Reassessment of the taxonomic structure of the diazotrophic genus *Azoarcus sensu lato* and description of three new genera and new species, *Azovibrio restrictus* gen. nov., sp. nov., *Azospira oryzae* gen. nov., sp. nov. and *Azonexus fungiphilus* gen. nov., sp. nov.

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The taxonomic structure of members of the genus *Azoarcus sensu lato* was reassessed in a polyphasic approach. Two species, *Azoarcus communis* and *Azoarcus indigenus*, three unnamed species containing diazotrophs associated with Kallar grass roots (groups C, D) and a group of strains (E) isolated from fungi were analysed. They were compared by PAGE analyses of cellular proteins, genomic fingerprints, morphological and nutritional features to new isolates from rice roots. All strains within groups C, D and E containing 5–12 isolates showed group-specific cell and colony morphology and carbon source utilization patterns, with exception of the obligately microaerobic strain BS20-3, a member of group C. All strains, with this exception, also had almost indistinguishable electrophoretic protein patterns and genomic fingerprints generated with tDNA-directed primers, suggesting they belong to the same species. Phylogenetic analyses of almost complete 16S rDNA sequences carried out with three different algorithms (neighbour-joining, maximum-likelihood, parsimony) revealed that *Azoarcus sensu lato* is not monophyletic. Groups C, D and E formed three distinct lineages located between the *Azoarcus/Thauera* and the *Rhodocyclus* clusters. Phylogenetic distances between groups C, D and E were as large as between other genera (93–94% sequence similarity). This suggested they have the rank of three different genera. Since it was possible to differentiate them from each other and other related bacteria by phenotypic features, three new genera with one type species each are proposed: *Azovibrio restrictus* gen. nov., sp. nov., *Azospira oryzae* gen. nov., sp. nov. and *Azonexus fungiphilus* gen. nov., sp. nov.

Keywords: *Azoarcus*, nitrogen fixation, rice, phylogeny

INTRODUCTION

A genus of aerobic *Proteobacteria*, *Azoarcus*, was originally described with two valid diazotrophic species, *Azoarcus communis* and *Azoarcus indigenus* (Reinhold-Hurek *et al.*, 1993). Another diazotrophic strain (BH72), which clustered with *Azoarcus* spp. in rRNA–DNA homology studies (Reinhold-Hurek *et*

al., 1993) and 16S rRNA sequence analyses (Hurek *et al.*, 1993), was distinct at the species level according to DNA–DNA homology studies, but has not been named yet (Reinhold-Hurek *et al.*, 1993). Two other types of diazotrophs, group C and group D, were also distinct at the species level and clearly located on the *Azoarcus* rRNA branch according to rRNA–DNA hybridization studies, albeit at lower $T_{m(e)}$ values (71.4–71.6 °C). As they contained too few strains and their phenotypic features were not sufficiently discriminating, these species were not named but included in the genus *Azoarcus sensu lato* (Reinhold-Hurek *et*

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al., 1993). All strains included in the original description, except for two, shared a similar habitat: the interior of roots or stems of Kallar grass, a pioneer plant grown on a salt-affected field in the Punjab of Pakistan (Reinhold *et al.*, 1986; Reinhold-Hurek *et al.*, 1993). These bacteria are, in common with other diazotrophic endophytes of grasses, tightly associated with plants and have not been isolated from soil as yet (Boddey, 1995; Reinhold-Hurek & Hurek, 1998). In recent surveys, additional strains of plant-associated *Azoarcus* species have been cultivated from other sources, such as roots of several rice species (Engelhard *et al.*, 2000) and resting stages of a plant-associated basidiomycete (Hurek *et al.*, 1997). This fungus also harboured a new bacterial lineage, *Azoarcus* sp. group E (Hurek *et al.*, 1997). Availability of additional strains allowed us to reassess the taxonomic structure of members of the genus *Azoarcus sensu lato* in a polyphasic approach, based on phylogenetic analysis of 16S rDNA sequences, genomic fingerprints and phenotypic characteristics.

Knowledge about the diversity within *Azoarcus sensu stricto* was increased by the description of several new species with different ecological and physiological features. *Azoarcus tolulyticus* (Fries *et al.*, 1994) and *Azoarcus evansii* (Anders *et al.*, 1995), which have been isolated from soils or sediments, are capable of degrading aromatic hydrocarbons. *Azoarcus anaerobicus* (Springer *et al.*, 1998), which is a respiratory but strictly anaerobic bacterium, and strains degrading ethylbenzene (Rabus & Widdel, 1995) or cyclohexane-1,2-diol (Harder, 1997) were recently suggested to belong to the genus *Azoarcus*.

The availability of 16S rDNA sequences of *Azoarcus* spp. and related groups now allow a revision of the phylogenetic relationships within this branch. Analyses based on a 16S rDNA fragment (446 bp) have placed the species of *Azoarcus sensu lato* in one lineage (Hurek & Reinhold-Hurek, 1995). However, a recent analysis based on almost complete sequences suggested that only *Azoarcus sensu stricto* is monophyletic, whereas the other species represent different lineages and thus different genera (Hurek *et al.*, 1997). As a result of our polyphasic taxonomic studies, we here suggest splitting *Azoarcus sensu lato* into four genera, *Azoarcus* (previously *sensu stricto*), *Azovibrio* gen. nov., *Azonexus* gen. nov. and *Azospira* gen. nov.

METHODS

Bacterial strains. The strains used in this study are listed in Table 1. Reference strains of *Azoarcus tolulyticus* (Td-1) and *Azoarcus evansii* (KB740^T) were kindly provided by J. M. Tiedje and G. Fuchs, respectively. Type cultures of the new species were deposited at the BCCM/LMG culture collection, Belgium.

Media and growth conditions. Unless stated otherwise, cells were grown at 37 °C. For mass culture and maintenance, cells were grown on VM ethanol medium which consisted of (l⁻¹): K₂HPO₄, 0.6 g; KH₂PO₄, 0.4 g; NH₄Cl, 0.5 g;

MgSO₄·7H₂O, 0.2 g; NaCl, 1.1 g; CaCl₂·2H₂O, 0.026 g; MnSO₄·H₂O, 0.01 g; Na₂MoO₄·2H₂O, 0.002 g; Fe(III)-EDTA, 0.066 g; yeast extract, 1 g; Bacto-Tryptone, 3 g; pH 6.8; ethanol, 6 ml, sterilized by filtration. For determination of colony morphology, malic acid medium supplemented with Congo red (Cacarez, 1982) was used. Cells were also grown on semi-solid N-free SM medium (Reinhold *et al.*, 1986) for nitrogen fixation, or on SM medium with combined nitrogen (Reinhold *et al.*, 1985), both supplemented with 1 ml of a mixture of vitamins l⁻¹ (Hurek *et al.*, 1995).

Morphological and physiological tests. Cell dimensions and morphology were determined by phase-contrast microscopy after growth on N₂ and on SM medium with combined nitrogen. Microscopic images were recorded with a 3Chip RGB Colour Camera (Hamamatsu Photonic Systems). Electron micrographs were taken from cells negatively stained with phosphotungstate. Colony morphology was observed after growth on VM ethanol medium and Congo red agar. Salt and temperature tolerance were monitored in liquid VM ethanol medium inoculated with 1% (v/v) preculture, after 1 d incubation. Requirements for growth factors or tests for carbon source utilization were carried out in liquid SM medium as described previously (Hurek *et al.*, 1997). Potassium malate was replaced by the respective neutralized carbon source. Biotype 100 strips with medium 2 (bioMérieux) supplemented with the vitamin mixture (1 ml l⁻¹) were applied to determine nutritional profiles. The inoculum was grown for 2 d on VM ethanol agar plates at 37 °C and suspended in 0.9% (w/v) NaCl solution. Strips were inoculated according to manufacturer's instructions. Growth was recorded after 1, 2, 4 and 7 d incubation at 30 °C. The values obtained on day 4 are listed in Table 2 since recordings on day 7 did not differ from day 4.

Electrophoretic patterns of SDS-soluble proteins. Cells were grown on VM ethanol agar plates (diam. 14 cm) for 36 h at 30 °C after inoculation with a preculture grown overnight in the same liquid medium, washed and suspended in 0.9% (w/v) NaCl solution. Extracts of SDS-soluble proteins were prepared from whole cells as described by Kiredjian *et al.* (1986). Proteins were separated by SDS-PAGE on 12.5% (w/v) polyacrylamide gels (30:1, w/w, acrylamide/bisacrylamide) and stained with Coomassie brilliant blue R250.

DNA preparation. DNA was extracted from 1–2 ml of well-grown liquid cultures as described previously (Hurek *et al.*, 1993).

Genomic fingerprints by tDNA-PCR. PCR with tRNA-gene directed primers MS9 and MS10, which were 5'-labelled with the fluorescent dye CY-5, and analysis of amplification products on an automated sequencer (ALFexpress; Pharmacia) were carried out according to Hurek *et al.* (1997).

Phylogenetic analysis of 16S rRNA genes. Aligned sequences of 16S rRNA genes were loaded from the Ribosomal Database Project (www.cme.msu.edu/RDP/analyses.html) and put into GeneDoc software version 2.4.0 (www.cris.com/~ketchup/genedoc.shtml). Tree inference on 1338 sequence positions was carried out using the neighbour-joining algorithm of TREECON version 1.3b (Van de Peer & de Wachter, 1994) with a Jukes–Cantor correction distance matrix, using the complete deletion option. Alternatively, an unrooted maximum-likelihood quartet-puzzling tree was inferred using 10000 puzzling steps in the Tamura–Nei model (Tamura & Nei, 1993). Parsimony analysis was done with the DNAPARS program of the PHYLIP 3.5c package

Table 1. Strains used

Taxon	Strain	Other designation	Source of isolation and/or reference
<i>Azoarcus tolulyticus</i>	Td-1		Petroleum-contaminated soil, Western Washington (Fries <i>et al.</i> , 1994)
<i>Azoarcus evansii</i>	KB740 ^T	DSM 6898 ^T	Creek sediment, USA (Anders <i>et al.</i> , 1995)
<i>Azoarcus indigens</i>	VB32 ^T	LMG 9092 ^T	Surface-sterilized (SS) stem bases of Kallar grass, Pakistan (Reinhold-Hurek <i>et al.</i> , 1993)
	VW35a		SS roots of Kallar grass, Pakistan (Reinhold-Hurek <i>et al.</i> , 1993)
	VW34c		SS roots of Kallar grass, Pakistan (Reinhold-Hurek <i>et al.</i> , 1993)
	BS2-10		Fungal sclerotium from rice field, Pakistan (Hurek <i>et al.</i> , 1997)
	OSP14-2		SS roots of <i>Oryza sativa</i> , Khumaltar, Nepal (Engelhard <i>et al.</i> , 2000)
<i>Azoarcus communis</i>	SWub3 ^T	LMG 9095 ^T	Root piece of Kallar grass, Pakistan (Reinhold-Hurek <i>et al.</i> , 1993)
	S2	LMG 5514, BPD2	Refinery oily sludge, France (Laguerre <i>et al.</i> , 1987)
	LFN91		Root piece of Kallar grass, Pakistan (Engelhard <i>et al.</i> , 2000)
<i>Azoarcus</i> sp.	BH72		SS roots of Kallar grass, Pakistan (Reinhold-Hurek <i>et al.</i> , 1993)
<i>Azovibrio restrictus</i> (formerly <i>Azoarcus</i> sp. group C)	S5b2 ^T	LMG 9099 ^T	SS roots of Kallar grass, Pakistan (Reinhold-Hurek <i>et al.</i> , 1993)
	S5b1		SS roots of Kallar grass, Pakistan (Reinhold-Hurek <i>et al.</i> , 1993)
	SSa3		SS roots of Kallar grass, Pakistan (Reinhold-Hurek <i>et al.</i> , 1993)
	BS1-14		Fungal sclerotium from rice soil, Pakistan (Hurek <i>et al.</i> , 1997)
	OSB2-4		SS roots of <i>Oryza sativa</i> , Bulbuhle, Nepal (Engelhard <i>et al.</i> , 2000)
<i>Azovibrio</i> sp.	BS20-3		Fungal sclerotium from rice soil, Pakistan (Hurek <i>et al.</i> , 1997)
<i>Azospira oryzae</i> (formerly <i>Azoarcus</i> sp. group D)	6a3 ^T	LMG 9096 ^T	SS roots from Kallar grass, Pakistan (Reinhold-Hurek <i>et al.</i> , 1993)
	6a2		SS roots from Kallar grass, Pakistan (Reinhold-Hurek <i>et al.</i> , 1993)
	BS2-3		Fungal sclerotium from rice soil, Pakistan (Hurek <i>et al.</i> , 1997)
	OM8A-5		SS roots of <i>Oryza officinalis</i> , Kapilavastu, Nepal (Engelhard <i>et al.</i> , 2000)
	OO80-5		SS roots of <i>Oryza officinalis</i> , Nepal (Engelhard <i>et al.</i> , 2000)
	OMP5-6		SS roots of <i>Oryza minuta</i> , Pangil, Philippines (Engelhard <i>et al.</i> , 2000)
	OSB4-5		SS roots of <i>Oryza sativa</i> , Bulbuhle, Nepal (Engelhard <i>et al.</i> , 2000)
	OSL52-5		SS roots of <i>Oryza sativa</i> , Laha, Nepal (Engelhard <i>et al.</i> , 2000)
	OSP16-4S		SS roots of <i>Oryza sativa</i> , Khumaltar, Nepal (Engelhard <i>et al.</i> , 2000)
	OSP39		Root pieces of <i>Oryza sativa</i> , Khumaltar, Nepal (Engelhard <i>et al.</i> , 2000)
<i>Azonexus fungiphilus</i> (formerly <i>Azoarcus</i> sp. group E)	OSC17		Root pieces, <i>Oryza sativa</i> , Italy (Engelhard <i>et al.</i> , 2000)
	BS5-8 ^T	LMG 19178 ^T	Fungal sclerotium from rice soil, Pakistan (Hurek <i>et al.</i> , 1997)
	BS19-2		Fungal sclerotium from rice soil, Pakistan (Hurek <i>et al.</i> , 1997)
	BS19-5		Fungal sclerotium from rice soil, Pakistan (Hurek <i>et al.</i> , 1997)
	BS19-7		Fungal sclerotium from rice soil, Pakistan (Hurek <i>et al.</i> , 1997)
	BS22-6		Fungal sclerotium from rice soil, Pakistan (Hurek <i>et al.</i> , 1997)

(Felsenstein, 1993). Bootstrap values were calculated from 100 pseudoreplicates generated by the SEQBOOT program.

RESULTS AND DISCUSSION

Comparison of electrophoretic protein patterns

To determine interstrain variations within the different groups of *Azoarcus sensu lato*, patterns of SDS-soluble proteins were compared by SDS-PAGE (Fig. 1). In

each group, isolates from different sources, i.e. a representative *Azoarcus* strain originating from Kallar grass (Reinhold-Hurek *et al.*, 1993) and novel isolates from rice roots (Engelhard *et al.*, 2000 or fungal sclerotia (Hurek *et al.*, 1997) were analysed. They had almost indistinguishable protein patterns within *Azoarcus* sp. group C (lanes 1–4) and group D (lanes 5–14), except for strain BS20-3 (lane 4), which differed substantially from other members of group C. Within

Table 2. Characteristics of strains of the genera *Azoarcus*, *Azovibrio*, *Azospira* and *Azonexus*

Data from this study and from Reinhold-Hurek *et al.* (1993), Hurek & Reinhold-Hurek (1995); Hurek *et al.* (1997), Anders *et al.* (1995), Zhou *et al.* (1995), Rhee *et al.* (1997) and Engelhard *et al.* (2000). +, Positive for all strains; —, negative for all strains; w, weak reaction for all strains; r, rare; d, 11–89% of strains are positive; numbers, number of strains giving positive reactions; v, strain instability; ND, not determined. All strains have the following features: cells are straight to curved rods; growth on N₂ under microaerobic conditions; oxidase-positive; catalase-positive (not determined for BS20-3); no growth in the presence of 5% NaCl and no growth-rate increase when NaCl is added to medium; denitrification (not determined for *Azonexus* sp.); no spore formation; no starch hydrolysis (not determined for *Azoarcus evansii*).

Characteristic	<i>Azoarcus indigenes</i> (n = 5)	<i>Azoarcus communis</i> (n = 3)	<i>Azoarcus</i> sp. BH72 (n = 1)	<i>Azoarcus</i> <i>tolulyticus</i> *	<i>Azoarcus</i> <i>evansii</i> *	<i>Azovibrio</i> <i>restrictus</i> (n = 5)	<i>Azovibrio</i> sp. BS20-3 (n = 1)	<i>Azospira</i> <i>oryzae</i> (n = 12)	<i>Azonexus</i> <i>fungiphilus</i> (n = 5)
Cell width (µm)	0.5–0.7	0.8–1.0	0.6–0.8	0.8–1.0*	0.4–0.8	0.6–0.8	0.6–0.8	0.4–0.6	0.6–0.8
Cell length (µm)	2.0–4.0	1.5–3.0	1.5–4.0	1.4–2.8	1.5–3.0	1.5–3.5	1.5–3.5	1.1–2.5	1.5–4.0
Elongated cells in stationary cultures	r	r	r	—	—	r	ND	r	+
Colony diameter (mm; VM/CR)†	2–3	2–4	2–3/1	1–1.5*	0.2–0.7*	1.5–2/1	Negligible	1–2	1–2/0.7
Colony colour (VM)†	Opaque yellowish	Translucent yellowish	Translucent yellowish	Opaque yellowish*	Translucent beige*	Opaque beige	Negligible	Translucent pink-salmon	Opaque ochreous
Colony colour (CR)†	Whiteish pink, white margin	Whiteish pink, pink centre	Orange-red	Orange-red	Orange-red	Orange-red	Negligible	Translucent orange	Dark red
Colony surface	Rough	Smooth	Shiny	Shiny	Shiny	Shiny	—	Shiny	Shiny
Growth at 40 °C	+	+	+	—*	—*	4‡	ND	+	+
Growth in presence of 2% NaCl	w	1	w	d§	ND	w	ND	—	+
Requirement for <i>p</i> -aminobenzoic acid	+	—	—	—	—	—	—	—	—
Requirement for cobalamine	—	1	—	—	—	—	+	8	+
Oxidation/fermentation of glucose	—	—	—	ND	+¶	—	—	—	—
Sole carbon sources for growth:#									
<i>n</i> -Butylamine, 3-hydroxybenzoate	+	+	+	+	+	—	—	—	—
Benzylamine	1 ^a	v	+	+	+	—	—	—	—
Phenylacetate	+	+	+	—*	+	—	—	—	—
Benzoate	1 ^b	1 ^c	+	+	+	—	—	—	—
L-Phenylalanine	+	+	—	+	+	—	—	—	—
D(+)–Malate	+	+	+	+	—*	4 ^d	—	—	—
L-Alanine	2 ^e	+	+	+	+	2 ^e	+	—	—
2-Oxoglutarate	+	+	+	—*	+	—	—	+	+
D-Tartrate	+	1 ^c	+	—*	+	—	—	+	—
<i>n</i> -Caproate	—	+	+	—*	—*	—	—	+	—
Propionate	+	+	+	—*	+	+	+	+	—
Isovalerate	—	+	+	+	+	—	+	+	—
L-Proline	—	—	—	+	+	—	—	—	+
Malonate	—	—	—	—	—	—	—	—	1 ^f
D(+)–Glucose, maltose	—	—	—	+	1 ^g	—	—	—	—
D(+)–Fructose	—	—	—	+	+¶	—	—	—	—
D(+)–Galactose, sucrose, maltotriose, palatinose, D(+)–melezitose, maltitol, D(+)–turanose, <i>trans</i> -aconitate	—	—	—	+	+	—	—	—	—
D,L-Glycerate	3 ^h	—	—	+	+	—	—	—	—
Toluene (denitrifying)	—	—	—	+	ND	—	—	—	—
Protocatechuate, 4-hydroxybenzoate, L-mandelate	+	+	+	—*	—*	—	—	—	—
<i>m</i> -Coumarate	1 ⁱ	+	+	—*	—*	—	—	—	—
Tryptamine	+	v	+	—*	—*	—	—	—	—
Gentisate	+	+	+	—*	+	—	—	—	—
L(+)–Tartrate, <i>meso</i> -tartrate and betain*	—	—	—	—	+¶	—	—	—	—
<i>p</i> -Aminobenzoate	+	—	—	—*	+	—	—	—	—
Itaconate	+	—	—	—*	—*	—	—	—	—
Glutarate	—	+	+	+	+	—	—	—	—
D-Mandelate	+	+	—	—*	—*	—	—	—	—
Citrate	—	+	—	—	—	—	—	—	—
Glycerol, D-alanine	—	1 ^c	—	—	—	—	—	—	—
(–)-Quinate	—	1 ^c	—	+	—	—	—	—	—
Isobutyrate	—	+	+	+	+	1 ^j	+	v	—
L-Aspartate	+	v	+	+	+	+	—	+	+
3-Hydroxybutyrate	+	+	+	+	+	—	+	+	+

* Marked characters were determined in this study for *Azoarcus tolulyticus* Td-1 or *Azoarcus evansii* KB740^T.

† Growth on VM ethanol agar (VM) or Congo red agar (CR) at 37 °C for 4 d.

‡ Positive for *Azovibrio restrictus* except strain OSB2-4.

§ Positive in *Azoarcus tolulyticus* except strain Tol-4^T, and Td-1 in this study.

|| Requirement for *Azoarcus communis* LFN91 and all strains of *Azospira oryzae* except 6a3^T, 6a2, 5c1, and OO80-5.

¶ Not tested for *Azoarcus evansii* pF6.

Fig. 1. Normalized SDS-PAGE patterns of *Azoarcus sensu lato* strains group C, D and E, originating from the root interior of Kallar grass or rice, or from fungal sclerotia.

group D, strains OM8A-5, OSL52-5, OSP39 and OSC17 formed a subgroup by differing in one major protein band from the others. All strains of *Azoarcus* sp. group E, which had been isolated from fungal sclerotia (Hurek *et al.*, 1997) had nearly identical protein patterns (lanes 15–19) except for strain BS5-8^T which differed in one major band. Almost indistinguishable protein patterns indicate a high degree of overall genomic similarity (Kersters, 1985) which suggested that the respective strains within a group belonged to the same species, except for isolate BS20-3.

Genomic fingerprints by tDNA-PCR

For analysis of the genotypic variations within groups of *Azoarcus sensu lato*, PCR-based genomic fingerprints were compared. Primers to tRNA genes applied at low stringency were previously shown to amplify bands of different sizes, most likely clusters of tRNA genes (Hurek *et al.*, 1997). The tDNA-PCR profiles were shown to correlate well with the species affiliation in *Azoarcus* spp., except for group E in which only a single band was amplified (Hurek *et al.*, 1997). Fig. 2 shows genomic fingerprints generated by tDNA PCR for different strains of *Azoarcus indigenus* (lanes 1–5) as

well as *Azoarcus* sp. group C and group D (lanes 11–20). Most fingerprint profiles were group-specific. Strains of fungal or rice origin showed patterns almost identical to Kallar grass strains within a given group (lanes 4/2 or 5/3, respectively). Within group C, all strains, including BS20-3, shared three major bands (lanes 6–10); however, some variation occurred in minor bands. Banding patterns within group D were very homogeneous, with five major bands which almost all strains had in common (lanes 11–20). Strains OM8A-5, OSL52-5 and OSP39, which in protein patterns showed some deviation from other members of the group, were identical in fingerprints to, for example 0080-5 and OSP16-4, indicating high genomic similarities. Thus, PCR-based fingerprints confirmed that members within group C or D of *Azoarcus sensu lato* were likely to belong to the same species.

Phylogenetic analyses of 16S rDNA sequences

The 16S rRNA genes of *Azoarcus* spp. and representative genera of the β -subclass of the *Proteobacteria* were subjected to phylogenetic analysis using different methods. As a result of these and other studies, we propose three new genera (*Azovibrio*, *Azospira* and

All strains grow on L-malate, DL-lactate, succinate, fumarate, ethanol, acetate (except for *Azoarcus toluolyticus* Td-17 and Td-21), L-glutamate (no substrate except for L-malate has been tested for *Azoarcus evansii* pF6 according to the literature); no growth on D(+)mannose, L(+)arabinose, maltitol, N-acetyl-D-glucosamine, D-gluconate, caprate; *no growth on D(+)galactose, D(+)trehalose, L(+)sorbose, α -D(+)melibiose, D(+)raffinose, α -lactose, lactulose, 1-O-methyl- β -galactopyranoside, 1-O-methyl- α -galactopyranoside, D(+)cellobiose, β -gentiobiose, 1-O-methyl- β -D-glucopyranoside, D(–)-ribose, D(+)xylose, α -L-rhamnose, α -L-fucose, D(+)arabitol, L(–)-arabitol, xylitol, dulcitol, D-tagatose, myo-inositol, D-sorbitol, adonitol, hydroxyquinoline- β -glucuronide, D-lyxose, erythritol, 1-O-methyl- α -D-glucopyranoside, 3-O-methyl-glucopyranose, D-saccharate, mucate, D-glucuronate, D-galacturonate, 2-keto-D-gluconate, 5-keto-D-gluconate, 3-phenylpropionate, caprylate, 4-aminobutyrate, DL- α -amino-n-valerate, trigonelline, putrescine, histamine, ethanolamine, D-glucosamine, tyrosine. a, positive for strain BS2-10, weak for strain OSP14-2; b, positive for strain BS2-10; c, positive for strain LFN91; d, negative for strain BS1-14; e, positive for strains BS2-10, OSP14-2 (*Azoarcus indigenus*) and positive for strains OSB2-4, BS1-14 (*Azovibrio restrictus*); f, positive for strain BS19-5; g, negative for strain pF6; h, negative for strains VW35a and VW34c; i, positive for strain BS2-10; j, positive for strain BS1-14.

Fig. 2. Genomic fingerprints of *Azoarcus* spp. generated by tDNA-PCR. Amplification products were analysed on an ALFexpress automated sequencer, using the Fragment Manager program (Pharmacia). Lanes: 1–5, *Azoarcus indigenes* strains VB32^T, VW34c, VW35a, BS2-10 and OSP14-2, respectively; 6–10, *Azoarcus sensu lato* sp. group C strains S5b2^T, Ssa3, OSB2-4, BS1-14 and BS20-3, respectively; 11–20, *Azoarcus sensu lato* sp. group D strains 6a3^T, 6a2, OO80-5, OSB4-5, OSP16-4, BS2-3, OM8A5, OSL52-5, OSP39 and OSC17, respectively. Positions of size markers are indicated on the left.

Azonexus) for *Azoarcus sensu lato* sp. groups C, D and E, respectively. A neighbour-joining tree inference was carried out on a large set of sequence data containing all genera of the β -subclass of the *Proteobacteria*. It clustered the *Rhodocyclus* group and genera *Azoarcus*, *Thauera* and *Zoogloea* at a significant level (89%) in bootstrap analysis (not shown). However, the internal nodes separating the *Thauera*, *Zoogloea* and *Azoarcus* clades were not well resolved (not shown), as we had observed previously (Hurek *et al.*, 1997). Since the set of data were too large to carry out maximum-likelihood analysis with sufficient puzzling steps, an unrooted tree of the *Rhodocyclus* group was inferred with three different algorithms (maximum-likelihood, neighbour-joining and parsimony). The tree topology of the ML analysis is shown in Fig. 3, with confidence values for all three different algorithms given at the nodes when significant (larger than 50%).

The following conclusions can be drawn.

(i) *Azoarcus sensu lato* is not monophyletic. It was divided from the *Azoarcus sensu stricto* clade by the *Thauera* clade in all three analyses (Fig. 3): the node separating the genera *Thauera* and *Azoarcus sensu stricto* from *Azoarcus sensu lato* and *Zoogloea* was well supported in maximum-likelihood and neighbour-joining analyses (80/81%). On the other hand, the node grouping *Azoarcus sensu lato* (*Azovibrio*) and *Zoogloea* is well supported in parsimony analysis (89%). None of our analyses placed the entire

Azoarcus group unambiguously in one clade. Therefore, *Azoarcus sensu lato* sp. groups C, D and E are unlikely to belong to the genus *Azoarcus sensu stricto*, as suggested previously (Hurek *et al.*, 1997).

(ii) *Azoarcus sensu lato* sp. groups C, D and E represented different lineages, despite their exact phylogenetic relationships within the *Rhodocyclus* subgroup not being well resolved. The branch lengths and thus the phylogenetic distances for all three groups in maximum-likelihood and neighbour-joining analyses were similar to those of other genera such as *Thauera* or *Rhodocyclus*, indicating that groups C, D and E might have the rank of three different genera.

(iii) *Azoarcus sensu stricto* fell into two different clades. The analysis of phylogenetic relationships in the *Azoarcus/Thauera* subgroup was rendered difficult because the branching pattern at the *Thauera/Azoarcus* node was unstable and most likely multifurcated. The resolution of phylogenetic analyses of 16S rDNA in taxa closely related to each other is sometimes limited, for example in the genera previously collected in *Rhizobium*, the evolutionary relationships are not clear (Wang *et al.*, 1998; Young & Haukka, 1996). Despite this drawback, all named species and also strains of uncertain affiliation in the genus *Thauera* clustered in one clade, mostly with highly significant support levels (Fig. 3). This was also the case for most soil-borne species or strains of *Azoarcus* of uncertain affiliation, supporting the reliability of our analyses and their suitability to assign novel strains to known groups. Likewise, *Azoarcus anaerobicus*, whose phylogenetic position was not clear because tree inference had previously been carried out with only eight reference sequences (Springer *et al.*, 1998) could be clearly localized in this clade (Fig. 3). Another isolate, mXyN1 (Rabus & Widdel, 1995), was apparently misnamed in the database as *Azoarcus* and was clearly a member of *Thauera* (Fig. 3). In contrast, none of the *Azoarcus* species containing plant-associated strains was located in the clade of soil-borne species in any type of analysis we carried out. Since the phylogenetic distances within these clades and *Thauera* were similar, both subgroups of *Azoarcus sensu stricto* might also deserve the rank of different genera in future.

Morphological, nutritional and physiological features

Strains within the different groups of *Azoarcus sensu lato* showed a similar cell and colony morphology with pronounced intergroup differences (Table 2). All were highly motile, slightly curved rods with one polar flagellum (Fig. 4e), but cells of *Azoarcus* sp. group D were smaller in width than, for example those of group C (Fig. 4a, b). Cell morphology underwent typical changes especially in semi-solid, N-free medium which becomes alkaline after prolonged growth: in *Azoarcus* sp. group D, elongated cells of 4–5 μ m with one helical winding occurred (Fig. 4a), very rarely with several windings, and a cell length of 8 μ m in late-stationary-

Fig. 3. Phylogenetic analysis of 16S rDNA sequences (1338 positions) from the *Rhodocyclus*–*Azoarcus* subgroup of the β -subclass of the *Proteobacteria*. The genera *Azovibrio*, *Azospira* and *Azonexus* were formerly called *Azoarcus sensu lato* sp. group C, D and E, respectively. An unrooted maximum-likelihood quartet-puzzling tree based on 10000 puzzling steps is shown. The reliability of each internal branch is indicated by how often the corresponding cluster was found among 10000 intermediate trees expressed as a percentage. Maximum-likelihood branch lengths are proportional to the number of nucleic acid substitutions per site. The same set of sequences was also analysed by using the neighbour-joining algorithm or the parsimony algorithm with 100 bootstrap repetitions. Confidence values are given at nodes when all three analyses were giving values above 90% (> 90), 80% (> 80) and so on. a, Maximum-likelihood and neighbour-joining confidence values greater than 60%, not significantly supported by parsimony; b, maximum-likelihood and neighbour-joining confidence values greater than 50%, not significantly supported by parsimony. The differences in tree topology by parsimony are: *Azospira* and *Azonexus* branches interchanged, and *Azovibrio* and *Zoogloea* branches interchanged. GenBank accession numbers of 16S rDNA sequences are shown in parentheses.

phase culture. Members of *Azoarcus* group E formed some large-loop-like, coiled, extremely elongated cells of up to 50 μm in early-stationary-phase cultures (Fig. 4d), which sometimes appeared to fragment into halfmoon-shaped cells later (Fig. 4c). Both cell types were not observed for *Azoarcus* sp. group C (Fig. 4b). Strain BS20-3, which belongs to this group (Hurek *et al.*, 1997) had an identical cell shape but did not share colony morphology, since it does not grow on agar plates in air due to obligate microaerophily.

The physiological and nutritional features of the different species or groups of *Azoarcus sensu stricto* and *sensu lato* are shown in Table 2. Tests for all strains (including one strain each of *Azoarcus evansii* and *Azoarcus tolulyticus*) were carried out under identical conditions. In carbon source utilization patterns, only few interstrain differences were observed within *Azoarcus* sp. group D and group E (*Azospira* and *Azonexus*), respectively. This indicated that they are phenotypically homogeneous groups despite the minor

deviations in some electrophoretic protein patterns, which did not correlate with the small nutritional or physiological differences. This was also the case for *Azoarcus* sp. group C (*Azovibrio*), with the exception of strain BS20-3 that differed in cobalamine requirement and four of the carbon sources tested (Table 2). In addition, carbon utilization patterns were useful for differentiation of groups C, D and E from each other and from species of *Azoarcus sensu stricto* (Tables 2 and 3).

In *Azoarcus sensu stricto*, most of the tests carried out for the original genus description (Reinhold-Hurek *et al.*, 1993) and in the present study have not been reported for members of the novel soil-borne species *Azoarcus tolulyticus* and *Azoarcus evansii*. Therefore, we tested two representative strains (Table 2). They differed considerably from the plant-associated species in colony morphology, especially lacking the yellowish pigment which was observed in the original genus description. Moreover, they grew on several carbo-

Fig. 4. Cell morphology of *Azospira oryzae* (a), *Azovibrio restrictus* (b, e) and *Azonexus fungiphilus* (c, d). Phase-contrast images of cells grown under N_2 in semi-solid medium during exponential (b), stationary (d) and late-stationary growth phases (a, c), or transmission electron micrograph after negative staining with phosphotungstate (e). Bars, 5 μ m (a–c), 10 μ m (d) or 1 μ m (e).

hydrates, again deviating from the original description of *Azoarcus* (Reinhold-Hurek *et al.*, 1993).

The majority of the tests have not been carried out on the growing number of isolates from soil or sediment which have been reported to belong to *Azoarcus* (Harder *et al.*, 1998; Hess *et al.*, 1998; Rabus & Widdel, 1995; Rhee *et al.*, 1997; Springer *et al.*, 1998; Van Schie & Young, 1998), and for many strains it is not clear whether they fix nitrogen (Harder *et al.*, 1998; Rabus & Widdel, 1995; Rhee *et al.*, 1997; Van Schie & Young, 1998), a key feature in the original description of *Azoarcus* (Reinhold-Hurek *et al.*, 1993). Therefore, it is difficult to draw conclusions about the phenotypic homogeneity of the genus at this point and all bacteria of this genus should be subjected to an identical set of tests in future.

Conclusions

Based on our polyphasic taxonomic studies, we propose to split the genus *Azoarcus* into four genera: *Azoarcus* (previously *Azoarcus sensu stricto*), contain-

ing the validly described species *Azoarcus communis*, *Azoarcus indigenus*, *Azoarcus toluelyticus*, *Azoarcus evansii* and *Azoarcus anaerobicus*, as well as strain BH72 differing at the species level (Reinhold-Hurek *et al.*, 1993) and additional strains belonging to both *Azoarcus* 16S rDNA clusters (Fig. 3). Three groups of *Azoarcus sensu lato* sp., C, D and E, deserve the rank of three separate genera with one species in each, *Azovibrio restrictus* gen. nov., sp. nov., *Azospira oryzae* gen. nov., sp. nov. and *Azonexus fungiphilus* gen. nov., sp. nov., respectively. The following results support this proposal. (i) Strains can be assigned to these three groups by electrophoretic protein patterns, genomic fingerprints, cellular fatty acid compositions (Reinhold-Hurek *et al.*, 1993), morphological features and carbon source utilization patterns; genotypic and phenotypic homogeneity within these groups suggest that strains belong to the same species (except for strain BS20-3 in the genus *Azovibrio*, which might represent a separate species but was not analysed at present). (ii) Previous DNA–DNA hybridization studies (Reinhold-Hurek *et al.*, 1993) have shown that these groups differ at least at the species level from each other and other *Azoarcus* species. (iii) Phylogenetic analyses of 16S rDNA sequences using three different algorithms showed that *Azoarcus sensu lato* is not consistently monophyletic. Groups C, D and E form three lineages located between the *Azoarcus*/*Thauera* and the *Rhodocyclus* clusters. (iv) Phylogenetic distances between groups C, D and E are as large as between other genera, such as *Rhodocyclus* and group D, or *Thauera* and *Azoarcus sensu stricto* (Fig. 3). Accordingly, the percentage sequence similarity between them is only 93–94 %, values which also separate other proteobacterial genera from each other, e.g. *Thauera*/*Azoarcus* (Hurek *et al.*, 1997), *Telluria*/*Duganella* (Hiraishi *et al.*, 1997) or *Mesorhizobium*/*Phyllobacterium* (Jarvis *et al.*, 1997). (v) The new genera can be differentiated from each other and from other related bacteria by phenotypic features (Table 3).

The remaining genus, *Azoarcus*, consists of at least two different 16S rDNA clades, one of them containing a variety of isolates from soil and sediment which appear to differ considerably from the originally described, plant-associated species. To decide whether these soil-borne strains deserve the rank of a separate genus, more detailed comparative phenotypic studies under standardized conditions will be necessary.

The species delineation within the genus *Azoarcus* is supported by DNA–DNA homology studies for the named species *Azoarcus communis*, *Azoarcus indigenus* and the unnamed species represented by strain BH72 (Reinhold-Hurek *et al.*, 1993). *Azoarcus anaerobicus* has a maximal 16S rDNA identity of 97.1 % to other described species (Springer *et al.*, 1998), a value which can be regarded as sufficiently low to allow description of a new species (Stackebrandt & Goebel, 1994). According to this criterion, *Azoarcus toluelyticus* is

Table 3. Differential characteristics of the genera *Azoarcus*, *Azovibrio*, *Azospira*, *Azonexus* and morphologically similar diazotrophs of the *Proteobacteria*

Data from this study and from Gillis & Reinhold-Hurek (1994), Gillis *et al.* (1989) and Baldani *et al.* (1996). +, Positive for all strains; —, negative for all strains; D, differs among species; d, 11–89% of strains are positive; ND, not determined.

Character	<i>Azoarcus</i>	<i>Azovibrio</i>	<i>Azospira</i>	<i>Azonexus</i>	<i>Herbaspirillum</i>	<i>Burkholderia vietnamensis</i>	<i>Azospirillum</i>	<i>Acetobacter diazotrophicus</i>
Subclass	β	β	β	β	β	β	α	α
Cells curved	+	+	+	+	+	—	—	—
Cell width (μm)	0.4–1.0	0.6–0.8	0.4–0.6	0.6–0.8	0.6–0.7	0.3–0.8	0.8–1.4	0.7–0.9
Colony colour*	Yellowish to beige	Beige	Pinkish translucent	Ochreous	Cream	Cream	Pinkish opaque	Brown
Fermentative	—	—	—	—	—	—	D	+
Growth on sugars	D†	—	—	—	+	+	+	+
Requirement for cobalamine	—	—	—	+	—	—	—	—
Growth on:								
<i>n</i> -Butylamine	+	—	—	—	D	—	ND	ND
3-Hydroxybenzoate	+	—	—	—	+	+	ND	ND
L-Phenylalanine	+‡	—	—	—	—	+	ND	ND
Glutarate	+§	—	—	—	—	+	ND	ND
2-Oxoglutarate	D	—	+	+	+	d	D	ND
<i>n</i> -Caproate	D	—	+	—	—	+	ND	ND
Propionate	D	+	+	—	+	+	+	ND
L-Proline	D	—	—	+	+	+	+	+

* On VM ethanol agar or nutrient agar.

† Positive for soil-borne species, negative for grass-associated species.

‡ Negative for *Azoarcus* sp. BH72.

§ Negative for *Azoarcus indigenus*.

sufficiently different from the plant-associated species (Zhou *et al.*, 1995). However, *Azoarcus evansii* has a 16S rRNA identity as high as 99.4% to some strains of *Azoarcus toluyticus* (Anders *et al.*, 1995) and DNA–DNA homology studies have not been carried out.

Description of *Azovibrio* gen. nov.

Azovibrio (A.zo'vi.bri.o. Fr. n. *azote* nitrogen; L. v. *vibrare* move rapidly to and fro, vibrate; M.L. masc. n. *Azovibrio* nitrogen-fixing organism which vibrates; combination of French/Latin by analogy with *Azospirillum*).

Formerly *Azoarcus* sp. group C. Gram-negative, non-spore-forming, slightly curved rods; cells range from 0.6 to 0.8 μm in width and from 1.5 to 3.6 μm in length; elongated cells occur very rarely in stationary, alkalized cultures. Highly motile with a corkscrew-like motion by means of a single polar flagellum. Micro-aerophilic growth as veil-like pellicles which move up to the surface in semi-solid N-free media. Strictly respiratory type of metabolism with oxygen or nitrate as terminal electron acceptor; however, strictly micro-aerobic growth may occur. Chemorganoheterotrophic. Oxidase-positive. Catalase-weak. No dissimilatory nitrate reductase. Growth factor requirement varies. Capable of growth in atmospheric nitrogen (N_2) and reduction of acetylene to ethylene. Growth is restricted to a few carbon sources, like salts of organic acids such as L-malate, DL-lactate, succinate, fumarate, acetate, propionate and ethanol, and amino acids such as L-glutamate. No growth occurs on most other organic acids and amino acids, nor on any mono- or disaccharides (Table 2). Grow well at 37 °C.

Not halophilic or NaCl-dependent. Neutral pH optimal for growth. Major cellular fatty acids are *cis*-9 16:1, 16:0, 18:1, 14:0 and 3-OH-10:1. G + C content of DNA is 64–65 mol%. Member of the β -subclass of the *Proteobacteria*. Characteristics differentiating them from other diazotrophs given in Table 2 and 3. Type species is *Azovibrio restrictus*.

Description of *Azovibrio restrictus* gen. nov., sp. nov.

Azovibrio restrictus (re'stric.tus. L. adv. *restrictus* limited, restricted, referring to the restricted spectrum of carbon sources used for growth).

On VM ethanol medium, colonies are opaque beige, smooth, convex with an entire margin; on Congo red medium, colonies are orange-red, shining with otherwise similar characteristics. Grows weakly in presence of 2% NaCl. Most strains grow at 40 °C. No requirement for cobalamine. In addition to those described for the genus, can use L-aspartate as sole carbon source, but not isovalerate or 3-hydroxybutyrate. Source is surface-sterilized roots of gramineae such as Kallar grass [*Leptochloa fusca* (L.) Kunth] or rice (*Oryza sativa*), grown in the Punjab of Pakistan or Nepal, respectively, or resting stages (sclerotia) of a basidiomycete (Pakistan). Type strain, S5b2^T (= LMG 9099^T), has a G + C content of 64.8 mol% and was isolated from Kallar grass roots. It grows at 40 °C and on D(+)-malate.

Another strain of this genus is BS20-3. Differs from *Azovibrio restrictus* by obligately microaerobic growth, thus colony growth is negligible. Requires cobalamine. L-Alanine, isovalerate, isobutyrate and 3-hydroxy-

butyrate, but not L-aspartate, serve as sole carbon sources in addition to those mentioned in the genus description. Source: resting stages (sclerotia) of a basidiomycete (Pakistan).

Description of *Azospira* gen. nov.

Azospira (A.zo'spi.ra. Fr. n. *azote* nitrogen; L. fem. n. *spira* winding, turn; M.L. fem. n. *Azospira* nitrogen-fixing spiral).

Formerly *Azoarcus* sp. group D. Gram-negative, non-spore-forming, curved rods; cells range from 0.4 to 0.6 µm in width and from 1.1 to 2.5 µm in length; elongated cells with one to several spiral windings of up to 8 µm cell length occur rarely in late-stationary-phase, alkalized, nitrogen-fixing cultures. Highly motile with a corkscrew-like motion by means of a single polar flagellum. Microaerophilic growth as veil-like pellicles which move up to the surface in semi-solid N-free media. Strictly respiratory type of metabolism with oxygen or nitrate as terminal electron acceptor. Chemorganoheterotrophic. Oxidase-positive. Catalase-positive. No dissimilatory nitrate reductase. Growth factor requirement varies. Capable of growth in atmospheric nitrogen (N₂) and reduction of acetylene to ethylene. Grow mostly on salts of organic acids, such as L-malate, DL-lactate, succinate, fumarate, acetate, 2-oxoglutarate, *n*-caproate and propionate, and on ethanol; no growth occurs on most amino acids, nor on any mono- or disaccharides (Table 2). Grow well at 37 °C. Not halophilic or NaCl-dependent; no growth at 2% NaCl. Neutral pH optimal for growth. Major cellular fatty acids are *cis*-9 16:1, 16:0, 18:1 and 3-OH-10:1. G+C content of DNA is 65–66 mol%. Member of the β-subclass of the *Proteobacteria*. Characteristics differentiating them from other diazotrophs given in Table 2 and 3. Type species is *Azospira oryzae*.

Description of *Azospira oryzae* gen. nov., sp. nov.

Azospira oryzae (o'ry.zae. L. fem. n. *Oryza* genus name of rice; L. gen. n. *oryzae* from rice, referring to its frequent occurrence in association with rice roots).

On VM ethanol medium, colonies are translucent, pinkish to salmon coloured, smooth, convex with an entire margin; on Congo red medium, colonies are translucent orange, shining with otherwise similar characteristics. In addition to those described for the genus, can use D-tartrate, isovalerate, 3-hydroxybutyrate, L-glutamate and L-aspartate as sole carbon source. Growth optimum at 40 °C. Source is surface-sterilized roots of gramineae such as Kallar grass [*Leptochloa fusca* (L.) Kunth] or several species of rice (*Oryza*), grown in the Punjab of Pakistan or Nepal, Philippines and Italy, respectively, or resting stages (sclerotia) of a basidiomycete (Pakistan). Type strain is 6a3^T (= LMG 9096^T), which has a G+C content of 65.2 mol% and was isolated from Kallar grass. It has no requirement for cobalamine.

Description of *Azonexus* gen. nov.

Azonexus (A.zo'nex.us. Fr. n. *azote* nitrogen; L. masc. n. *nexus* coil; M.L. masc. n. *Azonexus* nitrogen-fixing coil).

Formerly *Azoarcus* sp. group E. Gram-negative, non-spore-forming, slightly curved rods; cells range from 0.6 to 0.8 µm in width and from 1.5 to 4.0 µm in length; elongated straight to coiled cells of up to 50 µm length occur in stationary-phase, alkalized, nitrogen-fixing cultures. Highly motile with a corkscrew-like motion by means of a single polar flagellum. Microaerophilic growth as veil-like pellicles which move up to the surface in semi-solid N-free media. Strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Chemorganoheterotrophic. Oxidase-positive. Catalase-positive. Growth requirement for cobalamine. Capable of growth in atmospheric nitrogen (N₂) and reduction of acetylene to ethylene. Grow mostly on salts of organic acids, such as L-malate, DL-lactate, succinate, fumarate, 2-oxoglutarate and acetate, and on ethanol; grows on only a few amino acids such as L-proline and none of the tested mono- or disaccharides serve as sole carbon sources (Table 2). Grows well at 37 °C; can grow at 40 °C. Not halophilic or NaCl-dependent, but growth occurs at 2% NaCl. Neutral pH optimal for growth. Member of the β-subclass of the *Proteobacteria*. Characteristics differentiating them from other diazotrophs given in Table 2 and 3. Type species is *Azonexus fungiphilus*.

Description of *Azonexus fungiphilus*, gen. nov., sp. nov.

Azonexus fungiphilus (fun.gi'phil.us. M.L. masc. n. *fungi* mushroom, fungi; Gr. adj. *philos* loving; M.L. masc. adj. *fungiphilus* loving mushrooms or fungi, referring to its source of isolation).

On VM ethanol medium, colonies are opaque ochreous, smooth, convex with an entire margin; on Congo red medium, colonies are dark red, shining with otherwise similar characteristics. In addition to those described for the genus, can use 3-hydroxybutyrate, L-glutamate and L-aspartate as sole carbon source. Source: resting stages (black sclerotia) of a basidiomycete related to *Ustilago* found in a rice field in Pakistan. Type strain is BS5-8^T (= LMG 19178^T).

ACKNOWLEDGEMENTS

We thank James M. Tiedje, Michigan State University, and Georg Fuchs, Universität Freiburg, Germany, for the gift of strains, and also Christiane Staubitz and Christina Neumann (both MPI) for assistance in some nutritional and biochemical tests.

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