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Azoarcus gen. nov., Nitrogen-Fixing Proteobacteria Associated with Roots of Kallar Grass (*Leptochloa fusca* (L.) Kunth), and Description of Two Species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov.

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Among the nitrogen-fixing bacteria associated with roots of *Leptochloa fusca* (L.) Kunth in saline-sodic soils in the Punjab of Pakistan, we repeatedly found yellow-pigmented, straight to curved, gram-negative rods. To group and identify these organisms, we examined morphological, nutritional, and biochemical features and performed polyacrylamide gel electrophoretic analyses of cellular proteins, gas chromatographic analyses of fatty acids, DNA-rRNA hybridizations, and DNA-DNA hybridizations. Our results showed that 11 isolates formed five groups distinct at the species level, with each group containing one to three members. These bacteria constituted a separate rRNA branch in rRNA superfamily III (corresponding to the beta subclass of the *Proteobacteria*) at a branching $T_{m(e)}$ level of 67.7°C [$T_{m(e)}$ is the temperature at which 50% of a hybrid is denatured under standard conditions]. On this branch, the five groups were located in two clusters with $T_{m(e)}$ values of 79.4 to 80.4°C and around 71.5°C. We propose a new genus, the genus *Azoarcus*, for these strains. *Azoarcus indigens* is the type species and has a growth factor requirement; its type strain is strain VB32 (= LMG 9092). A second named species, *Azoarcus communis*, includes a strain obtained from French refinery oily sludge, strain LMG 5514. Bacteria of this genus have a strictly aerobic type of metabolism, fix nitrogen microaerobically, and grow well on salts of organic acids but not on carbohydrates. Swedish isolates obtained from human sources (E. Falsen group 15 strains LMG 6115 and LMG 6116), as well as "[*Pseudomonas*] *gasotropha*" LMG 7583^T, were also located on this rRNA branch at a lower $T_{m(e)}$ level (70.4 to 71.2°C).

In the rhizospheres of tropical, subtropical, and temperate grasses, nitrogen-fixing bacteria belonging to at least 11 genera have been found (see reference 15). Often, several species can be isolated from one plant (34, 44). A genus which has been isolated frequently (13) and has been studied intensively with respect to its associations with grasses is the genus *Azospirillum*.

Kallar grass (*Leptochloa fusca* (L.) Kunth) is associated with several different nitrogen-fixing bacteria (44). This grass, which tolerates soil salinity and waterlogged conditions, is widely distributed in tropical to subtropical regions ranging from Australia to Africa. In the Punjab of Pakistan, it is used as a pioneer plant on saline-sodic, alkaline soils having low fertility (47). Because of its luxuriant growth without the addition of any nitrogenous fertilizer, giving harvests of 20 to 40 metric tons (2×10^4 to 4×10^4 kg) of hay per ha per year (46), we became interested in the nitrogen-fixing bacteria colonizing Kallar grass roots.

Studies of rhizospheric populations were carried out on a field in Punjab which had been under Kallar grass cultivation for more than 10 years. Nitrogen-fixing bacteria were found to form root-zone-specific associations, with different populations colonizing the surface and the interior of roots (44). Two *Azospirillum* species were dominant on the rhizoplane, and one of these organisms has been described as a new, salt-tolerant species, *Azospirillum halopraeferens* (42). However, the diazotrophs predominating inside the roots were gram-negative rods which could not be assigned to previously described taxa on the basis of phenotypic characteristics (44). Indirect evidence for the colonization of the root interior by these organisms was confirmed by the results of immunofluorescence studies (41) and immunogold electron microscopy (23). The higher probability of metabolite transfer between host and diazotrophs inside roots than between host and root surface bacteria focused our attention on the diazotrophs inside roots (39).

In this paper we describe the repeated isolation of one group of diazotrophic rods (44) from Kallar grass roots and the results of a polyphasic taxonomic study that led to taxonomic description of these organisms. On the basis of our results, we propose the new genus *Azoarcus*, consisting of two named species, *Azoarcus communis* and *Azoarcus indigens*, and three additional unnamed groups which are also distinct at the species level. Bacteria from other sources could also be assigned to the *Azoarcus* rRNA branch.

(A preliminary account of this study was presented previously [43].)

MATERIALS AND METHODS

Isolation of diazotrophic strains. Samples of Kallar grass were collected from the same field in the Punjab region of Pakistan in 1984 and 1988. Kallar grass grew almost exclusively on the saline-sodic soil at this site. Samples were subjected to isolation procedures either immediately (in 1984) or within 4 days after transport at 4°C to Europe. A sample of Kallar grass from a mangrove-covered, brackish sandy river bank near Brisbane, Australia, was processed 2

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days after collection. Enrichment cultures were prepared by using pieces of roots with adhering rhizosphere soil, surface-sterilized root pieces, and serial dilutions of preparations containing surface-sterilized, homogenized roots and stem bases. Surface sterilization, homogenization, and serial dilution were carried out as described previously (44). Putative nitrogen-fixing bacteria were enriched on two semisolid, nitrogen-free, malate-containing media: SM medium supplemented with vitamins (44) and SSM medium (44). SSM medium was modified to contain the salt concentrations commonly found in soil saturation extracts of the saline-sodic soils in the Punjab. After two successive enrichment steps of 48 to 72 h, bacteria from subsurface pellicles were isolated on the media, which contained 0.8% agar instead of 0.2% agar and 20 mg of yeast extract per liter. For up to 7 days after inoculation, single colonies were transferred to semisolid medium. Strains which exhibited growth as subsurface pellicles were further purified. Nitrogenase activity was assayed by the acetylene reduction test (42) with a model IGC 120DFL gas chromatograph (Intersmat, Suresnes, France). Bacteriological purity was checked on plates containing VM medium, a modification of SM medium which contains half as much potassium DL-malate, 0.3% beef extract (Oxoid), 0.1% yeast extract (Oxoid), 0.1% NaCl, and 0.05% NH_4Cl. The growth temperature used for enrichment and isolation was 30°C, and the temperature used for further cultivation was 37°C unless stated otherwise. Except for strains BH72 and Bb6c1, the designations for strains that were isolated from salt-enriched (SSM) medium begin with S. Pure cultures of N_2-fixing strains were maintained at 30°C in semisolid SM medium supplemented with vitamins or SSM medium with biweekly transfers. Strains were also kept for 1 to 2 months at 4°C on slants of VM medium after 2 days of growth at 37°C. Long-term preservation was achieved by placing the organisms in liquid nitrogen as described previously (40) or by lyophilization.

Bacterial strains. The strains used in this study are listed in Table 1. In addition to our own isolates, other free-living N_2 fixers and reference organisms belonging to rRNA superfamilies II and III were included. Reference strains were routinely grown on nutrient agar, which contained (per liter of distilled water) 5 g of bacteriological peptone, 2 g of yeast extract, 1 g of beef extract, 5 g of NaCl, and 15 g of agar.

Azotobacter sp. and *Azomonas* sp. strains were grown on medium containing (per liter of distilled water) 10 g of glucose, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 5 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.9 g of K_2HPO_4, 10 mg of $FeSO_4 \cdot 7H_2O$, and 5 g of $CaCO_3$.

PAGE of SDS-soluble cell proteins. All strains were grown at 28°C for 36 h in one or two Roux flasks on VM medium modified by 2.5-fold-stronger buffering with potassium phosphate buffer (pH 6.8). Extracts of sodium dodecyl sulfate (SDS)-soluble proteins were prepared from whole cells as described previously (27). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) by using a slight modification (27) of the method described by Laemmli (29).

Gas chromatography of cellular fatty acids. Bacterial strains were precultured on VM agar slants and streaked onto plates containing 3.0% (wt/vol) Trypticase soy broth (BBL) supplemented with 1.5% (wt/vol) Bacto Agar (Difco) by a streak-plate method to obtain well-separated colonies, and the plates were incubated at 28°C for 24 h. Approximately 60 mg (wet weight) of cells was harvested from the most dilute quadrant exhibiting confluent growth. The methods used for extraction of fatty acids, preparation of fatty acid methyl esters (FAMEs), and separation of FAMEs by gas-liquid chromatography have been previously described (51). FAME fingerprints were identified by using the Microbial Identification System software package (MIS version 3.2; Microbial ID, Inc., Newark, Del.). A statistical program developed by Microbial ID, Inc., was used for cluster analysis of the strains.

Preparation of high-molecular-weight DNA. Cells were grown in Roux flasks on the appropriate growth medium. DNA was prepared by the method of Marmur (31) or by a slightly modified procedure (42). When available, DNAs obtained from members of our research group were used (Table 1).

DNA base composition. The average guanine-plus-cytosine (G+C) contents of the DNAs from isolates were determined by the thermal denaturation method and were calculated by using the equation of Marmur and Doty (32), as modified by De Ley (6). The base composition was determined for one representative strain of each group.

DNA-rRNA hybridization. Single-stranded high-molecular-weight DNA was fixed onto membrane filters (type SM 11358; Sartorius, Göttingen, Germany) as described by De Ley and De Smedt (9). ^3H-labeled rRNAs from isolates BH72 and H6a2 were prepared and purified (9) by using $[^3H]$adenine and $[^3H]$uracil as precursors; the specific activities of the 23S rRNA preparations were 21×10^3 and 11×10^3 cpm μg^{-1}, respectively. Labeled rRNAs from the following reference organisms were available from members of our research group: *Alteromonas haloplanktis* LMG 2852^T (T = type strain) (50), *Xanthomonas campestris* LMG 568^T (4), *Pseudomonas fluorescens* LMG 1794^T (11), *Oceanospirillum linum* LMG 5414^T (36), *Azorhizobium caulinodans* LMG 6465^T (14), *Aquaspirillum serpens* biovar bengal strain LMG 6234^T (37), [*Pseudomonas*] *rubrisubalbicans* LMG 2286^T (18), *Hydrogenophaga palleronii* LMG 2366^T (52), and *Oligella urethralis* LMG 5304 (45). Hybridizations between filter-fixed DNA and labeled 23S rRNA were carried out as described previously (9). As a measure of the thermal stability of each hybrid, we determined its $T_{m(e)}$ (the temperature at which 50% of the hybrid was denatured under standard conditions).

DNA-DNA hybridization. The degrees of DNA binding were determined spectrophotometrically from initial renaturation rates (8). Renaturations were carried out by using approximately 50 μg of DNA per ml at 79.8°C since this was the optimal renaturation temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; pH 7.0). The molecular complexity of each genome was estimated from the renaturation reaction constants by using the genome size of *Escherichia coli* as a reference (17).

Morphological and physiological tests. Cell dimensions and morphology were determined by phase-contrast microscopy. Cells of strain BH72 negatively stained with uranyl acetate were checked electron microscopically for the type of flagellation. Cells were grown on medium containing combined nitrogen (liquid SM medium supplemented with 0.05% NH_4Cl and 0.01% yeast extract) at 28°C with shaking and with N_2 as nitrogen source in semisolid SM medium supplemented with vitamins or SSM medium at 37°C to evaluate cell morphology.

Unless stated otherwise, cells were incubated at 37°C for physiological tests. Colony morphology was determined at 30°C on Congo red agar (4) and nutrient agar, and color development was determined at 37°C on agar plates containing VM medium with potassium DL-malate replaced by 0.6% (vol/vol) filter-sterilized ethanol. Salt tolerance was tested in liquid VM medium containing 2 or 5% NaCl at 28°C with

TABLE 1. Strains used

Taxon	Strain designation as received[a]	Other designation[a]	Source and year isolated[b]
Strains assigned to:			
Azoarcus indigens	VB32^{T}[c]	LMG 9092^{T}	Surface-sterilized stem bases, Pakistan, 1988
	VW34c[c]		Surface-sterilized roots, Pakistan, 1988
	VW35a[c]		Surface-sterilized roots, Pakistan, 1988
Azoarcus communis	SWub3^{T}[c]	LMG 9095^{T}	Root piece, Pakistan, 1988
	LMG 5514	BPD2, S2	Refinery oil sludge, France, 1982, G. Laguerre
Azoarcus spp.	BH72		Surface-sterilized roots, Pakistan, 1984, B. Reinhold
	S5b2[c]		Surface-sterilized roots, Pakistan, 1988
	S5b1[c]		Surface-sterilized roots, Pakistan, 1988
	SSa3[c]		Surface-sterilized roots, Pakistan, 1988
	6a3[c]		Surface-sterilized roots, Pakistan, 1988
	6a2[c]		Surface-sterilized roots, Pakistan, 1988
	5c1[c]		Surface-sterilized roots, Pakistan, 1988
Unidentified EF group 15	LMG 6115	CCUG 10488	Wound, Göteborg, Sweden, 1981, E. Falsen
Unidentified EF group 15	LMG 6116	CCUG 13232	Blood, Göteborg, Sweden, 1983, E. Falsen
"[*Pseudomonas*] *gasotropha*"	LMG 7583^{T}	DSM 1085^{T}	Mud, River Moskwa, Russia, DSM
Reference and other strains			
Pseudomonas fluorescens	LMG 1794^{T}	MMCA 40^{T}[d]	
Flavimonas oryzihabitans	LMG 7040^{T}	IAM 1568^{T}	
Chryseomonas luteola	LMG 7041^{T}	IAM 13000^{T}	
Azomonas macrocytogenes	LMG 8755^{T}	NCIB 8700^{T}	
Neisseria gonorrhoeae	NCTC 8375^{T}[d]		
Janthinobacterium lividum	Sneath HB^{T}[d]	LMG 2892^{T}	
Comamonas acidovorans	Stanier 14^{T}[d]	LMG 1226^{T}	
Herbaspirillum seropedicae	Z78	LMG 6514	
Herbaspirillum seropedicae	Bb6c1		Surface-sterilized roots, Australia, 1988[c]
Azotobacter chroococcum	NCIB 11694^{T}	LMG 8756^{T}	
Alcaligenes xylosoxidans subsp. *denitrificans*	ATCC 15173^{T}[d]	LMG 1231^{T}	
[*Pseudomonas*] *solanacearum*	NCPPB 325^{T}[d]	LMG 2299^{T}	
Chromobacterium violaceum	NCTC 9757^{T}[d]	LMG 1267^{T}	
Derxia gummosa	NCIB 9064^{T}	LMG 3977^{T}	
[*Pseudomonas*] sp.	DC		Roots of *Deschampsia caespitosa*, Finland, K. Haathela
Pseudomonas sp.	4B	ATCC 43038	Forest soil, Canada, Y.-K. Chan
Unidentified	H6a2		Surface-sterilized roots, Pakistan, 1984
	S7a1[c]		Surface-sterilized roots, Pakistan, 1988
	S6c1[c]		Surface-sterilized roots, Pakistan, 1988
	G3		Rice field in Ganges Valley, Bangladesh, R. Bally

[a] The original strain designations are given for the Kallar grass isolates. ATCC, American Type Culture Collection, Rockville, Md.; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; LMG, Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; MMCA, Medical Microbiology Culture Collection, Aarhus, Denmark; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; G. Laguerre, strain LKG5514 is deposited as S2 in the Centre de Biorecherches de Elf-Labège (France).

[b] Source, place and/or year of isolation, and investigator for strains assigned to *Azoarcus* spp., other isolates originating from Kallar grass, and for unidentified strains.

[c] Isolated from Kallar grass (*L. fusca* (L.) Kunth) by B. Reinhold-Hurek in this study.

[d] Strains from which we used DNAs available in our research group for DNA-rRNA hybridizations.

shaking. Growth was monitored by measuring turbidity after 3 days of incubation. Optimum salt concentrations for growth were determined for strains SWub3^{T}, VB34c, BH72, S5b2, and 6a3 in liquid SM medium supplemented with vitamins, yeast extract (0.05%), and NH_4Cl (0.01%) by using NaCl concentrations of 0, 0.25, 0.5, and 0.75%. Exponential growth in 250 ml-Erlenmeyer flasks containing 25 ml of medium was monitored turbidimetrically at 40°C with reciprocal shaking (150 strokes per min), and generation times were compared. The optimal growth temperatures were determined for the same strains in a similar way by using liquid VM medium and temperatures of 33.5, 36.5, 40, and 45°C. For strains H6a2 and BH72, tolerance of N_2-dependent growth to NaCl, different temperatures, and different pH values was also determined by measuring acetylene reduction rates as described previously (42). Requirements for growth factors were checked on medium containing combined nitrogen (liquid SM medium supplemented with 0.05% NH_4Cl) and with N_2 as nitrogen source (semisolid SM medium) at 28 and 37°C, respectively.

Gram staining and tests for cytochrome *c* oxidase activity, catalase activity, starch hydrolysis, and oxidation or fermentation of glucose and fructose on Hugh-Leifson medium were carried out by using standard methods (48) and reference strains as positive controls. Other biochemical data were obtained from API 20E strips (bioMérieux, Montalieu Vercieu, France) used according to the manufacturer's directions. Inocula were obtained from fresh VM medium plates. Strips were interpreted after 22 h of incubation at 37°C.

Carbon substrate assimilation tests. API 50CH, API 50AO, and API 50AA galleries (bioMérieux) were used to test for growth on 147 different carbon sources (carbohydrates and organic and amino acids). Inocula were obtained from fresh VM agar slants. After inoculation as described by Kersters et al. (26), the galleries were incubated at 30°C, and values were recorded after 1, 2, 4, and 7 days. The values obtained on day 7 are listed in Table 5.

Enzymatic tests. To detect constitutive enzymatic activities on 19 substrates, API ZYM galleries (bioMérieux) were used; cells grown for 20 h on nutrient agar in Roux flasks were used as inocula. Inoculation and evaluation were carried out according to the manufacturer's instructions. The galleries were incubated at 37°C for 4 h.

RESULTS AND DISCUSSION

Isolation and initial selection of strains. Two strains of diazotrophic, slightly yellowish rods (strains H6a2 and BH72) were obtained from the interior of roots of Kallar grass grown in the Punjab of Pakistan during the first survey in 1984 (44). According to preliminary DNA-rRNA hybridization data, these two strains were located in separate rRNA superfamilies (superfamilies II and III, respectively). Additional isolation experiments were carried out in March and November 1988 in order to (i) elucidate whether these bacteria occurred repeatedly in this habitat and in what proportions they occurred and (ii) determine their exact taxonomic positions. Isolates obtained from the first and second surveys and one isolate obtained from Australian Kallar grass roots were screened for similarities in cell morphology, colony morphology, and the ability to form a subsurface pellicle in semisolid nitrogen-free medium, which is indicative of microaerobic N_2 fixation. Strains producing growth as a subsurface pellicle were similar in cell morphology to strain BH72; they were slightly curved rods with corkscrew-like motility. Strain BH72 produced intracellular reserve granules (Fig. 1b) which consisted of poly-β-hydroxybutyrate (22). A total of 16 isolates obtained from roots of Kallar grass (Table 1) were selected for further study; 11 of these isolates were assigned to the new genus *Azoarcus* in this study. Strains belonging to rRNA superfamily III that had $T_{m(e)}$ values similar to those of strain BH72 were also selected for further study; these strains were unidentified strain LMG 5514, "[*Pseudomonas*] *gasotropha*" LMG 7583^T, E. Falsen (EF) group 15 strains LMG 6115 and LMG 6116, *Derxia gummosa* LMG 3977^T, and *Herbaspirillum seropedicae* LMG 6514.

Comparison of protein electropherograms. Figure 2 shows the SDS-PAGE patterns of the strains studied. The protein electropherograms of 10 of the new isolates fell into four groups, groups A to D; the electropherograms of these groups clearly differed but exhibited some overall similarities with each other and with the electropherogram of strain BH72 (whose electropherogram was unique). Within each group the protein electropherograms were almost indistinguishable, indicating that the members of each group had high overall levels of genomic similarity (25). The protein pattern of Kallar grass isolate SWub3^T was very similar to that of strain LMG 5514, which originated from a petroleum refinery oily sludge in France (30). Some similarities were also obvious between the group D pattern and the patterns of strains LMG 6115 and LMG 6116, which were isolated from human clinical sources in Sweden (EF group 15) (16). The protein electropherograms of isolates S7a1, S6c1, and H6a2 and "[*Pseudomonas*] *gasotropha*" LMG 7583^T were distinct. The protein pattern of strain Bb6c1, which was obtained from an Australian Kallar grass site, exhibited so many similarities with the protein pattern of *Herbaspirillum seropedicae* LMG 6514 that strain Bb6c1 may be a member of this species. This possibility was supported by the results of carbon substrate assimilation tests (data not shown).

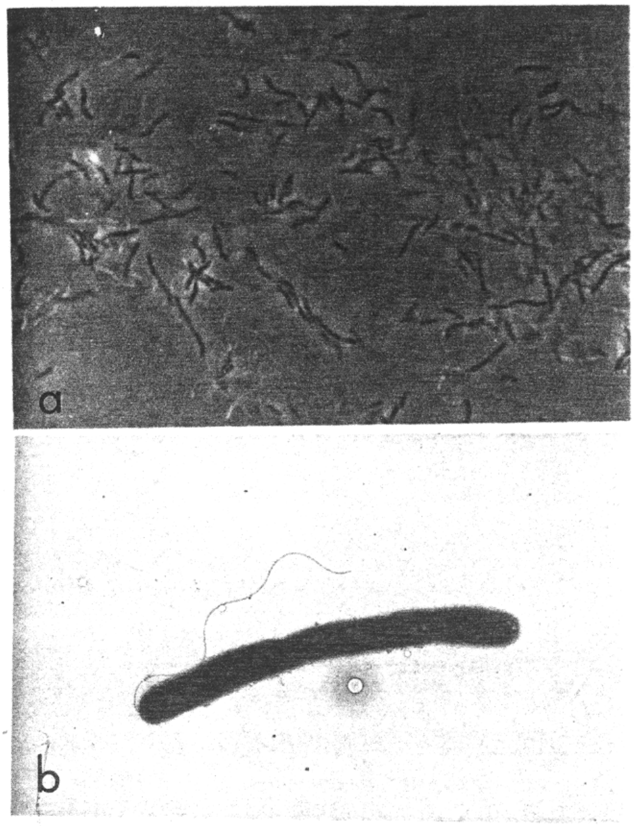

FIG. 1. Phase-contrast (a) and electron (b) microscopic images of *Azoarcus indigens* VB32^T (a) and *Azoarcus* sp. strain BH72 (b). *Azoarcus indigens* VB32^T and strain BH72 cells were grown in the presence of N_2 in semisolid SM medium supplemented with vitamins for 24 h and in liquid VM medium to late exponential phase, respectively.

Gas chromatographic analysis of cellular fatty acid patterns (FAMEs). The cellular fatty acid compositions of 11 isolates obtained from Kallar grass and strain LMG 5514 are shown in Table 2. A numerical comparison of the fatty acid profiles is shown as a two-dimensional plot in Fig. 3. Three clusters are recognized; these three clusters contain strains belonging to protein electrophoretic groups A, B plus C, and D. Again, strain BH72 occupies a separate position. The major fatty acids of all strains studied are 16:0, *cis*-9 16:1, and 18:1. Group A strains are differentiated by the presence of significant relative amounts of 12:0 (6.5 to 6.8%) and cyclo 17:0 (7.9 to 11.3%). Characteristic of strains belonging to groups B and C and strain BH72 is a higher relative percentage of 14:0 (3.1 to 4.3%). The fatty acid patterns of strains belonging to groups B and C are very similar; only minor quantitative differences distinguish these electrophoretic groups. Strain BH72 is separated from the cluster containing groups B and C by a higher mean percentage of 18:1 (17.8%). The absence of 3-OH-10:0 and the presence of small amounts of 3-OH-8:0 (1.4 to 1.6%) yield the characteristic fingerprint of group D strains.

DNA-rRNA hybridization. In order to detect generic and suprageneric relationships with other gram-negative bacte-

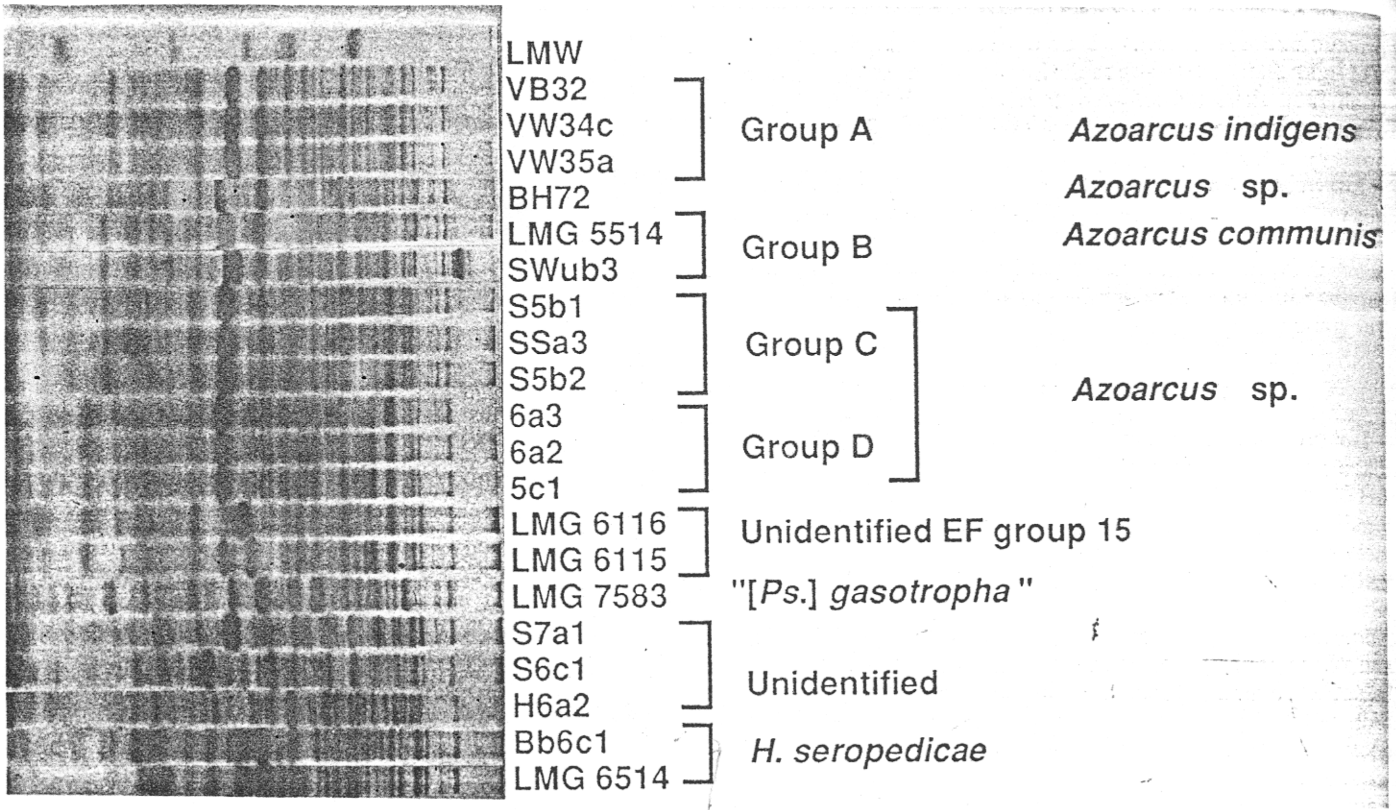

FIG. 2. Normalized SDS-PAGE patterns of *Azoarcus* strains, other isolates originating from Kallar grass, reference strain *Herbaspirillum seropedicae* LMG 6514, unidentified clinical isolates LMG 6115 and LMG 6116, and "[*Pseudomonas*] *gasotropha*." LMW, low-molecular-weight protein markers.

ria, we hybridized the DNAs of strains BH72 and H6a2 with labeled rRNAs of reference strains. The results of these hybridizations, expressed as $T_{m(e)}$ values, are shown in Table 3. The reference strains used included representative members of rRNA superfamilies I (*Alteromonas haloplanktis* LMG 2852^T), II (*Xanthomonas campestris* LMG 568^T, *P. fluorescens* LMG 1794^T, *Oceanospirillum linum* 5214^T), III (*Aquaspirillum serpens* biovar bengal strain LMG 6234^T, [*Pseudomonas*] *rubrisubalbicans* LMG 2286^T, *Hydrogenophaga palleronii* LMG 2366t1^T, *Oligella urethralis* LMG 5304), and IV (*Azorhizobium caulinodans* LMG 6465^T). $T_{m(e)}$ values of more than 68°C were obtained with members of rRNA superfamily III for strain BH72 and with members of rRNA superfamily II for strain H6a2. With a $T_{m(e)}$ of 73.8°C versus rRNA from *P. fluorescens* LMG 1794^T, strain H6a2 is located in the rRNA complex containing the authentic genus *Pseudomonas*, as well as *Azotobacter* and *Azomonas*.

In order to confirm these results and to detect the specific positions of the new isolates within rRNA superfamilies II and III, we prepared radioactively labeled rRNAs from strains BH72 and H6a2. The results of DNA-rRNA hybridizations are shown in Table 4. The data are also shown as a dendrogram based on $T_{m(e)}$ values in Fig. 4. The affiliation of

TABLE 2. Cellular fatty acid compositions of *Azoarcus* spp. strains

Taxon	Protein group	Strain	% of total fatty acids										
			3-OH-8:0[a]	10:0	3-OH-10:0	12:0	14:0	15:0	*cis*-9 16:1	16:0	cyclo 17:0	18:1	18:0
Azoarcus indigens	A	VB32^T		Tr[b]	5.0	6.5	Tr		33.5	34.6	11.3	8.3	
	A	VW35a		Tr	4.6	6.5	Tr		36.4	35.4	8.6	7.8	
	A	VW34c		Tr	4.7	6.8	Tr		37.4	35.4	7.9	6.9	
Azoarcus sp.		BH72			4.5	Tr	3.1		47.8	26.2		17.8	
Azoarcus communis	B	SWub3^T		Tr	6.5	Tr	4.1	Tr	51.4	25.8	Tr	9.7	Tr
	B	LMG 5514		Tr	6.0	Tr	4.3	Tr	50.9	28.7	Tr	8.1	
Azoarcus sp.	C	S5b2			5.2		3.9		51.9	29.9		9.1	
	C	S5b1			5.6		4.1		52.8	29.4		8.0	
	C	SSa3			5.4		4.1		52.7	32.0		5.8	
	D	6a3	1.4		Tr		Tr		50.6	28.2		18.6	Tr
	D	6a2	1.4				Tr		50.0	28.6		19.1	Tr
	D	5c1	1.6				Tr		51.4	28.3		18.2	

[a] Number of carbon atoms:number of double bonds.

[b] Tr, trace (less than 1%).

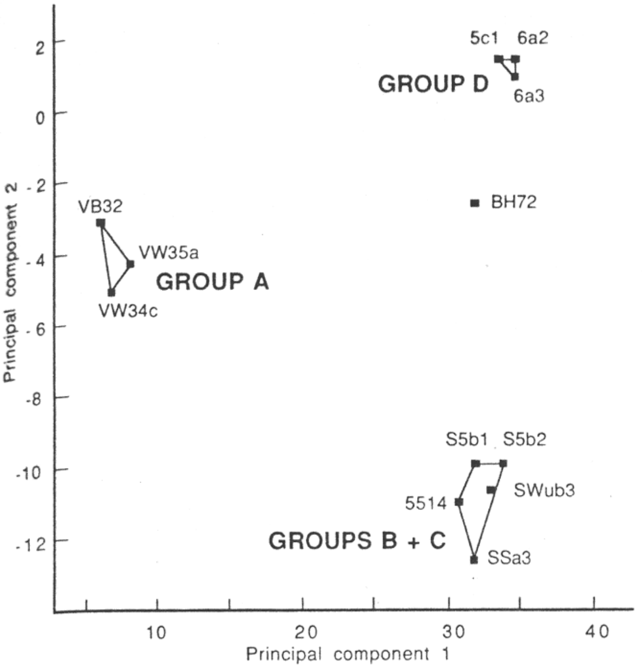

FIG. 3. Two-dimensional plot of principal-component analysis data for cellular fatty acid profiles of *Azoarcus* strains isolated from Kallar grass and strain LMG 5514.

our isolates with superfamilies III and II was corroborated by the results of reciprocal hybridizations. $T_{m(e)}$ values between 65.4 and 70.0°C were obtained for hybridizations between strain BH72 rRNA and DNAs from representative members of all known branches of rRNA superfamily III. Hybridizations with DNAs from representative strains belonging to SDS-PAGE (Fig. 2) groups A (VB32^T), C (S5b2), and D (6a3), both Kallar grass isolates belonging to group B, and strains S6c1 and S7a1 showed that all Kallar grass isolates except S6c1 and S7a1 are members of a separate new rRNA branch linked to the base of rRNA superfamily III at an average $T_{m(e)}$ of 67.1 ± 1.6°C. Because groups A, C, and D are very homogeneous as determined by gel electrophoresis, we concluded that 11 of the Kallar grass isolates are located on the new branch. Members of groups A and B clustered at the top of this rRNA branch, with $T_{m(e)}$ values of 80.4 and 79.4°C, respectively. Isolate LMG 5514 from oily sludge in France, which belongs to group B together with Kallar grass strain SWub3^T from Pakistan according to protein and fatty acid patterns, also fell into this top cluster [$T_{m(e)}$, 79.7°C], confirming the phenotypic data (see below). Representatives of groups C and D are also located on this branch, albeit at lower $T_{m(e)}$ values (71.4 and 71.6°C). The rRNA branch does not include Kallar grass isolates S6c1 and S7a1, which have $T_{m(e)}$ values at the base level of RNA superfamily III (around 67.7°C). These organisms definitely belong in this rRNA superfamily; whether they belong to another rRNA branch has not been investigated yet.

TABLE 3. $T_{m(e)}$ values of hybrids between DNAs from *Azoarcus* sp. strain BH72 and strain H6a2 and radioactively labeled rRNAs from reference strains

Labeled rRNA from:	Radioactive label	$T_{m(e)}$ (°C) of hybrid with DNA from: *Azoarcus* sp. strain BH72	$T_{m(e)}$ (°C) of hybrid with DNA from: Strain H6a2
Alteromonas haloplanktis LMG 2852^T	^{14}C	57.6	60.9
Xanthomonas campestris LMG 568^T	^{14}C	61.9	59.2
Pseudomonas fluorescens LMG 1794^T	^{14}C	61.4	73.7
Oceanospirillum linum LMG 5414^T	^{3}H	59.7	66.9
Azorhizobium caulinodans LMG 6465^T	^{3}H	57.8	59.3
Aquaspirillum serpens biovar bengal strain LMG 6234^T	^{3}H	67.7	
[*Pseudomonas*] *rubrisubalbicans* LMG 2286^T	^{3}H	68.4	62.0
Hydrogenophaga palleronii LMG 2366t1^T	^{14}C	68.5	
Oligella urethralis LMG 5304	^{3}H	68.2	

TABLE 4. $T_{m(e)}$ values of hybrids between DNAs from reference and unidentified strains and radioactively labeled rRNAs from *Azoarcus* sp. strain BH72 and strain H6a2

DNA from:	$T_{m(e)}$ (°C) of hybrid with [^{3}H]rRNA from: *Azoarcus* sp. strain BH72	$T_{m(e)}$ (°C) of hybrid with [^{3}H]rRNA from: Strain H6a2
Neisseria gonorrhoeae NCTC 8375^T	66.9	
Janthinobacterium lividum LMG 2892^T	67.6	
Comamonas acidovorans LMG 1226^T	67.5	
Herbaspirillum seropedicae LMG 6514	67.3	
Alcaligenes xylosoxidans subsp. *denitrificans* LMG 1231^T	65.4	
[*Pseudomonas*] *solanacearum* LMG 2299^T	66.7	
Chromobacterium violaceum LMG 1267^T	70.0	
Derxia gummosa LMG 3977^T	69.9	
Flavimonas oryzihabitans LMG 7040^T	60.8	76.6
Pseudomonas fluorescens LMG 1794^T		73.6
Chryseomonas luteola LMG 7041^T		75.1
Azomonas macrocytogenes LMG 8755^T		77.5
Azotobacter chroococcum LMG 8756^T		77.8
Unidentified strain G3		75.1
[*Pseudomonas*] sp. strain DC	61.5	59.6
Pseudomonas sp. strain 4B	67.1	
"[*Pseudomonas*] *gasotropha*" LMG 7583^T	71.2	
Unidentified strain LMG 6115 (EF group 15)	71.1	
Unidentified strain LMG 6116 (EF group 15)	70.4	
Azoarcus communis LMG 5514	79.7	
Azoarcus communis SWub3^T	79.4	
Azoarcus indigens VB32^T	80.4	
Azoarcus sp. strain 6a3	71.6	
Azoarcus sp. strain S5b2	71.4	
Unidentified strain S7a1	67.9	
Unidentified strain S6c1	66.0	
Azoarcus sp. strain BH72	81.5	
Unidentified strain H6a2		81.4

It is our experience that organisms belonging to a single rRNA branch having $T_{m(e)}$ values ranging from 71 to 81°C versus the same labeled reference rRNA genotypically constitute a taxonomic unit corresponding to a typical bacterial family (7). Within such an rRNA branch intrageneric similarities generally correspond to a $T_{m(e)}$ span of 81.5 to approximately 76°C (7); consequently, groups A and B and strain BH72 belong in a new genus. We propose the name *Azoarcus* for these organisms because they are slightly curved, diazotrophic rods. The new rRNA branch containing these bacteria is the *Azoarcus* rRNA branch.

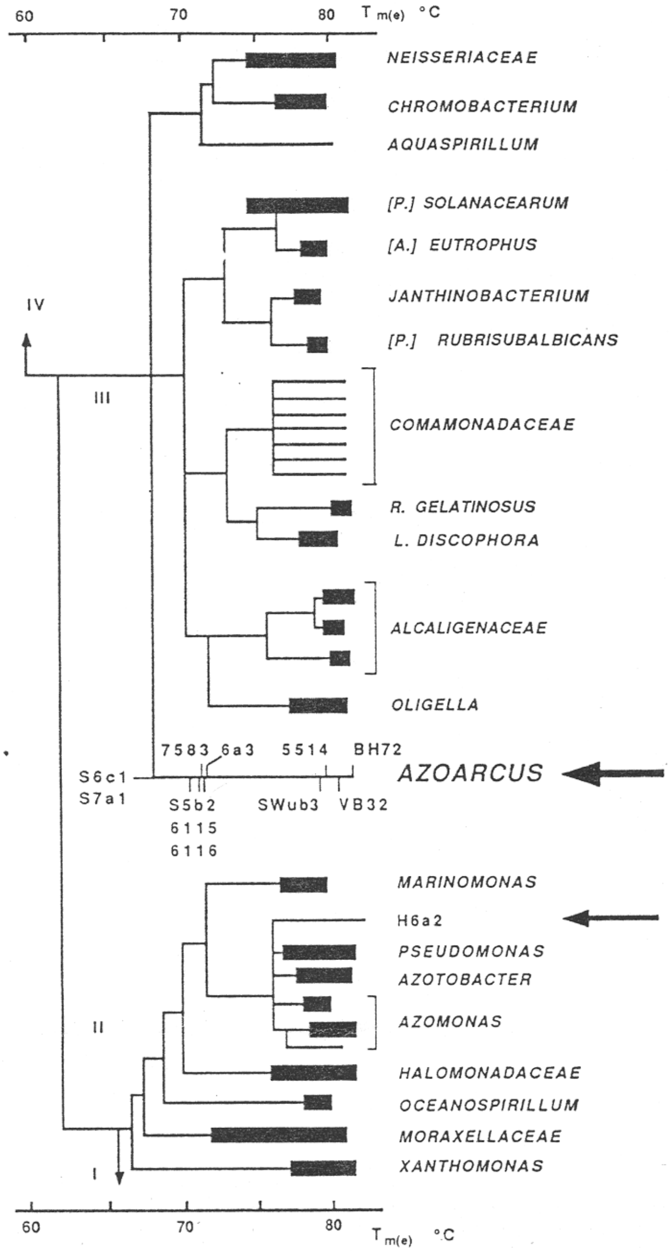

FIG. 4. Simplified rRNA cistron similarity dendrogram of parts of rRNA superfamilies III and II sensu De Ley (7), which correspond to the beta and gamma subclasses of the *Proteobacteria* (49), respectively. $T_{m(e)}$ values are from Table 4 and from references. The solid bars indicate the ranges observed within genera or groups. Arrows indicate the positions of Kallar grass isolates. [A.], *Alcaligenes*; R., *Rubrivivax*; L., *Leptothrix*.

Herbaspirillum seropedicae LMG 6514, a grass-associated diazotroph (1) with some phenotypic similarities to the Kallar grass isolates, belongs to the same superfamily but occupies a separate position [$T_{m(e)}$, 67.3°C]. Additional DNA-rRNA hybridization experiments (data not shown) proved that this organism belongs on the [*Pseudomonas*] *rubrisubalbicans* rRNA branch (18). In addition, we studied other straight to curved, yellowish rods which were located at the base $T_{m(e)}$ level in rRNA superfamily III. Our results are as follows: (i) [*Pseudomonas*] sp. strain 4B (5) could not be assigned to the new rRNA branch; and (ii) two clinical isolates from Sweden (LMG 6115 and LMG 6116) and the facultatively lithoautotrophic organism "[*Pseudomonas*] *gasotropha*" LMG 7583^{T} (54) yielded $T_{m(e)}$ values ranging from 70.5 to 71.2°C and thus belong on this branch, as confirmed by the results of hybridizations with other labeled rRNAs from members of superfamily III (data not shown). Indeed, strains LMG 6115 and LMG 6116 have protein electropherograms similar to those of group D organisms, whereas strain LMG 7583^{T} could not be assigned to any SDS-PAGE group (Fig. 2). *Derxia gummosa* LMG 3977^{T} had a $T_{m(e)}$ of 69.9°C versus members of the new rRNA branch in rRNA superfamily III, but later it was shown that this strain is a member of the [*Pseudomonas*] *rubrisubalbicans* rRNA branch (18).

So far, isolate H6a2 remains unique. Reciprocal DNA-rRNA hybridizations with labeled rRNA from this strain confirmed its position in rRNA superfamily II. With $T_{m(e)}$ values of 77.8 and 77.5°C versus DNAs of *Azotobacter chroococcum* LMG 8756^{T} and *Azomonas macrocytogenes* LMG 8755^{T}, strain H6a2 seems more closely related to the *Azotobacter chroococum* and *Azomonas macrocytogenes* rRNA branches than to the *P. fluorescens* rRNA branch [$T_{m(e)}$, 73.6°C]. We could not confirm these results by reciprocal DNA-rRNA hybridizations because labeled *Azotobacter chroococcum* and *Azomonas macrocytogenes* rRNAs were not available. Consequently, strain H6a2 is preliminarily located at the branching level of the three rRNA branches in this rRNA complex (Fig. 4). The smaller cell width of strain H6a2 (44) does not suggest a close relationship between this organism and the genus *Azotobacter* or *Azomonas*. *Flavimonas oryzihabitans* LMG 7040^{T}, another plant-associated species (21, 28), *Chryseomonas luteola*, a species containing yellowish rods (28), and an unidentified nitrogen-fixing organism isolated from rice, strain G3 (38), have also been assigned to the *Pseudomonas-Azotobacter-Azomonas* rRNA complex (19a). Hybridizations with labeled rRNA from strain H6a2 showed that strain LMG 7040^{T}, the type strain of *Chryseomonas luteola*, and strain G3 were no more closely related to H6a2 than they were to other members of this rRNA complex. A grass-associated diazotroph from Finland, [*Pseudomonas*] sp. strain DC (20), did not fall into rRNA superfamily I, II, or III.

DNA-DNA hybridization. Since the protein electrophoretic homogeneity of *Azoarcus* groups A, C, and D suggested that the levels of genomic similarity were high (25), only representatives of these groups were hybridized. No meaningful DNA-DNA binding could be detected between strains BH72, VB32^{T}, SWub3^{T}, S5b2, and 6a3 ($\leq$25%). Thus, genetically, all of these organisms differ at least at the species level. The 72% level of DNA-DNA binding between strain SWub3^{T} and LMG 5514 confirmed the high level of similarity between the protein electropherograms of these organisms (Fig. 2, group B); they belong in the same species.

Members of the five species differ significantly in the complexity of their genomes. The strains belonging to the top cluster of the *Azoarcus* rRNA branch had slightly higher genome molecular weights than the other strains, as follows: strain SWub3^{T}, $3.30 \times 10^9 \pm 0.22 \times 10^9$; strain LMG 5514, $3.33 \times 10^9 \pm 0.51 \times 10^9$; strain VB32^{T}, $3.35 \times 10^9 \pm 0.32 \times 10^9$; and strain BH72, $2.97 \times 10^9 \pm 0.15 \times 10^9$. In contrast, the genome molecular weights of strains S5b2 and 6a3 are $2.49 \times 10^9 \pm 0.07 \times 10^9$ and $2.38 \times 10^9 \pm 0.10 \times 10^9$, respectively. Whether this is due to additional plasmid DNA or to a difference in genome size could not be determined because of the method used, which measured the total number of unique sequences.

Physiological, biochemical, enzymatic, and nutritional features. Characteristic features of *Azoarcus* strains are shown

TABLE 5. Characteristics of *Azoarcus* strains[a]

Characteristic	Group A: *Azoarcus indigens* VB32^{T}, VW35a, and VW34c	*Azoarcus* sp. strain BH72	Group B: *Azoarcus communis* SWub3^{T} and LMG 5514	Group C: *Azoarcus* sp. strains S5b2, S5b1, and SSa3	Group D: *Azoarcus* sp. strains 6a3, 6a2, and 5c1
Cell width (μm)	0.5–0.7	0.6–0.8	0.8–1.0	0.6–0.8	0.4–0.6
Cell length (μm)	2.0–4.0	1.5–4.0	1.5–3.0	1.5–3.5	1.1–2.5
Elongated cells (8–12 μm) occur in stationary cultures	r	r	r	r	–
Motility	+	+[b]	+	+	+
Optimum temp for growth (°C)	40	40	37	ND	40
Growth in the presence of 2% (wt/vol) NaCl	w	w	+/w[c]	w/+/w[d]	–
Requirement for *p*-aminobenzoic acid	+	–	–	–	–
Nitrate reductase	+	+	–/+[c]	–	–
Catalase	+	+	+	w	+
Voges-Proskauer reaction	+	–	–	–	+
Urease	+	–	–	–	–
Esterase (C4)	±	+	+	±	w
Leucine arylaminase	+	±	w	±	±
Valine arylaminase	w	w	–	–	w
Sole carbon sources used for growth					
n-Valerate and 2-ketoglutarate	+	+	+	–	+
DL-Hydroxybutyrate	+	+	+	w	+
D-Tartrate	+	+	–	–	+
L-Aspartate	+	+	–	w/w/–[d]	+
Isobutyrate, isovalerate, *n*-caproate, and D-aspartate	–	+	+	–	+
D-Malate	+	+	+	±	–
Glutarate	–	+	+	–	–
Itaconate, mesaconate, and *p*-aminobenzoate	+	–	–	–	–
Citrate and D-alanine	–	–	+	–	–
DL-Glycerate	w/–/–[e]	–	–	–	–
L-Proline	–	–	–/+[c]	–	–
Phenylacetate	+	+	±	–	–
Benzoate and benzylamine	–	+	–	–	–
m-Hydroxybenzoate, *p*-hydroxybenzoate, L-mandelate, amylamine, L-phenylalanine, butylamine, and tryptamine	+	+	+	–	–
D-Mandelate	+	–	+	–	–
Terephthalate	–	–	–	±/–/–[d]	–/–/+[f]
DL-5-Aminovalerate	–	±	–	–	–
DNA G+C content (mol%)	66.6	67.6	62.4	64.8	65.2

[a] All strains have the following features in common: cells are straight to curved rods; microaerophilic growth occurs on N_2; oxidase positive; weak reactions for alkaline phosphatase, acid phosphatase, esterase (C8), lipase, and phosphoamidase; no spore formation; no growth in the presence of 5% NaCl and no increase in the growth rate when NaCl is added; no denitrification; no fermentation or oxidation (medium is alkalinized) of glucose or fructose; no starch hydrolysis or gelatin liquefaction; no cystine arylaminase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α- and β-glucuronidase, α- and β-galactosidase, and α-fucosidase activities; the sole carbon sources used for growth include acetate, propionate, butyrate, succinate, fumarate, DL-lactate, L-malate, pyruvate, ethanol, L-glutamate (except strain SSa3), and *meso*-tartrate (except strain VW34c); no growth occurs on heptanoate, caprylate, pelargonate, caprate, oxalate, malonate, maleate, adipate, pimelate, suberate, azelate, sebacate, glycolate, L-tartrate, levulinate, citraconate, aconitate, *o*-hydroxybenzoate, phthalate, iso-phthalate, glycine, D-α-alanine, L-leucine, L-isoleucine, L-norleucine, L-valine, DL-norvaline, DL-aminobutyrate, L-serine, L-threonine, L-cysteine, L-methionine, L-tyrosine, L-histidine, DL-tryptophan, trigonelline, ornithine, L-lysine, L-citrulline, L-arginine, DL-kynurenine, betaine, creatine, β-alanine, DL-3-aminobutyrate, DL-4-aminobutyrate, DL-2-aminobutyrate, DL-3-aminobutyrate, urea, acetamide, sarcosine, ethylamine, ethanolamine, diaminobutane, spermine, histamine, glucosamine, glycerol, *meso*-erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methylxyloside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl-D-mannoside, methyl-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, maltose, lactose, D-melibiose, sucrose, trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, gluconate, 2-ketogluconate, and 5-ketogluconate. For all characteristics except the enzyme tests: +, positive for all strains; –, negative for all strains; ±, intermediate reaction; w, weak reaction; r, rare; ND, not determined. For the enzyme tests: w, reaction value of 1 or 2; ±, reaction value of 3; +, reaction value of 4 or 5; –, no reaction.

[b] Motile by means of one flagellum or (very rarely) two monopolar flagella.

[c] Reaction of strain SWub3^{T}/reaction of strain LMG 5514.

[d] Reaction of strain S5b2/reaction of strain S5b1/reaction of strain SSa3.

[e] Reaction of strain VB32^{T}/reaction of strain VW35a/reaction of strain VW34c.

[f] Reaction of strain 6a3/reaction of strain 6a2/reaction of strain 5c1.

in Table 5. Additional tests were carried out with strains BH72 and H6a2. Both of these strains are capable of N_2-dependent growth and acetylene reduction at 40°C, just as *Azospirillum halopraeferens* is, which originated from the same Kallar grass field (42). No growth occurs at 45°C. Nitrogen fixation activity (acetylene reduction) occurred between pH 6.0 and 7.8, with optima at pH 6.5 and 6.8 for strains H6a2 and BH72, respectively. Acetylene reduction activity decreased sharply when 0.5 g of NaCl per liter was added to the growth medium of strain BH72, whereas the acetylene reduction levels were not affected by up to 2.5% NaCl in strain H6a2, which thus is salt tolerant. Diazotrophy has been demonstrated unequivocally for both strains by performing $^{15}N_2$ incorporation experiments (44).

Remarkable among the nutritional features of members of the genus *Azoarcus* (Table 5) is the fact that their use of carbon sources for growth is very restricted. Growth is mainly supported by organic acids and aromatic compounds,

TABLE 6. Differential characteristics of the genus *Azoarcus* and morphologically similar diazotrophs[a]

Taxon	Subclass of *Proteobacteria* or superfamily sensu De Ley[b]	Cells curved or vibroid	Cell width (μm)	Colonies yellowish	Strictly microaerobic	Growth at 40°C	Fermentative ability	Good growth on malic acid	Growth on sugars	G+C content (mol%)
Azoarcus	Beta	+	0.4–1.0	+	−	+	−	+	−	62–68
Herbaspirillum	Beta	+	0.6–0.7	−	−	−	−	+	+	66–67
Derxia	Beta	−	1.0–1.2	−	−	+	−	−	+	69–73
Azospirillum	Alpha	+	0.08–1.4	−	−	−[c]	D[d]	+	+	68–71
Xanthobacter agilis	Alpha	−	0.7	+	−	−	−	ND	−	66–67
Acetobacter diazotrophicus	Alpha	−	0.7–0.9	−	−	−	+	w	+	61–63
Arcobacter nitrofigilis	VI	−	0.2–0.9	−	+	−	−	+	−	28
Sphingomonas paucimobilis	Alpha	−	1.0	+	−	ND	+	ND	+	67.4

[a] Data from this study and references 1–3, 12, 18, 19, 24, 33, and 42. +, mainly positive; −, mainly negative; D, differs among species; w, weak reaction; ND, not determined.

[b] See reference 7.

[c] *Azospirillum halopraeferens* grows at 41°C.

[d] Present in oxidation-fermentation tests.

whereas none of 49 carbohydrates tested are used. The same tendency is evident for some classical biochemical and enzymatic features tested, for most of which we obtained weak or negative responses. Thus, the phenotypic criteria which distinguish *Azoarcus* species from each other are very limited.

The conclusions of our polyphasic studies are as follows: (i) the Kallar grass isolates belonging to rRNA superfamily III constitute a totally separate rRNA branch linked to the base of rRNA superfamily III with an average $T_{m(e)}$ of 67.7°C; (ii) strains can be assigned to five groups on the basis of protein electropherograms and cellular fatty acid compositions; (iii) these groups differ from each other at least at the species level since there was no meaningful DNA-DNA binding detected between them; (iv) there is no doubt that organisms belonging to groups A and B and strain BH72 belong in one new genus, *Azoarcus*; (v) the nomenclature of the other members of the *Azoarcus* rRNA branch, including group C and D strains, strains LMG 6115 and LMG 6116, and "*[Pseudomonas] gasotropha*," is less clear. On the one hand, the low $T_{m(e)}$ values of these organisms (around 70 to 71°C) provide a way to exclude them from the genus *Azoarcus* sensu stricto. On the other hand, the members of groups C and D are very similar in their morphological, nutritional, and biochemical characteristics, in their FAMEs, and in their protein electropherograms to members of the top $T_{m(e)}$ level. Because if they are left unnamed, no indication about their phylogenetic relationship is given and they will disappear in the very large group of unnamed strains, and because not enough data are available to determine their precise taxonomic position, we prefer to stress the phenotypic similarities by preliminarily including the members of groups C and D in the genus *Azoarcus* as *Azoarcus* sp. Useful features for differentiating the genus *Azoarcus* from other nitrogen-fixing, gram-negative bacteria are shown in Table 6. There are not enough phenotypic data available to compare strains LMG 6115 and LMG 6116 and "*[Pseudomonas] gasotropha*" with the genus *Azoarcus*, and therefore we do not propose any changes in the nomenclature of these organisms.

The authentic azoarci consist of three species, corresponding to groups A and B, and strain BH72. For group A, which is characterized by a growth factor requirement, we propose the name *Azoarcus indigens*. For group B, which includes strains that originated from two different habitats and continents, indicating that these organisms might have a wide distribution, we propose the name *Azoarcus communis*. These two species can be differentiated from each other by cell width. Although all Kallar grass isolates belonging to this genus originated from one field, the occurrence of the genus *Azoarcus* is not unique to this habitat since a soil bacterium from France was also assigned to this taxon. The close relationship between microorganisms obtained from entirely different environments (i.e., roots, oily sludge, and human sources) is striking but has also been observed for the genera *Agrobacterium* (10, 35) and *Acidovorax* (53). However, it is remarkable that the habitat which we investigated, grass roots in one particular field, harbors such a variety of phylogenetically related microorganisms.

Description of *Azoarcus* gen. nov. *Azoarcus* (A.zo'ar.cus. Fr. n. *azote*, nitrogen; L. masc. n. *arcus*, arch, bow; M. L. masc. n. *Azoarcus*, nitrogen [-fixing] bow). Cells are straight to slightly curved rods that occur singly or in pairs. Cell pairs often appear to be slightly S shaped (Fig. 1a). Cells range from 0.4 to 1.0 μm wide by 1.1 to 4.0 μm long. In most strains, elongated cells (8 to 12 μm) occur rarely in late-log- or stationary-phase cultures on semisolid or liquid SM medium supplemented with combined nitrogen (Fig. 1b). The cells are all motile; strain BH72 is usually motile by means of a single polar flagellum (Fig. 1b); rarely there are two flagella. Gram negative. On nitrogen-free, semisolid media the strains exhibit microaerophilic growth; first veil-like pellicles are developed several millimeters below the medium surface, and later the pellicles move to the surface. On Congo red agar, opaque pink colonies with translucent or whitish entire margins are formed; these colonies have a round, convex shape and a smooth texture. Colonies develop a nondiffusible yellowish pigment, particularly on VM agar supplemented with ethanol instead of potassium malate. Yellow pigmentation is less pronounced in strains belonging to groups C and D. Chemoorganoheterotrophic. Strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. No denitrification. Oxidase positive. Catalase positive. The presence of dissimilatory nitrate reductase and urease varies among strains. Except for one species, no growth factor is required. Capable of growth on atmospheric nitrogen (N_2) and reduction of acetylene to ethylene. Grows well on salts of organic acids like L-malate, acetate, proprionate, succinate, fumarate, DL-lactate, and pyruvate and on ethanol. Growth on amino acids is restricted to L-glutamate and a few other amino acids and amines which can be used by some strains. No growth occurs on mono- or disaccharides (Table 5). The optimal growth temperature is between 37 and 40°C. No growth occurs at 45°C. N_2-dependent growth occurs at 41°C. Not halophilic. Growth is not enhanced by addition of moderate concentrations of NaCl (0.25 to 0.75%). Strains grow well at a neutral pH. All strains have *cis*-9 16:1, 16:0, and 18:1 as their major cellular fatty acids. The G+C content of the DNA is between 62 and 68 mol%. The members of this genus belong to the beta subclass of the *Proteobacteria*. Some characteristics which differentiate them from other morphologically similar diazotrophs are shown in Table 6. The genus *Azoarcus* consists of five groups (Table 5) which differ at least at the species level. Below we describe two groups that consist of several members and are located at the top of the rRNA branch as named species: *Azoarcus indigens* and *Azoarcus communis*.

Description of *Azoarcus indigens* sp. nov. *Azoarcus indigens* (in 'di.gens. L. v. *indigere*, to be in need of; M. L. part. pres. *indigens*, being in need of, referring to the vitamin requirement). Type species. The description of *Azoarcus indigens* is the same as the description of the genus. This species can be differentiated from the other groups by its requirement for *p*-aminobenzoic acid when it is grown on N_2, by the presence of urease, and by growth on itaconate, mesaconate, and *p*-aminobenzoate. Growth in liquid media is clumpy; aggregation is very strong on peptone media. Additional morphological, biochemical, and nutritional features are shown in Table 5. The major fatty acids are 3-OH-10:0, 12:0, *cis*-9 16:1, cyclo 17:0, and 18:1. Source: isolated from roots and stem bases of *L. fusca* (L.) Kunth grown on saline-sodic soils in Pakistan. The type strain is strain VB32 (= LMG 9092), which has a G+C content of 66.6 mol%.

Description of *Azoarcus communis* sp. nov. The description of *Azoarcus communis* (com'mu.nis. L. masc. adj. *communis*, common, referring to diverse habitats) is the same as the description of the genus. This species can be distinguished from the other groups by its greater cell width (0.8 to 1.0 μm) and its growth on citrate and D-alanine. Other morphological, biochemical, and nutritional characteristics are shown in Table 5. The major fatty acids are 3-OH-10:0, 14:0, *cis*-9 16:1, 16:0, and 18:1. Source: isolated from roots of *L. fusca* (L.) Kunth grown on saline-sodic soils in the Punjab of Pakistan and from refinery oily sludge in France. The type strain is strain SWub3 (= LMG 9095), which has a G+C content of 62.4 mol%.

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