 \cdot 5H_2O$ (0.1 mg). Nitrate was not added to control medium. To test for anaerobic growth on various carbon sources, potassium malate was omitted from SM medium and 0.01% (wt/vol) yeast extract and 5 mM KNO_3 were added. As a carbon source, 0.25% (wt/vol) malic acid (neutralized with KOH), 3 mM benzoic acid, 1 mM phenol, 0.26 mM toluene, or 0.23 mM benzene was added. Media were by strict anaerobic protocol dispensed into 15-ml tubes or 50-ml serum bottles, and vials were flushed with N_2 and sealed with butyl stoppers. Cultures were incubated at 37°C, and after 5 days of incubation, toluene and benzene were again added to the flasks at the same amount. Growth was monitored by observing the turbidity of the cultures. The capacity for denitrification was determined by measuring the accumulation of nitrous oxide in test tubes after 10% (vol/vol) acetylene had been added to the headspace. Nitrous oxide was determined gas chromatographically according to the method of Seiler and Conrad (25).

Preparation of DNA samples. High-molecular-weight DNA of *Azoarcus* strains was isolated according to the method of Marmur (17) with modifications (19). Quick lysates for PCRs were prepared by boiling; a freshly grown small colony of approximately 1-mm diameter was suspended in 50 μl of distilled water, boiled for 10 min, and slowly cooled to room temperature. After centrifugation in a microcentrifuge (13,000 × *g*), the supernatant was used for PCRs without further purification. DNA from plant roots was extracted as described previously (11).

Design of oligonucleotides. Primers were targeted to eubacterial 16S rDNAs with different degrees of specificity. Numbers given after the primer sequences are the primer positions corresponding to the numbering of the *Escherichia coli* 16S rRNA sequence (2). Forward primers for PCRs were TH1 (5′-TGGCTCA GATCGAACGCTGGCGGC-3′; positions 20 to 43) (9), a modification of a primer originally designed for amplification of 16S rDNA of *Proteobacteria* alpha subdivision members (30); TH14 (5′-GCTAATACCGCATACGTCCTGAG GG-3′; positions 168 to 192) (9); and TH3 (5′-GATTGGAGCGGCCGATGTC-3′; positions 222 to 240) (9). Reverse primers for PCR (9) were TH2 (5′-AAC GCTCGCACCCTCGTATTACCGC-3′; positions 552 to 527) and TH5 (5′-CT GGTTCCCGAAGGCACCC-3′; positions 1040 to 1022). Primers TH3 and TH5 specifically amplify 16S rDNA fragments of *Azoarcus* spp., whereas primers TH14 and TH2 are slightly less specific (9). In addition, for sequencing, primer 342fw (5′-CTACGGGRSGCAGCAG-3′; positions 342 to 357) adapted from the work of Lane (15) was used. To generate templates for in vitro transcription by PCR, primer BR45, which consisted of a fusion of a reverse primer complementary to 16S rDNA (boldface; positions 357 to 343) with the promoter region recognized by T7 RNA polymerase (from reference 27) (5′-GTAATACGACT CACTATAGGG**CTGCTGCCTCCCGTA**-3′), was used.

Conditions of PCRs. Amplification of DNA by PCR was carried out as described by Hurek et al. (9). For primer pairs TH14-TH2, TH3-TH5, and TH14-BR45, buffers of condition B were used; for primer pair TH1-TH5, condition A was used. In a 100-μl reaction mixture, 1 or 10 ng of chromosomal DNA template was used for amplification with primer pair TH14-TH2 or primer pairs TH3-TH5, TH1-TH2, and TH14-BR45, respectively. In the case of quick lysates, 5 μl was applied. Thermocycling was carried out as follows: initial denaturation at 94°C for 5 min; then 30 cycles with 1 min of denaturation at 94°C, annealing at 70°C for 2 min, and extension at 72°C for 2 min; and an additional extension step at 72°C for 10 min. For primer pair TH1-TH5, we used a 64°C annealing temperature and 30 cycles; for primer pair TH14-BR45, we used a 48°C annealing temperature and 33 cycles.

DNA sequencing. When PCR amplifications yielded a single distinct band in agarose gel electrophoresis, amplification products from three 100-μl reaction mixtures were once chloroform extracted to remove residual mineral oil and then purified with P5 columns (Quiagen Inc., Chatsworth, Calif.) according to the manufacturer's instructions. Precipitated DNA was dissolved in 18 μl of distilled water. Four to seven microliters was used for sequencing of double-stranded DNA by the dideoxy method with ^{35}S-labeled dATP. DNA was denatured by boiling and quick cooling in dry ice-ethanol. Sequencing with primers TH14 and 342fw was carried out with a Sequenase sequencing kit (U.S. Biochemicals, Cleveland, Ohio), the annealing buffer being replaced by the dimethyl sulfoxide-containing buffer proposed by Winship (29).

Phylogenetic analysis. The bootstrap consensus tree from 100 resamplings was inferred by the neighbor-joining method (24) from pairwise evolutionary distances (13) in Felsenstein's computer package PHYLIP, version 3.5.c (5). The tree is based on 446 aligned nucleotide positions. No parts of the sequence were masked out and excluded from phylogenetic analysis. The reference sequences from the EMBL database were *Azoarcus* sp. strain BH72 (L15530) (β), *Azoarcus* sp. strain S5b2 (L15532), *A. indigens* VB32^T (L15531) (β), *Azoarcus denitrificans* Td-1 (L33687) (β), *E. coli* (M24996) (τ), *Neisseria gonorrhoeae* (X07714) (β), *Azoarcus* sp. strain KB740 (X77679) (β), *Thauera* sp. strain K172 (X77118) (β), *Rhodocyclus purpureus* (M34132) (β), *Rubrivivax gelatinosus* (M60682) (β), *Spirillum volutans* (M34131) (β), and *Thauera selenatis* (X68491) (β). Greek letters indicate the corresponding subclass of *Proteobacteria.*

In vitro transcription. PCR products generated with primer pair TH14-BR45 in two 100-μl reaction mixtures were separated from primers and nucleotides with Quiagen P5 columns (see "DNA sequencing" above), with an additional phenol-chloroform extraction prior to precipitation. DNA was dissolved in 10 μl of diethylpyrocarbonate-treated H_2O containing 1 mM EDTA, pH 8.0. One microliter was employed for in vitro transcription with T7 RNA polymerase by using an in vitro transcription kit (Stratagene), with 30% UTP replaced by fluorescein-12-UTP (Boehringer, Mannheim, Germany). RNA precipitated from a 4-μl reaction mixture was dissolved in 20 μl of diethylpyrocarbonate-treated H_2O. To control generation of RNA, a 0.5-μl sample was subjected to denaturing polyacrylamide gel electrophoresis (23) and a fluorescent band was visualized by UV irradiation.

Whole-cell in situ hybridization. Cells grown in liquid medium as for DNA isolation were fixed, spotted on glass slides, and dehydrated as described by Trebesius et al. (26), except that fixation was with 1% (vol/vol) glutaraldehyde instead of paraformaldehyde. Samples of bacteria to be compared with the same probe were treated on the same slide. Hybridization with 0.7 μl of fluorescing probe per spot was carried out for 6 h at 53°C in a humid chamber in hybridization buffer which consisted of 20 mM Tris-HCl buffer (pH 7.4), 0.01% (wt/vol) sodium dodecyl sulfate, 0.01 M sodium chloride, and 90% (vol/vol) formamide. Hybridization was terminated by immersion of slides in distilled water; two additional wash steps were carried out in hybridization buffer for 20 min at 53 and 68°C. Samples were mounted in phosphate-buffered saline (3) containing 10% (vol/vol) glycerol and examined with an Axiophot microscope (Zeiss, Oberkochem, Germany) with epifluorescence illumination (filter 488010), and results were recorded on Agfapan 400 black-and-white film.

Nucleotide sequence accession numbers. The partial 16S rDNA sequences of isolates and reference strains have been placed in EMBL under the accession numbers X85432 (*A. communis* SWub3^T), X85433 (*A. communis* KGP1), X85435 (*Azoarcus* sp. strain 6a3), and X85434 (PCRbacteria).

RESULTS AND DISCUSSION

Strategy for identification of *Azoarcus* spp. The strategy which we found suitable to identify members of the genus *Azoarcus* is illustrated in Fig. 1. As proposed previously (9), 16S rDNA-targeted, genus-specific PCR of bacterial DNA (amplification product, approximately 820 bp) and oligonucleotide hybridization with oligonucleotide TH15 to amplification products of PCR with primer pair TH14-TH2 or TH1-TH5 (amplification product, approximately 390 or 1,020 bp, respectively) indicate affiliation with the genus *Azoarcus*. To verify assignment to the genus and to allow grouping, we attempted to use partial 16S rDNA sequences. Overlapping amplification products from primer pairs TH14-TH2 and TH3-TH5 were applied for direct double-strand sequencing with various primers, including TH14, TH2, TH3, TH5, and other primers binding to eubacterial 16S rDNA. Primers TH14 and 342fw proved to be most efficient in generating well readable sequences with the dimethyl sulfoxide buffer system applied by us. The use of primers which will specifically amplify *Azoarcus* 16S rDNA may allow direct identification without prior isolation of pure cultures from environmental samples, if one strain is predominant (see below).

Phylogenetic relationship of *A. communis* SWub3^T, 6a3, and cultured and uncultured strains from Kallar grass as well as toluene-degrading isolates with other members of the genus *Azoarcus*. Sequence fragments of 446 bp corresponding to po-

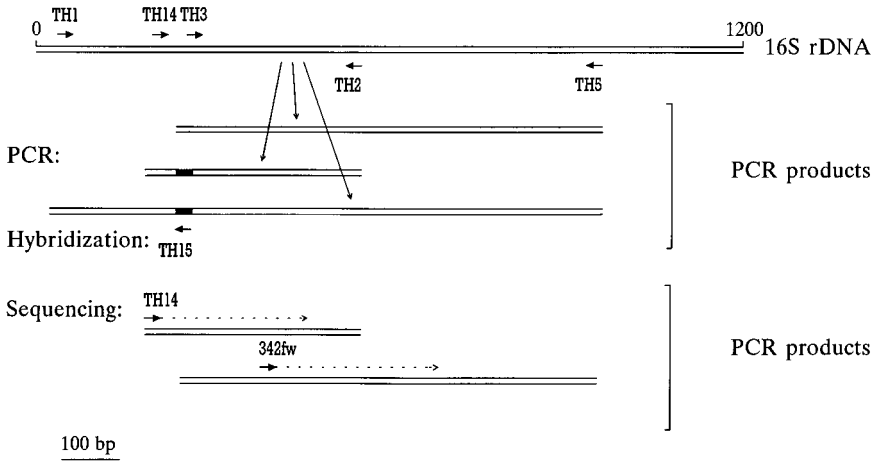

FIG. 1. Strategy of retrieval and sequencing of 16S rDNA. Arrows indicate the annealing position of each primer and the direction of elongation. PCR products can be used as targets for oligonucleotide hybridization with TH15 or directly used for sequencing with primers TH14 and 342fw.

sitions 220 to 664 of the *E. coli* 16S rRNA sequence (2) were obtained from direct PCR sequencing using the strategy mentioned above and were used for alignments. A phylogenetic tree based thereon by using distance statistics is shown in Fig. 2. It also includes data available for other strains. Tree inference by the maximum parsimony and likelihood methods revealed similar topologies. With *Azoarcus* sp. strain 6a3 and *A. communis* SWub3^T included in analysis, representatives of all five *Azoarcus* species clustered. In good agreement with DNA-rRNA hybridization data (22), *A. communis* was more closely related to *A. indigens* and strain BH72 than to strains S5b2 and 6a3. Using a short 446-bp segment, we obtained good concurrence with previous analysis of complete 16S rRNA sequences (9), since both methods revealed a close relationship of *Azoarcus* strains with *Rhodocyclus purpureus*; a newly described organism, *T. selenatis* (16), appeared to be even more closely related. Analysis of the published 16S rDNA sequence of *T. selenatis* revealed that the supposedly *Azoarcus*-specific primers for PCR and oligonucleotide hybridization had only very few or no mismatches with it (TH2, one; TH3, none; TH5, one; TH14, none; TH15, none). Strains of *Thauera* might therefore give false-positive results, stressing the importance of sequence analysis.

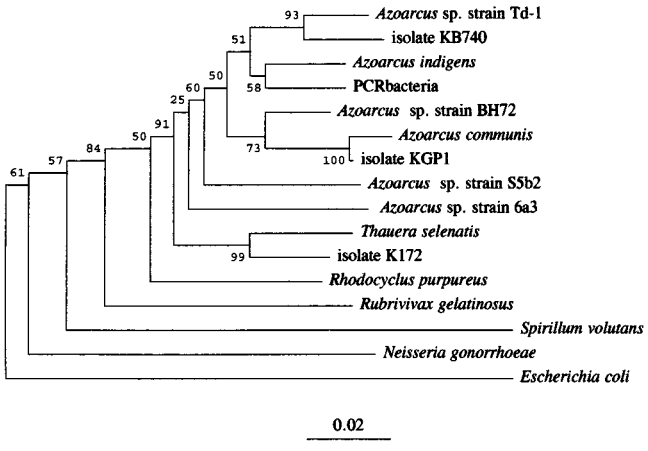

FIG. 2. Phylogenetic relationships of *Azoarcus* species with other members of the *Proteobacteria* beta subclass. The phylogenetic tree was reconstructed with the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE in PHYLIP, version 3.5.c, on the basis of a 446-bp 16S rDNA segment. Values at the nodes represent the bootstrap confidence estimates on the branches in 100 replicates. PCRbacteria represents not an isolate but rather a segment generated by PCR from an environmental sample. Bar = phylogenetic distance, representing accumulated changes per nucleotide.

Additionally, our approach was suitable to assign a new isolate to a known species, *A. communis*. Strain KGP1 was isolated from a fruiting body of a rhizospheric ascomycete from a Kallar grass pot culture and showed typical, slightly yellowish colonies on VM medium plates supplemented with ethanol instead of malate; cells were slightly curved, motile rods, and a typical subsurface pellicle was formed on semisolid nitrogen-free medium. Quick lysates yielded DNA of sufficient purity to carry out PCR amplifications. All primer pairs applied for PCR, TH3-TH5, TH14-TH2, and TH1-TH5, yielded amplification products of the expected size, which hybridized with oligonucleotide TH15 (not shown). Partial 16S rDNA sequences subjected to phylogenetic analysis showed that strain KGP1 belongs into the genus *Azoarcus*, being related to *A. communis* SWub3^T (Fig. 2).

Roots of the same plants from which strain KGP1 originated were used for enrichment of *Azoarcus* spp. and for isolation of DNA, which was subjected to PCR and sequence analysis according to the strategy mentioned above. Amplification with primer pairs TH3-TH5 and TH14-TH2 yielded products of the expected size (not shown). Partial 16S rDNA sequences were unambiguously readable and clustered within the genus *Azoarcus* according to phylogenetic analysis (PCRbacteria, Fig. 2), being related to *A. indigens* but probably composing a new group. Enrichment studies did not yield isolates belonging to the genus *Azoarcus*. Therefore, the sequence obtained stems either from a physiologically active, noncultured strain or from nonviable bacteria. This is of particular interest, since even unculturable bacteria appear to be easily detectable by our approach. It is well known from habitats other than plant roots that it seems to be currently not possible to grow the vast majority of bacteria in culture: from lakewater (12) or marine water (6) samples, only 0.1 or 12.5%, respectively, of the directly counted bacteria could be cultured. We cannot exclude the possibility that a similar situation will occur for diazotrophs in roots of grasses.

Moreover, bacteria degrading aromatic hydrocarbons were identified as *Azoarcus* spp., which had not been isolated from plants. Strain Td-1 was enriched on toluene as a carbon source and was assigned to *Azoarcus* sp. according to a 280-bp 16S

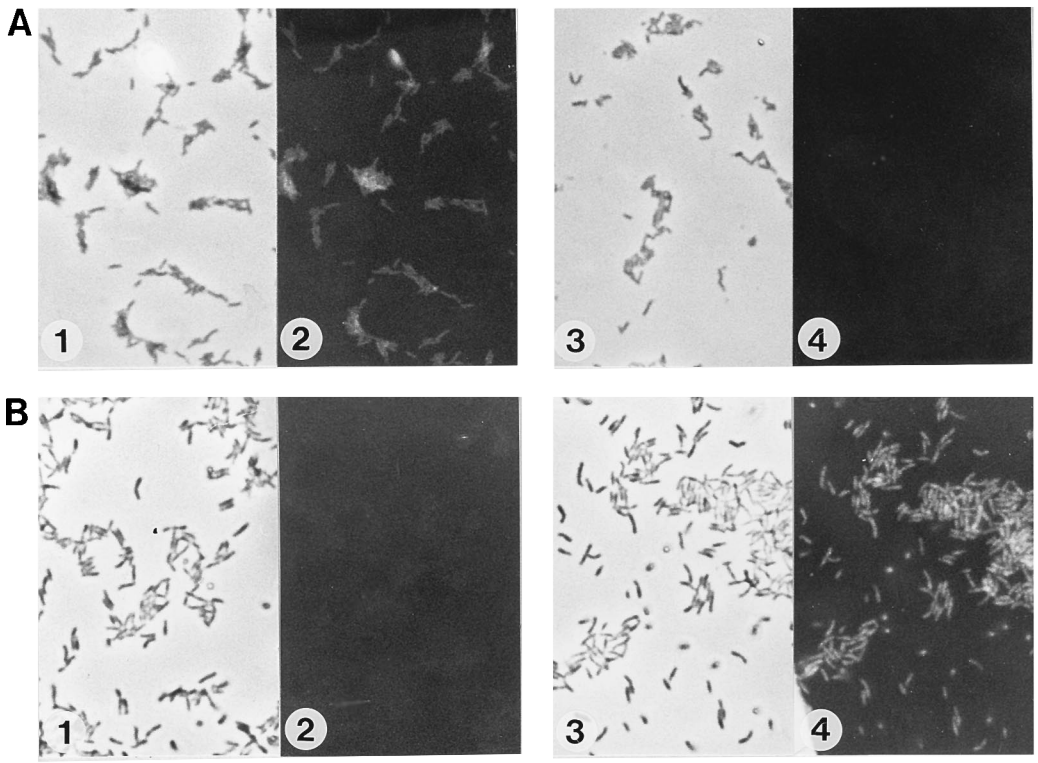

FIG. 3. Specificity of in situ hybridization with fluorescent probes generated by in vitro transcription against 16S rDNA of *A. indigens* VB32^T and *A. communis* SWub3^T. Fixed cells of *A. indigens* VB32^T (A) and *A. communis* SWub3^T (B) were hybridized with a homologous probe (A, panels 1 and 2; B, panels 3 and 4) and a heterologous probe (A, panels 3 and 4; B, panels 1 and 2). Phase-contrast (panels 1 and 3) and epifluorescence (panels 2 and 4) micrographs are shown for identical fields.

rDNA segment (corresponding to positions 820 to 1090 of the *E. coli* 16S rDNA) (7). Sequences generated by our approach corroborated this finding and indicated that Td-1 belongs to a separate group, *A. indigens* being most closely related. Strains KB740 and K172, both denitrifying strains degrading hydrocarbons such as benzoate and 4-hydroxybenzoate (8), were classified as well. Strain KB740 clustered with the toluene-degrading isolate Td-1 within the genus *Azoarcus*, whereas strain K172 was related to *T. selenatis* (Fig. 2).

These data indicate that partial 16S rDNA sequences of a 446-bp segment were suitable to differentiate members of the genus *Azoarcus* from other, related members of the *Proteobacteria* and to group species within this genus. Sequences of 16S rDNA segments have successfully been used to unravel phylogeny of other eubacterial groups, e.g., the genus *Rhizobium* and related genera in the alpha subdivision of *Proteobacteria* (30). In this case, a 260-bp segment was long and variable enough to provide sufficient resolution, with a short highly variable region being omitted from the analyses. In our case, segments shorter than the one proposed here, e.g., from positions 206 to 460, did not provide distance matrices which were robust enough to differentiate the genus *Azoarcus* from related genera, whether highly variable regions were omitted from analysis or not (not shown).

Denitrification and use of aromatic hydrocarbons for growth of *Azoarcus* spp. Assignment of denitrifying bacteria that anaerobically degrade toluene (7) or benzoate and 4-hydroxybenzoate (8) to the genus *Azoarcus* prompted us to evaluate these features in plant-associated *Azoarcus* strains. Tests for denitrification carried out previously were based on observation of N_2 formation in commercial test systems (API 20E; bioMérieux, Montalieu Vercieu, France) and had yielded negative results (22). When we applied strictly anaerobic protocols, growth was observed for strains VB32^T, SWub3^T, BH72, 6a3, and S5b2 within 3 days in medium containing 5 mM KNO_3 in contrast to medium containing no nitrate. After 2 days of growth, we added acetylene to the headspace to inhibit nitrous oxide reductase; subsequently, accumulation of nitrous oxide was detected in all strains when nitrate was present in the medium, indicating that denitrification occurred. Among the carbon sources tested for anaerobic denitrifying growth, benzoate, toluene, benzene, and phenol were not used and malate was a good substrate for growth of all strains. Aerobic growth on monoaromatic compounds like benzoate, benzylamine, and *m*- and *p*-hydroxybenzoate has been described for some plant-associated strains of *Azoarcus* spp. previously (22). The capability to attack aromatic compounds might be an advantage for *Azoarcus* spp. to cope with phenolic substances involved in plant defense mechanisms.

Whole-cell hybridization with fluorescent probes. In order to visualize bacteria specifically, fluorescently monolabeled, 16S rRNA-targeted oligonucleotide probes have been successfully used in in situ hybridization studies (1, 4). However, for some applications, e.g., when the content of rRNA is low in cells (4), a more densely labeled probe is an advantage. We tested homogeneously labeled antisense probes complementary to 16S rRNA, which were generated by in vitro transcription from PCR products. Probes spanning positions 168 to 357 according to the numbering of the *E. coli* 16S rRNA gene covered the variable region V2, which might allow specific detection of different strains of *Azoarcus* spp. We therefore tested the specificity of probes generated against two different species. Fluorescing antisense probes of an initial length of approximately 210 nucleotides directed against *A. indigens*

VB32^T or *A. communis* SWub3^T (Fig. 3) detected only the homologous strain in whole-cell hybridization. Despite its length, the probe penetrated cells, as has been described previously for 23S rRNA-based probes which were 315 nucleotides in length (26). Hybridization was specific, although the probe also spans conserved regions of the 16S rRNA; however, the specificity will have to be tested with appropriate, highly related strains of *Azoarcus* spp. prior to each new application. The use of a PCR primer carrying the promoter region of T7 RNA polymerase has the advantage that a cloning step into a vector carrying these RNA polymerase promotors is avoided. Another advantage of using PCR for template generation is that probes which are directed against uncultured strains, which are detected by PCR with *Azoarcus*-specific primers, can easily be produced; amplification products can be employed for a second PCR step, which provides the template for in vitro transcription.

Conclusions. The usefulness of partial sequences of 16S rDNA for differentiating the genus *Azoarcus* from other genera and for intrageneric grouping has been demonstrated. By using genus-specific PCR-primers to generate templates for sequencing, an uncultured strain could be identified in environmental samples without construction of a clone library. The proposed approach was also successfully applied to denitrifying isolates which anaerobically degrade monoaromatic hydrocarbons; they cluster within the genus *Azoarcus*, albeit not with the plant-associated strains, which correlates with differences in the substrate spectrum for anaerobic growth. Thus, the diversity observed within the genus *Azoarcus* has increased; the genotypically rather divergent members of this genus might be defined as several genera within the family *Azoarcaceae*, when further knowledge on phenotypic features has been gathered. Taking into account that all isolates originate from one Kallar grass field, except for one member of the species *A. communis* and the group of aromatic hydrocarbon-degrading strains, the diversity is at first sight surprising. However, it is consistent with the observation that often the diversity of microorganisms appears in large measure to reflect obligate or facultative associations with higher organisms and to be determined by spatial-temporal diversity of their hosts or associates (28). Such a temporal variation might be reflected in the observation that we were unable to isolate similar strains at different times: from two surveys that we conducted in 1984 and 1988 on the same field and in the same season of the year, *Azoarcus* strains cultured from within a radius of 5 m were entirely different: none of the genotypes distinct at the species level isolated in 1988 were isolated in 1984 (20, 22). The presence of several similar but not identical genotypes with similar functions might impart to the ecosystem a buffering capacity against the loss of species (18) or an adaptation of a functionally similar microflora to slight variations in the environment. The proposed method will facilitate studies on the ecological distribution, host range, and diversity of *Azoarcus* spp.

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