Description of *Azospira restricta* sp. nov., a nitrogen-fixing bacterium isolated from groundwater

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A novel, Gram-negative bacterial strain, SUA2^T, isolated from groundwater, was characterized using a polyphasic approach. Cells are Gram-negative, non-spore-forming, straight to curved rods with a single polar flagellum. Strain SUA2^T is oxidase- and catalase-positive and is able to fix nitrogen. Poly- β -hydroxybutyrate storage granules are produced. Dominant fatty acids when grown in R2A and VM ethanol media for 72 h at 37 °C are C_{16:0}, C_{16:1 ω 7c}, C_{17:0} cyclo, C_{10:0} 3-OH, C_{18:1 ω 7c}, C_{12:0} and C_{15:0}. DNA G + C content is 67.9 mol%. Phenotypic and phylogenetic data indicate that strain SUA2^T is related to, but clearly differentiated from *Azospira oryzae*. Strain SUA2^T is thus proposed as a novel species of the genus *Azospira* with the name *Azospira restricta* sp. nov. The description of the genus *Azospira* is emended to include the characteristics of this novel species. The type strain of *Azospira restricta* is SUA2^T (=NRRL B-41660^T = DSM 18626^T = LMG 23819^T).

The genus *Azospira* was described by Reinhold-Hurek & Hurek (2000) to accommodate a lineage of nitrogen-fixing bacteria within the *Betaproteobacteria* that could be phylogenetically and phenotypically differentiated from other strains originally assigned to the genus *Azoarcus sensu lato* (Reinhold-Hurek *et al.*, 1993). At present, the genus *Azospira* includes a single validly published species, *Azospira oryzae*, strains of which have been isolated from surface-sterilized roots of gramineae such as Kallar grass (*Leptochloa fusca*) and rice (*Oryza*), or resting stages (sclerotia) of a basidiomycete (Reinhold-Hurek & Hurek, 2000). The description of *Azospira oryzae* sp. nov. was recently emended to accommodate perchlorate-reducing strains isolated from a swine-waste treatment lagoon that were originally described as *Dechlorosoma suillum* (Achenbach *et al.*, 2001), but which were later shown to be phylogenetically and phenotypically

similar to and represent a later subjective synonym of *Azospira oryzae* (Tan & Reinhold-Hurek, 2003).

During a study aimed at characterizing the microbial population in groundwater from an uncontaminated groundwater well located upgradient from a hazardous waste site near Baton Rouge, Louisiana, USA (Bowman *et al.*, 2006), a novel bacterial strain, designated SUA2^T, was isolated on R2A agar (Difco) incubated aerobically at 23 °C. Purity of the strain was verified by microscopy after multiple transfers.

Colony morphology was observed after aerobic growth on plates of VM ethanol medium (Reinhold-Hurek & Hurek, 2000) incubated at 37 °C for 4 days, or R2A (Difco) at 30 °C for 7 days. Cell morphology was observed by phase-contrast microscopy (Nikon Optiphot), differential interference contrast microscopy (Nikon microphot-Fxa) and transmission electron microscopy (JEOL 1000CX TEM). Electron micrographs were taken from cells negatively stained with uranyl acetate (2%, v/v). Gram staining and catalase tests were performed as described by Smibert & Krieg (1981). Oxidase tests were performed as described by Tarrand & Gröschel (1982). NaCl tolerance was tested in liquid VM

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *nifH* sequences of strain SUA2^T are DQ974114 and EF158046, respectively.

Supplementary figures showing morphological features and whole-cell soluble protein patterns (SDS-PAGE), and a supplementary table of fatty acid composition data are available with the online version of this paper.

ethanol medium supplemented with 1.0 and 2.0% (w/v) NaCl. The temperature range for growth was tested in liquid VM ethanol medium incubated at temperatures of 15, 20, 23, 30, 37 and 40 °C. Additional biochemical and physiological properties were determined using API 20 NE (bioMérieux) test kits, according to the manufacturer's instructions. Substrate metabolism was determined on Biolog GN and GP MicroPlates inoculated with cells grown in R2A liquid broth overnight at 23 °C. Cells were washed with a 0.9% (w/v) NaCl solution, centrifuged and resuspended in the inoculation fluid provided with the kit. Substrate metabolism was determined by purple colour development after incubation of the plate at 30 °C for 85 h followed by measurement of A_{595} using an automated plate reader (Multiskan Ascent, version 4.0; Labsystems).

The ability of SUA2^T cells to use nitrate or perchlorate as alternative electron acceptors was tested using anoxic DSMZ medium 908 (www.dsmz.de/media/med908.htm) with acetate replaced by lactate (10 mM) and supplemented with either sodium nitrate (10 mM) or sodium perchlorate (10 mM) as potential electron acceptors. For both perchlorate and nitrate, electron acceptor utilization was assessed by comparing growth of cells in the perchlorate- or nitrate-containing medium with control cultures prepared in media without the supplemental electron acceptor after 8 days incubation at 30 °C. Nitrate reduction and denitrification activities were further assessed as described by Smibert & Krieg (1981).

Nitrogen-fixing ability was determined by growth and acetylene reduction in semi-solid (0.2% w/v, agar), nitrogen-free SM medium (Reinhold *et al.*, 1986), in which malate was replaced by 10 mM lactate. Cells grown on R2A for 3 days were inoculated vertically into 17 ml Hungate tubes containing 10 ml of the semi-solid SM medium using a sterile loop. The headspace was supplemented with 0.2 ml acetylene prior to incubation at 30 °C for 8 days. Uninoculated tubes served as negative controls. Acetylene reduction (ethylene production) was determined by analysis of 0.1 ml gas headspace samples using a gas chromatograph (HP6890; HP) equipped with a capillary column (GS-Gaspro, 60 m × 0.32 mm i.d.; J&W P/N 113-4362) and a flame-ionization detector. Helium was used as carrier gas at a flow rate of 45 ml min⁻¹. The injector and detector temperature was 225 °C. The oven temperature was increased from 50 to 200 °C at a rate of 50 °C min⁻¹. A PCR-based assay using the PolF/PolR PCR primer set and a PCR protocol as described by Poly *et al.* (2001) was used to detect presence of the *nifH* gene.

For the preparation of soluble whole-cell proteins, cells were cultured in liquid R2A medium overnight and washed with 20 mM potassium phosphate buffer (pH 7.0). Cells were disrupted by sonication (Fisher Scientific Sonic Dismembrator) at 20 kHz in an ice bath. After removal of cell debris by centrifugation at 16 000 g for 15 min, soluble proteins were separated by SDS-PAGE on a 12.5% (w/v) polyacrylamide gel (30:1, w/w, acrylamide/bisacrylamide).

Protein bands were visualized by staining with Coomassie brilliant blue R250. For comparison purposes, *Azospira oryzae* strains 6a3^T (= LMG 9096^T), obtained from BCCM/LMG (the Belgian Co-ordinated Collections of Microorganisms/Laboratorium voor Microbiologie) and PS (syn. *Dechlorosoma suillum*), kindly provided by Dr John Coates of the University of California at Berkeley, were also analysed. These two reference strains were grown and processed in exactly the same manner as strain SUA2^T.

Cellular fatty acids were extracted, saponified and methylated according to the protocol of the Sherlock Microbial Identification System (MIDI) after growth in R2A (this study) and VM ethanol (DSMZ Identification Service) media for 72 h at 37 °C. The fatty acids were analysed by a gas chromatograph equipped with the Microbial Identification software package (Sasser, 1990). Genomic DNA G+C content was determined by HPLC as described by Mesbah *et al.* (1989).

Poly-β-hydroxybutyrate (PHB) extraction, collection and conversion to crotonic acid were performed according to the protocol of Smibert & Krieg (1981). Strain SUA2^T and *Azospira oryzae* strains 6a3^T and PS were grown in liquid R2A medium for 5 days at 30 °C on a rotary shaker prior to biomass collection for this analysis. The presence of crotonic acid was detected spectrophotometrically at 235 nm (Smibert & Krieg, 1981).

Extraction of genomic DNA, PCR amplification and sequencing of 16 rRNA genes was carried out as described by Rainey *et al.* (1996). Purified sequencing-reaction products were electrophoresed using a model 3100 DNA sequencer (Applied Biosystems). The 16S rRNA gene sequence of strain SUA2^T was aligned against previously determined sequences from the NCBI database (www.ncbi.nlm.nih.gov) using the BioEdit (version 4.7.8; Hall, 1999) and CLUSTAL_X programs (Thompson *et al.*, 1997). Phylogenetic analyses were performed using components of the MEGA 3.1 package (Kumar *et al.*, 2004). The neighbour-joining algorithm was used to build the phylogenetic tree from distance matrices calculated with Jukes–Cantor correction (Jukes & Cantor, 1969). Bootstrap analysis was performed using PHYLIP 3.62 (Felsenstein, 2004; <http://evolution.genetics.washington.edu/phylip.html>) with 1000 resamplings.

The characteristics of strain SUA2^T are provided in the emended genus and species description (Table 1, and Supplementary Fig. S1, available with the online version of this paper). Strain SUA2^T grew in semi-solid, nitrogen-free medium, forming pellicles that moved up to the surface, indicating that strain SUA2^T was able to fix N₂ gas as a nitrogen source. After 8 days incubation in nitrogen-free medium, reduction of acetylene (C₂H₂) to ethylene (C₂H₄) was observed via GC, further indicating that strain SUA2^T exhibits nitrogenase activity. The presence of the *nifH* gene was detected by gel electrophoresis following PCR amplification using the PolF/PolR PCR primer set described by

Table 1. Characteristics of strain SUA2^T and *Azospira oryzae*

Data for *Azospira oryzae* were taken from Reinhold-Hurek & Hurek (2000) and Tan & Reinhold-Hurek (2003), except for fatty acid data which were determined in the present study using methods and growth conditions identical to those used for strain SUA2^T. All are Gram-negative, non-spore-forming, straight to curved rods with a single polar flagellum, oxidase- and catalase-positive, and able to fix nitrogen. All are unable to grow in the presence of 2% NaCl (w/v). All utilize β -hydroxybutyric acid and D,L-lactic acid, but do not utilize N-acetyl-D-glucosamine, adonitol, L-alanine, γ -aminobutyric acid, L-arabinose, D-arabitol, D-cellobiose, erythritol, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, D-glucose, γ -hydroxybutyric acid, *m*-inositol, α -ketoglutaric acid, D-lactose, lactulose, D-maltose, D-mannose, D-melibiose, L-phenylalanine, L-proline, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, D-tagatose, D-trehalose, turanose, xylitol or D-xylose.

Characteristic	SUA2 ^T	<i>Azospira oryzae</i> 6a3 ^T (=LMG 9096 ^T)	<i>Azospira oryzae</i> PS (=DSM 13638 ^T)
Origin	Groundwater	Kallar grass	Swine-waste treatment lagoon
Cell dimension (μ m)	0.5 $\times$ 1.2–2.5	0.4–0.6 $\times$ 1.1–2.5	0.3 $\times$ 1.0
Colony colour on VM ethanol medium	Greenish-yellow	Orange	Orange
Nitrate reduction	–	+	+
Perchlorate utilization	–	–	+
DNA G+C content (mol%)	67.9	65.2	65.8
Fatty acids (%)			
C _{8:0} 3-OH	–	1.6	0.9
C _{10:0}	0.6	–	–
C _{10:0} 3-OH	6.5	–	0.1
C _{12:0}	3.8	–	–
C _{14:0}	1.2	0.6	0.8
C _{15:0}	2.4	0.4	0.6
C _{16:1} ω 7c	32.0	43.6	41.8
C _{16:0}	37.4	43.9	48.5
C _{17:1} ω 8c	0.4	0.1	0.1
C _{17:0} cyclo	6.6	–	–
C _{17:0}	0.6	0.3	0.4
C _{18:1} ω 7c	6.2	8.3	5.4
C _{18:0}	0.6	0.6	0.5
Utilization of:			
Acetic acid	–	+	+
α -Ketoglutaric acid	–	+	+
Propionic acid	–	+	+
Succinic acid	–	+	+
L-Aspartic acid	–	+	+
L-Glutamic acid	–	+	+
L-Malic acid	–	+	+
Putrescine	+	–	–
γ -Hydroxybutyric acid	+	–	–

Poly *et al.* (2001). The gene sequence was verified by sequencing of the PCR product.

Comparison of the fatty acid composition of strain SUA2^T with that described for *Azospira oryzae* strain 6a3^T in the description of the genus *Azospira* (Reinhold-Hurek & Hurek, 2000) showed large differences in the major fatty acids detected. In the description of the genus *Azospira*, which was based on *Azospira oryzae* 6a3^T, the major fatty

acids were described as C_{10:1} 3-OH, C_{16:0}, C_{16:1} ω 9c and C_{18:0}. However, reanalysis of the fatty acids of strains 6a3^T (=LMG 9069^T) and PS showed that three of the four fatty acids, namely C_{10:1} 3-OH, C_{16:1} ω 9c and C_{18:0}, described as major fatty acids in the description of the genus *Azospira* (Reinhold-Hurek & Hurek, 2000) are in fact not detected in the *Azospira oryzae* strains (Table 1). These data were reanalysed in two independent laboratories after the cells had been grown on VM ethanol medium under the

Fig. 1. Neighbour-joining tree showing the phylogenetic relationship of SUA2^T, related taxa and environmental clone sequences on 16S rRNA gene sequences. Bootstrap values expressed as a percentage of 1000 replications are shown at the branching points. Bar, 1 inferred nt substitution per 100 nt.

conditions described by Reinhold-Hurek & Hurek (2000) and also on R2A medium (Supplementary Table S1, available with the online version of this paper).

Nearly complete 16S rRNA gene sequences were used to reconstruct a phylogenetic dendrogram showing the relationship between strain SUA2^T, *Azospira oryzae* strains, other genera in the family *Rhodocyclaceae* and environmental 16S rRNA gene sequences (Fig. 1). Sequence similarity between strain SUA2^T and the closest relative for which a sequence is available from the public databases, the uncultured groundwater bacterium 249ds10 (AY212697; Simpson *et al.*, 2004), was 98.1 %. 16S rRNA gene sequence similarities between strain SUA2^T and the four closest cultured relatives, *Azospira oryzae* strains 6a3^T, PS, GR-1 and R-25019 (AF011347, AF170348, AY277622 and AM084030, respectively), were in the range 95.7–95.8 %, indicating that strain SUA2^T represents a distinct, but related lineage to this species. High bootstrap values support the novelty of the lineage represented by strain SUA2^T. Strain SUA2^T is the first cultured member of this lineage which is currently composed of environmental 16S rRNA gene sequences from groundwater and activated sludge samples. The 16S rRNA gene sequence of strain SUA2^T shares between 97.1 and 98.1 % similarity with the sequences of these as yet uncultured organisms. The clear differentiation of strain SUA2^T from *Azospira oryzae* at the gene level was also seen in the comparison of the partial *nifH* gene sequence which showed only 81.5 % nucleotide similarity over 269 positions compared to those of *Azospira oryzae* strains 6a3^T and PS. The highest similarity between the *nifH* gene sequence of strain SUA2^T was to that of the unrelated taxon *Azonexus caeni*

(DQ029203) at 94 %. The DNA G+C content of strain SUA2^T was 67.9 mol%.

To further investigate differences between strain SUA2^T and the related *Azospira oryzae* strains, whole cell soluble proteins were compared by SDS-PAGE (Supplementary Fig. S2, available with the online version of this paper). The protein patterns of *Azospira oryzae* strains 6a3^T and PS were quite similar to one another, whereas the protein pattern of strain SUA2^T was clearly different. Strain SUA2^T and *Azospira oryzae* strain 6a3^T all produced polyhydroxybutyrate when grown in liquid R2A medium for 5 days at 30 °C.

Morphological characteristics and some phenotypic properties of strain SUA2^T confer with those described for the genus *Azospira* (Reinhold-Hurek & Hurek, 2000). These phenotypic features include the Gram-negative reaction, heterotrophic metabolism, non-spore-forming, curved rods with cells ranging from 0.4 to 0.6 µm in width and from 1.1 to 2.5 µm in length, motility by means of a single polar flagellum, production of catalase and oxidase, ability to grow well at 37 °C, absence of growth in the presence of 2 % (w/v) NaCl, capacity for nitrogen (N₂) fixation and reduction of acetylene to ethylene. Strain SUA2^T also matches the phenotypic feature of the *Azospira* genus description in that no growth occurs on mono- or disaccharides, nor on most amino acids.

However, some of the phenotypic characteristics determined for strain SUA2^T do not confer with the current description of the genus *Azospira*. Specifically, strain SUA2^T is unable to utilize nitrate as a terminal electron acceptor and does not grow on organic acids such as L-malate, succinate,

fumarate, acetate, 2-oxoglutarate, *n*-caproate and propionate, nor on ethanol. In addition, the G+C content of genomic DNA from strain SUA2^T, 67.9 mol%, is outside of the range of 65–66 mol% for the genus.

Strain SUA2^T is clearly discriminated from *Azospira oryzae*, the only validly published species of the genus *Azospira*, by substrate utilization, inability to utilize nitrate or perchlorate as electron acceptors and colony colour when grown on VM ethanol medium (Table 1). Fatty acid composition also distinguishes strain SUA2^T from *Azospira oryzae* strains, based on the presence of C_{17:0} cyclo, C_{10:0} 3-OH, C_{12:0} and C_{10:0} in the former and absence or presence of only trace quantities in the latter when grown at 37 °C on R2A medium. Whole-cell soluble protein patterns analysed by SDS-PAGE (supplementary Fig. S1) demonstrated that strain SUA2^T is distinct from *Azospira oryzae* in overall protein profiles, and *nifH* gene sequences also differ, supporting the classification of strain SUA2^T as a novel species.

On the basis of phylogenetic, chemotaxonomic and phenotypic features obtained in this study, strain SUA2^T is clearly discriminated from the one validly published species of the genus *Azospira*. Considering the lack of genus-level characteristics for differentiation of strain SUA2^T from *Azospira oryzae* as well as the relatively high 16S rRNA gene sequence similarities, we propose strain SUA2^T as a novel species which we have named *Azospira restricta* sp. nov. In addition, we have emended the genus description for *Azospira* to include the characteristics of this novel species and to clarify the fatty acid content.

Emended description of the genus *Azospira* Reinhold-Hurek & Hurek 2000; Tan & Reinhold-Hurek 2003

The emended description is based on the results of Reinhold-Hurek & Hurek (2000), Tan & Reinhold-Hurek (2003) and this study. Gram-negative, non-spore-forming, curved rods. Motile by means of a single polar flagellum. Aerobic, capable of microaerophilic growth in semi-solid, N-free media. Variable with respect to use of nitrate and perchlorate as terminal electron acceptors. Heterotrophic. Oxidase-positive. Catalase-positive. Capable of nitrogen fixation. Growth does not occur on most amino acids, nor on mono- or disaccharides. Optimum growth temperature about 37 °C. Produces polyhydroxybutyrate storage granules. Neutral pH optimal for growth. Major cellular fatty acids are C_{16:1}ω7c, C_{16:0} and C_{18:1}ω7c. G+C content of DNA is 65–68 mol%. Type species is *Azospira oryzae*.

Description of *Azospira restricta* sp. nov.

Azospira restricta (res.tric'ta. L. fem. part. adj. *restricta* limited, restricted, referring to the restricted spectrum of metabolized substrates).

On VM ethanol agar colonies are 1–2 mm diameter, translucent, greenish-yellow, smooth, convex with entire margins. On R2A agar colonies are cream-coloured, smooth,

convex with entire margins. Straight or curved rods, 0.5 × 1.2–2.5 µm. Inclusion granules are observed. Growth occurs from 20 to 40 °C with optimal growth at 37 °C. Growth occurs at neutral pH. No growth is observed in the presence of 2 % NaCl (w/v). Aesculin hydrolysis, gelatin hydrolysis, H₂S production and acetoin production do not occur. Negative for β-galactosidase, arginine dihydrogenase and lysine decarboxylase. Strain SUA2^T metabolizes β-hydroxybutyrate, γ-hydroxybutyrate, D,L-lactate and putrescine as growth substrates, but does not metabolize other substrates in Biolog GN and GP microplates. Nitrate and perchlorate are not used as electron acceptors. Growth occurs on β-hydroxybutyric acid, γ-hydroxybutyric acid, D,L-lactic acid and putrescine. Dominant cellular fatty acids are C_{16:0}, C_{16:1}ω7c, C_{17:0} cyclo, C_{10:0} 3-OH, C_{18:1}ω7c, C_{12:0} and C_{15:0}. DNA G+C content is 67.9 mol%.

The type strain SUA2^T (=NRRL B-41660^T=DSM 18626^T=LMG 23819^T) was isolated from groundwater.

Acknowledgements

This research was funded by the Governor's Biotechnology Initiative of the Louisiana Board of Regents grant BOR#015 (Enhancement of the LSU Hazardous Substance Research Center Environmental Biotechnology Initiative) and NPC Services, Inc. The authors thank Cindy Henk of the LSU Socolofsky Microscopy Center for assistance with microscopy. The authors thank Jean Euzéby for assistance with the etymology of the new species.

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