

## ***Azospirillum doebereineriae* sp. nov., a nitrogen-fixing bacterium associated with the C<sub>4</sub>-grass *Miscanthus***

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**A new group of nitrogen-fixing *Azospirillum* sp. bacteria was isolated from the roots of the C<sub>4</sub>-gramineous plant *Miscanthus*. Polyphasic taxonomy was performed, including auxanography using API galleries, physiological tests and 16S rRNA sequence comparison. The ability of the isolates to fix dinitrogen was evaluated by amplification of the *nifD* gene, immunodetection of the dinitrogenase reductase and acetylene-reduction assay. On the basis of these results, the nitrogen-fixing isolates represent a new species within the genus *Azospirillum*. Its closest phylogenetic neighbours, as deduced by 16S rDNA-based analysis, are *Azospirillum lipoferum*, *Azospirillum largimobile* and *Azospirillum brasilense* with 96.6, 96.6 and 95.9% sequence similarity, respectively. Two 16S rRNA-targeting oligonucleotide probes were developed which differentiate the new species from the other *Azospirillum* species by whole-cell fluorescence hybridization. Strains of the new species are curved rods or S-shaped, 1.0–1.5 µm in width and 2.0–3.0 µm in length, Gram-negative and motile with a single polar flagellum. Optimum growth occurs at 30 °C and at pH values between 6.0 and 7.0. No growth takes place at 37 °C. They have a respiratory type of metabolism, grow well on arabinose, D-fructose, gluconate, glucose, glycerol, malate, mannitol and sorbitol. They differ from *A. largimobile* and *A. lipoferum* by their inability to use N-acetylglucosamine and D-ribose, from *A. lipoferum* by their ability to grow without biotin supplementation and from *A. brasilense* by their growth with D-mannitol and D-sorbitol as sole carbon sources. Nitrogen fixation occurs in microaerobic nitrogen-limited conditions. For this species, the name *Azospirillum doebereineriae* sp. nov. is suggested, with strain GSF71<sup>T</sup> as the type strain (= DSM 13131<sup>T</sup>; reference strain Ma4 = DSM 13400). Its G+C content is 70.7 mol%.**

**Keywords:** *Azospirillum*, *Miscanthus*, plant-associated bacteria, diazotroph

### **INTRODUCTION**

Since the first detailed description of the genus *Azospirillum* with two species, *Azospirillum lipoferum* and *Azospirillum brasilense* (Tarrand *et al.*, 1978), members of *Azospirillum* has been isolated from the roots of numerous wild and cultivated grasses, cereals,

food crops and soils from tropical, subtropical and temperate regions all over the world (Döbereiner *et al.*, 1976; Bally *et al.*, 1983; Ladha *et al.*, 1987; Kirchhof *et al.*, 1997a; Gunarto *et al.*, 1999). Semi-solid nitrogen-free medium (Döbereiner, 1980) was the key to the successful isolation of microaerophilic N<sub>2</sub>-fixing bacteria. Using this technique, it was possible to isolate and describe three more species: *Azospirillum amazonense* (Magalhães *et al.*, 1983), *Azospirillum halopraeferens* (Reinhold *et al.*, 1987) and *Azospirillum irakense* (Khammas *et al.*, 1989). On the basis of 16S rDNA and DNA–DNA hybridization studies, the

The EMBL accession numbers for the partial 16S rDNA sequence and the partial *nifD* sequence of *Azospirillum doebereineriae* sp. nov. are AJ238567 and AJ238568, respectively.

former *Conglomeromonas largomobilis* subsp. *largomobilis* (Skerman *et al.*, 1983) was recently transferred to the genus *Azospirillum* as *Azospirillum largomobile* (Ben Dekhil *et al.*, 1997) and subsequently the name was corrected to *Azospirillum largimobile* by Sly & Stackebrandt (1999).

It is generally accepted that members of the genus *Azospirillum* can enhance the growth of plants in different modes, triggered, for example, by the production of phytohormones, which are signal molecules that interfere with plant metabolism (Bashan & Holguin, 1997). It is also possible that these bacteria transfer nitrogen to their host plants (Döbereiner, 1983; Okon, 1985). Although the N<sub>2</sub>-fixation *in planta* has not yet been shown unequivocally in azospirilla, the contribution of biological fixed nitrogen to the total plant nitrogen uptake is estimated as being between 40 and 80% in some varieties of sugar cane (Boddey *et al.*, 1995; Yoneyama *et al.*, 1997). In sorghum (Giller *et al.*, 1986), maize (Boddey, 1987) and rice (Baldani *et al.*, 1995), the range was 0–30% depending on the cultivar tested.

*Miscanthus* is a perennial lignocellulose-producing grass and a possible alternative crop for farmers using fields taken out of grain-crop production. Originally grown in southeastern Asia, *Miscanthus* can be established and cultivated under European temperate climatic conditions (Hartley, 1958). The plant fibre is utilized for the production of paper pulp or for replacing fossil energy in biomass power stations. *Miscanthus* is characterized by its highly efficient photosynthesis, like *Zea mays* and *Saccharum notatum*, which use a C<sub>4</sub> type of photosynthate carbon assimilation involving oxaloacetate and the Calvin cycle. In these plants, wasteful photorespiration is absent and therefore rates of photosynthesis and plant production are higher. In various field experiments, it has been demonstrated that the biomass yield (20–30 tons per hectare per year) does not increase with the application of mineral nitrogen fertilizer (Schwarz *et al.*, 1994). Christian *et al.* (1997) analysed the nitrogen balance of *Miscanthus* in <sup>15</sup>N-amended soil. In 2 years, the nitrogen uptake of the plant was greater than the amount supplied as fertilizer and as soil nitrogen sources, indicating that the plant was able to obtain substantial amounts of nitrogen from other sources.

The lack of response to nitrogen fertilization, combined with high biomass production, suggested that biological nitrogen fixation may be involved and that diazotrophic bacteria could be associated with this plant. The use of specific enrichment procedures and oligonucleotide probes made it possible to isolate and characterize various diazotrophic bacteria from *Miscanthus* plant tissues (Kirchhof *et al.*, 1997a, b). After physiological and phylogenetical analyses, most of these isolates were assigned to the genus *Herbaspirillum*, in which they constitute a new species (Kirchhof *et al.*, 2001), and the genus *Azospirillum*.

In this report, we present the phylogenetic position and physiological properties of a new species, for which the name *Azospirillum doebereineriae* is proposed, and two species-specific oligonucleotide probes for the identification and detection of this new species by *in situ* fluorescence hybridization.

## METHODS

**Isolation.** All of the strains used in this study are listed in Table 1. Roots of *Miscanthus sinensis* cv. 'Giganteus' and rhizosphere soil were collected from fields, on which there had been continuous *Miscanthus* cultivation since 1990, at the LBP (Bayerische Landesanstalt für Bodenkultur und Pflanzenbau), Freising, Germany. The roots were washed with sterile water and then crushed in 4% sucrose solution by using a mortar and pestle. Small vessels (approx. 10 ml) containing 5 ml NFb semi-solid nitrogen-free medium (Döbereiner, 1995) were inoculated with serial dilutions of crushed roots or rhizosphere soil suspensions. The composition of the NFb medium is as follows (l<sup>-1</sup>): malate (5.0 g); K<sub>2</sub>HPO<sub>4</sub> (0.5 g); MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2 g); NaCl (0.1 g); CaCl<sub>2</sub>·2H<sub>2</sub>O (0.02 g); bromothymol blue 0.5% in KOH 0.2 M (2 ml); sterile, filtered vitamin solution (1 ml); sterile, filtered micronutrient solution (2 ml); 1.64% FeEDTA solution (4 ml); KOH (4.5 g). The pH was adjusted to 6.5 and 1.8 g agar l<sup>-1</sup> was added. The vitamin solution contains, in 100 ml, biotin (10 mg) and pyridoxol-HCl (20 mg), dissolved at 100 °C in a water bath. The micronutrient solution consists of the following (l<sup>-1</sup>): CuSO<sub>4</sub>·5H<sub>2</sub>O (40 mg); ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.12 g); H<sub>3</sub>BO<sub>3</sub> (1.4 g); Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (1.0 g); MnSO<sub>4</sub>·H<sub>2</sub>O (1.175 g). JNFb contains extra K<sub>2</sub>HPO<sub>4</sub> (0.6 g) and KH<sub>2</sub>PO<sub>4</sub> (1.8 g) to stabilize the pH at about 5.8.

After 3–5 d incubation at 30 °C, one loop of pellicle-forming culture was transferred into fresh semi-solid medium. Further purification was done on NFb (supplemented with 50 mg yeast extract l<sup>-1</sup>) and half-strength DYGS medium (modified from that described by Rodrigues Neto *et al.*, 1986) agar plates. Pellicle forming cultures were maintained on half-strength DYGS medium containing the following (l<sup>-1</sup>): glucose (1.0 g); malate (1.0 g); yeast extract (2.0 g); peptone (1.5 g); MgSO<sub>4</sub>·7H<sub>2</sub>O (0.5 g); L-glutamic acid (1.5 g). The pH was adjusted to 6.0.

**Morphological examination.** Morphology and Gram-type were determined by using an Axioplan phase-contrast microscope (Zeiss). The type of flagellation and the cell dimensions were determined by electron microscopy using preparations negatively stained for 30 min with uranyl acetate (Ultrastain 1; Leica) or for 4 min with lead citrate (Ultrastain; Leica).

**Phenotypic characterization.** Carbon-source utilization was tested by using the API50 CHE and API20 NE gallery methods (bioMérieux). Cells from liquid overnight cultures in half-strength DYGS medium were harvested and washed twice with sterile PBS. The bacterial inoculum was diluted in minimal medium, according to the manufacturer's instructions, incubated aerobically at 30 °C and examined visually for growth daily over a period of 5 d. Additionally, the API50 CHE gallery method was performed anaerobically and acid production was recorded.

Furthermore, pellicle-forming ability and microaerobic N<sub>2</sub>-dependent growth were assessed in semi-solid JNFb medium

**Table 1.** Origins of the bacterial strains used in this study

Strain*	Source†	Reference	Origin
<i>Azospirillum</i> sp. GSF21	GSF/ISE	Kirchhof <i>et al.</i> (1997a)	Washed roots of <i>Miscanthus sacchariflorus</i>
<i>Azospirillum</i> sp. GSF65	This report		Washed roots of <i>Miscanthus sinensis</i> cv. 'Giganteus'
<i>Azospirillum</i> sp. GSF66	This report		Washed roots of <i>Miscanthus sinensis</i> cv. 'Giganteus'
<i>Azospirillum</i> sp. GSF67	This report		Washed roots of <i>Miscanthus sinensis</i> cv. 'Giganteus'
<i>Azospirillum</i> sp. GSF68	This report		Washed roots of <i>Miscanthus sinensis</i> cv. 'Giganteus'
<i>Azospirillum</i> sp. GSF69	This report		Washed roots of <i>Miscanthus sinensis</i> cv. 'Giganteus'
<i>Azospirillum</i> sp. GSF70	This report		Washed roots of <i>Miscanthus sinensis</i> cv. 'Giganteus'
<i>Azospirillum</i> sp. GSF71 <sup>T</sup>	This report		Washed roots of <i>Miscanthus sinensis</i> cv. 'Giganteus'
<i>Azospirillum</i> sp. GSF76	This report		Rhizosphere soil ( <i>Miscanthus sinensis</i> )
<i>Azospirillum</i> sp. GSFc	This report		Rhizosphere soil ( <i>Miscanthus sinensis</i> )
<i>Azospirillum</i> sp. Ma4	This report		Washed roots of <i>Miscanthus sinensis</i> cv. 'Giganteus'
<i>Azospirillum lipoferum</i> GSF1	GSF/ISE	Kirchhof <i>et al.</i> (1997a)	Washed roots of <i>Spartina pectinata</i>
<i>Azospirillum lipoferum</i> GSF5	GSF/ISE	Kirchhof <i>et al.</i> (1997a)	Washed roots of <i>Spartina pectinata</i>
<i>Azospirillum lipoferum</i> GSF15	GSF/ISE	Kirchhof <i>et al.</i> (1997a)	Washed roots of <i>Spartina pectinata</i>
<i>Azospirillum lipoferum</i> GSF18	GSF/ISE	Kirchhof <i>et al.</i> (1997a)	Washed stem of <i>Miscanthus sinensis</i> cv. 'Giganteus'
<i>Azospirillum lipoferum</i> GSF26	GSF/ISE	Kirchhof <i>et al.</i> (1997a)	Washed roots of <i>Miscanthus sacchariflorus</i>
<i>Azospirillum lipoferum</i> SpA3a	DSM 1838	Tarrand <i>et al.</i> (1978)	Grass roots
<i>Azospirillum lipoferum</i> Sp59b	DSM 1691 <sup>T</sup>	Tarrand <i>et al.</i> (1978)	Wheat roots
<i>Azospirillum lipoferum</i> SpRG6	DSM 1841	Tarrand <i>et al.</i> (1978)	Wheat roots
<i>Azospirillum lipoferum</i> SpRG20a	DSM 1840	Tarrand <i>et al.</i> (1978)	Wheat roots
<i>Azospirillum largimobile</i>	ACM 2041 <sup>T</sup>	Skerman <i>et al.</i> (1983)	Fresh water
<i>Azospirillum brasiliense</i> Wa3	C. Christiansen-Weniger	Christiansen-Weniger & van Ven (1991)	Rhizosphere of wheat
<i>Azospirillum brasiliense</i> Sp7	DSM 1690 <sup>T</sup>	Tarrand <i>et al.</i> (1978)	<i>Digitaria decumbens</i> roots
<i>Azospirillum brasiliense</i> Cd	DSM 1843	Tarrand <i>et al.</i> (1978)	<i>Cynodon dactylon</i> roots
<i>Azospirillum brasiliense</i> JM 6A2	DSM 1858	Tarrand <i>et al.</i> (1978)	Maize roots
<i>Azospirillum amazonense</i> Y-1	DSM 2787 <sup>T</sup>	Magalhães <i>et al.</i> (1983)	<i>Digitaria decumbens</i> roots
<i>Azospirillum amazonense</i> Y-6	DSM 2789	Magalhães <i>et al.</i> (1983)	<i>Pennisetum purpureum</i> roots
<i>Azospirillum irakense</i> KBC1	DSM 11586 <sup>T</sup>	Khammas <i>et al.</i> (1989)	Rice roots
<i>Azospirillum halopraefereans</i> AU4	DSM 3675 <sup>T</sup>	Reinhold <i>et al.</i> (1987)	<i>Leptochloa fusca</i> , root
<i>Azospirillum</i> sp. 4 AZ	DSM 4834	Malik (1988)	Fresh water
<i>Azospirillum</i> sp. 5 AZ	DSM 4835	Malik (1988)	Fresh water
<i>Azospirillum</i> sp.	DSM 1726	Malik & Schlegel (1980)	Plant litter
<i>Azospirillum</i> sp.	DSM 1727	Malik & Schlegel (1980)	Plant litter
<i>Skermanella parooensis</i>	ACM 2042 <sup>T</sup>	Skerman <i>et al.</i> (1983)	Fresh water
<i>Rhodospirillum centenaria</i>	DSM 9894 <sup>T</sup>	Favinger <i>et al.</i> (1989)	Hot spring

\* All *Azospirillum* sp. isolates were obtained from fields of *Miscanthus* at the Bayerische Landesanstalt für Bodenkultur und Pflanzenbau (LBP), Freising, Germany.

† DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany; ACM, Australian Collection of Microorganisms, Department of Microbiology, The University of Queensland, St Lucia, Australia; GSF/ISE, GSF-Institute of Soil Ecology, Neuherberg/Munich, Germany.

with different carbon sources. For this purpose, malate was replaced by fructose, glucose, glycerol, *myo*-inositol, maltose, D-mannitol, D-ribose, D-sorbitol or sucrose (Döbereiner, 1995). Three replicates were inoculated with 10 µl washed overnight culture in half-strength DYGS and incubated over a period of 3–4 d at 30 °C. To test the ability of the cells to grow with NaCl, 3% NaCl was added to semi-solid NFB medium.

The oxidase test was performed with Bactident–Oxidase test strips (Merck). Analyses for urease activity (API20 NE) and the biotin requirement (Tarrand *et al.*, 1978) were carried out as described in the literature.

The influences of temperature and pH on bacterial growth were analysed by measuring the optical density at 436 nm in liquid half-strength DYGS or minimal medium for *Azospirillum* (Tarrand *et al.*, 1978), respectively, with shaking at 150 r.p.m. The test for scarlet coloration of colonies on Congo red medium was performed as described by Caceres (1982).

**Phylogenetic analysis.** Approximately 1300 bp of the 16S rDNA of strain GSF71<sup>T</sup> was amplified by a PCR with primers 63f and 1387r (Marchesi *et al.*, 1998). The purified product was sequenced by Eurogentec by using the ABI

PRISM Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems). The new sequence was added to an alignment of approximately 6000 published homologous sequences from bacteria, using the alignment tool of the software package ARB (O. Strunk & W. Ludwig; <http://www.mikro.biologie.tu-muenchen.de/pub/ARB/>). Phylogenetic analyses were performed by applying distance-matrix (ARB; Felsenstein, Jukes–Cantor), maximum-parsimony (ARB) and maximum-likelihood methods (fastDNAMl; Maidak *et al.*, 1994) as described previously (Wittke *et al.*, 1997). The data sets used for the calculations varied with respect to the reference sequences and with respect to the alignment positions included. These alternative treeing methods generated locally differing trees and the consensus tree was constructed using the results of different methods.

**Oligonucleotide probes.** On the basis of the 16S rDNA sequence, new specific probes for *in situ* hybridization were designed using the PROBE DESIGN tool of the software package ARB. Oligonucleotide probes labelled with the fluorescent dyes Cy3 and FLUOS were purchased from Interactiva.

**Cell fixation.** Cells from exponentially growing cultures were fixed with 4% (w/v) paraformaldehyde according to Amann *et al.* (1990). Cell suspensions were immobilized on micro-

scope slides by air-drying. The cells were further fixed and dehydrated by immersing the slides in 50, 80 and 96% ethanol (Amann *et al.*, 1995).

**In situ hybridization and microscopy.** *In situ* whole-cell hybridization was performed as described by Snaidr *et al.* (1997). Slides were examined with an Axioplan microscope (Zeiss) using filter sets 09 and 15.

**Probe evaluation.** *In situ* hybridization conditions for the new oligonucleotide probes were optimized by gradually increasing the formamide concentration in the hybridization solution as described by Manz *et al.* (1992).

**G + C content.** The mean G + C content (mol%) of the DNA of the new bacterial isolates was determined by using the thermal denaturation method (Johnson, 1989). The melting profiles were recorded photometrically with a programmable thermophotometer (Gilford 250) and the G + C content (mol%) was calculated using the equation of Owen & Lapage (1976).

**Estimation of nitrogen-fixing ability using *nifD* PCR-amplification.** Bacterial isolates were analysed for the presence of the *nifD* gene by using a PCR approach with universal *nifD* primers (Stoltzfus *et al.*, 1997). The reaction products were separated by agarose gel electrophoresis, stained with ethidium bromide and the band size calculated by comparison with the 100 bp ladder length standard (Gibco-BRL). Finally, the purified *nifD*-PCR product was subjected to direct sequencing by TOPLAB (Martinsried, Germany). The resulting sequence was compared with sequences in the EMBL database.

**Immunological detection of dinitrogenase reductase.** The presence of nitrogenase was detected by SDS-PAGE and Western blot analyses with a polyclonal antiserum directed to the dinitrogenase reductase of *Rhodospirillum rubrum* and *Azotobacter vinelandii* (a kind gift from P. W. Ludden, University of Wisconsin, Madison, USA). This antiserum was purified with a protein A column (Bio-Rad). Cells were grown either for 3 d in semi-solid nitrogen-free NFB medium or overnight in liquid half-strength DYGS medium supplemented with 10 mM NH<sub>4</sub>Cl, then harvested and washed with sterile PBS. The protein content was determined by the method of Harlow & Lane (1988). A quantity of bacteria, representing 1.5 µg total protein, was boiled for 10 min in SDS-PAGE sample buffer (Qiagen) and the proteins were separated by SDS-PAGE according to the method of Laemmli (1970). Electroelution on nitrocellulose sheets (Hybond ECL; Amersham) was carried out with a semi-dry electroblotter (Bio-Rad). The immunological detection was performed according to Schloter *et al.* (1995).

**Acetylene-reduction assay.** The ability to fix nitrogen was tested by using the acetylene-reduction assay. For this purpose, bacterial cultures were grown in five vials of 15 ml volumes of semi-solid NFB medium and the 23 ml vials were sealed with rubber septa; 36 h after the inoculation, a 1/10 volume (air volume) of acetylene was added (Burris, 1972). The amount of ethylene was measured every 30 min for a total of 4 h. All incubations were done at 30 °C in the dark, avoiding any movement of the vials. Ethylene was measured using a Hewlett Packard 5890A gas chromatograph equipped with a flame-ionization detector and a packed column (1.83 m long, 0.318 cm i.d., stainless steel, packed with HayeSep N; Supelco). Calculations were based on peak area. The software CHEMSTATION (Hewlett Packard) was used for data analysis. To determine the cell number, serial

dilutions of the culture were performed at the end of the incubation, plated on half-strength DYGS medium and then counted after incubation at 30 °C for 5 d.

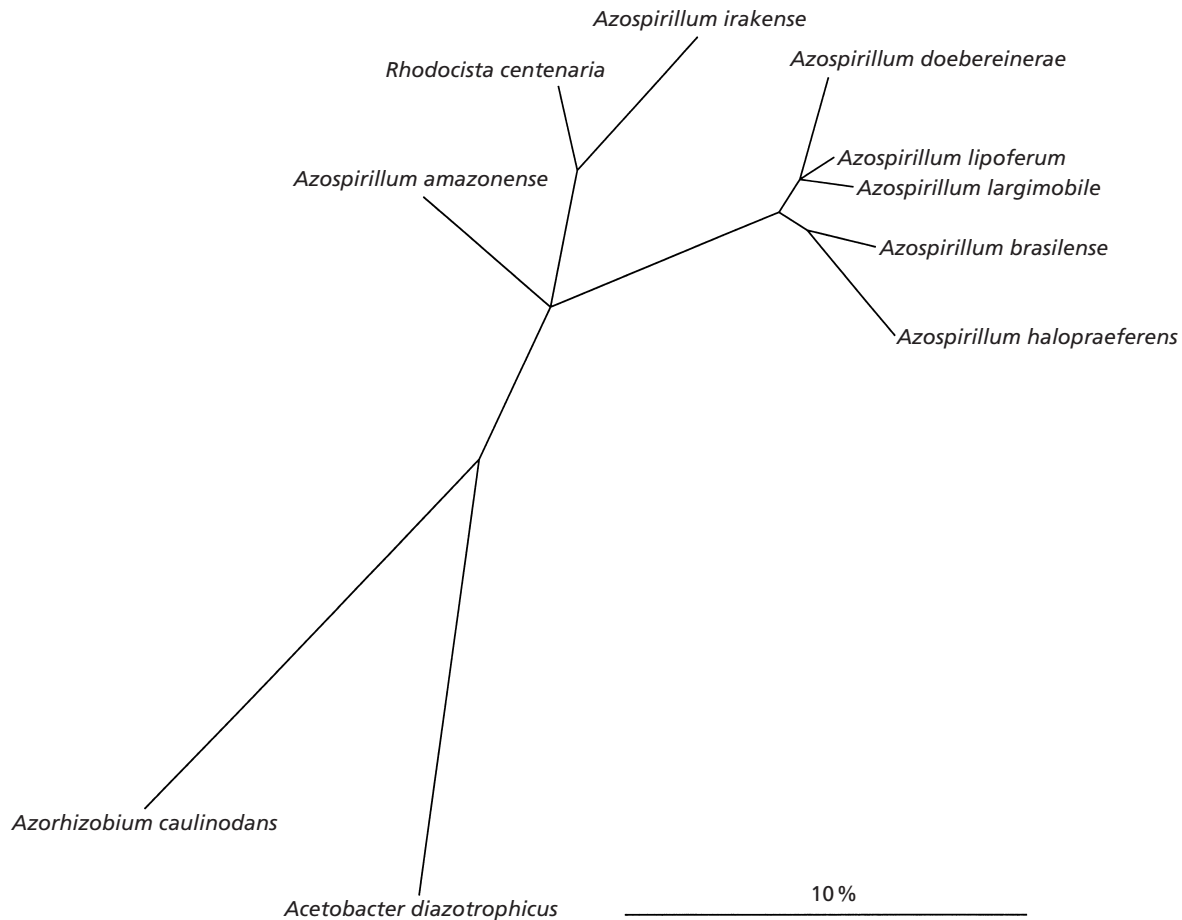
## RESULTS AND DISCUSSION

### Phylogenetic relationship

The 16S rRNA sequence analysis of *A. doebereineriae* sp. nov. GSF71<sup>T</sup> revealed that it is a member of the  $\alpha$ -subclass of the *Proteobacteria*, clustering with other *Azospirillum* species. The species *A. lipoferum*, *A. largimobile*, *A. brasilense* and *A. halopraeferens* showed the highest 16S rDNA sequence similarity with *A. doebereineriae* strain GSF71<sup>T</sup>, i.e. 96.6, 96.6, 95.9 and 93.6%, respectively. *A. irakense* and *A. amazonense* showed 90.6 and 90.2% 16S rDNA sequence similarity. The phylogenetic position of strain GSF71<sup>T</sup> within the genus *Azospirillum* is shown in Fig. 1. The phylogenetic tree is based on a combination of the results of three treeing methods, namely maximum-likelihood, maximum-parsimony and distance-matrix analyses of 50  $\alpha$ -proteobacterial 16S rDNA sequences. This approach was used to enable estimation of the robustness of the tree topology. It indicates that *A. lipoferum* and *A. largimobile* are the closest relatives of *A. doebereineriae* GSF71<sup>T</sup>. *A. brasilense* also shows a high level of sequence similarity, but the branches clearly separate. These results were confirmed by maximum-parsimony and maximum-likelihood analyses. The exact branching order of strain GSF71<sup>T</sup> with respect to its nearest neighbours (*A. largimobile* and *A. lipoferum*) in the phylogenetic tree could not be determined unambiguously, because it changed according to different tree calculation methods and different alignment positions. Therefore the branches of these three species were arbitrarily shifted in such a way that they originate in one multifurcation. The DNA G + C content of strain GSF71<sup>T</sup> is 70.7 mol%, which is in accordance with the values for the genus *Azospirillum* (64–71 mol%; Ben Dekhil *et al.*, 1997).

### Design and optimization of oligonucleotide probes specific for *A. doebereineriae* isolates

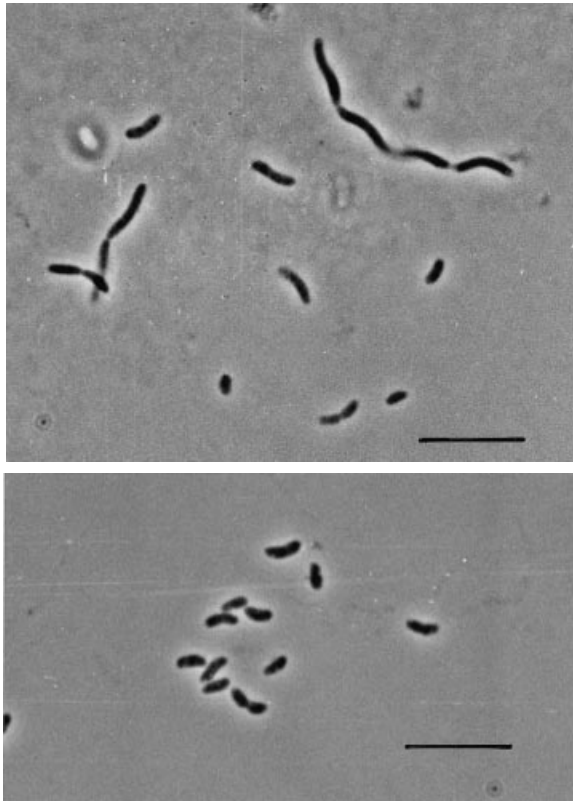
On the basis of the use of the PROBE DESIGN tool of the software package ARB, two new probes (Adoeb94 and Adoeb587) specific for the new *Azospirillum* sp. isolates were designed. Both probes are targeted to the 16S rRNA. The Adoeb587 probe has the sequence 5'-ACTTCCGACTAAACAGGC-3' at positions 587–604 and the Adoeb94 probe has the sequence (5'–3') 5'-CGTGCGCCACTGTGCCGA-3' at positions 94–111 (according to *Escherichia coli* numbering of the 16S rDNA sequence; Brosius *et al.*, 1981). Optimal hybridization conditions were evaluated by *in situ* whole-cell hybridization of reference cells with fluorescently labelled oligonucleotide probes. The formamide concentration of 30% (v/v) in the standard hybridization solution at 46 °C is recommended for both probes, because this is the highest concentration



**Fig. 1.** Phylogenetic relationship between strain GSF71<sup>T</sup> and other *Azospirillum* species and selected representatives of the  $\alpha$ -subclass of the *Proteobacteria*, based on the results of a maximum-likelihood analysis of nearly complete 16S rRNA sequences. Only sequence positions that share common residues in at least 50% of the members of the  $\alpha$ -*Proteobacteria* were included for the calculation of this tree. The tree topology was evaluated and corrected according to the results of distance and maximum-parsimony analysis. Multifurcations indicate branches for which a relative order of the branching cannot be determined unambiguously or for which a common order is not supported when different treeing methods are applied. For the sake of clarity, only a selection of the reference sequences is shown. Bar, 10% estimated base changes. Strain and accession numbers of the species shown in this tree: *A. amazonense* DSM 2787<sup>T</sup>, Z29616; *R. centenaria*, IAM 14193<sup>T</sup>, D12701; *A. irakense* DSM 11586<sup>T</sup>, Z29583; *A. doebereinae* DSM 13131<sup>T</sup>, AJ238567; *A. lipoferum* ATCC 29707, M59061; *A. largimobile* ACM 2041<sup>T</sup>, X90759; *A. brasilense* ATCC 29145<sup>T</sup>, X79739; *A. halopraeferens* DSM 3675<sup>T</sup>, X79731; *A. caulinodans*, LMG 6465<sup>T</sup>, X67221; *A. diazotrophicus* ATCC 49037<sup>T</sup>, X75618.

that still yielded good hybridization signals with the target cells and additionally allowed a discrimination of non-target cells. Oligonucleotide Adoeb587 has only a single destabilizing C–A mismatch at probe position 6 (5′–3′) in the target sequences of two *A. lipoferum* strains (DSM 1840 and DSM 1841) and *A. largimobile*. The binding of probe Adoeb587 to the 16S rDNA of these bacteria could be completely prevented by the addition of 30% formamide to the hybridization solution. Oligonucleotide Adoeb94 has at least two mismatches with respect to all other known 16S rDNA sequences and also shows bright and specific hybridization signals with a formamide concentration of 30% in the hybridization solution. The two new probes, together with probe EUB338, which is specific for all *Eubacteria* (Amann *et al.*, 1990), were suc-

cessfully used for a fast, whole-cell, *in situ* characterization of bacterial isolates. All strains, except the new ones belonging to *A. doebereinae* sp. nov. (GSF21, GSF65, GSF66, GSF67, GSF68, GSF69, GSF70, GSF71<sup>T</sup>, GSF76, GSFe, Ma4), were negative for hybridization with Adoeb94 or Adoeb587, and all strains were positive for hybridization with EUB338. After screening of nearly all of the *Azospirillum* isolates from *Miscanthus* (Kirchhof *et al.*, 1997a), it was found that a single previous isolate, GSF21, from *Miscanthus sacchariflorus*, could also be assigned to the new group. This affiliation could be confirmed by carbon-utilization pattern analysis. The combined application of both probes offers the possibility for *in situ* identification of this new group of *Azospirillum* strains in environmental samples. Combined application of



**Fig. 2.** (top) Micrograph of pleomorphic cells of *A. doebereinae* sp. nov. strain GSF71<sup>T</sup> grown for 5 d in semi-solid Nfb medium. (bottom) Micrograph of uniform small cells of *A. doebereinae* sp. nov. strain GSF71<sup>T</sup> grown in half-strength DYGS-medium liquid culture overnight. Bar, 10 µm.

both probes with the same specificity ensures reliable identification of the species by double staining.

#### Cell morphology and growth characteristics

Cells of *A. doebereinae* sp. nov. isolates are curved rods or S-shaped, 1.0–1.5 µm wide and 2.0–30 µm long. After growth overnight in liquid medium cells are small and vibroid (Fig. 2, bottom), whereas after prolonged growth in semi-solid N-free Nfb medium the cells are long and pleomorphic (Fig. 2, top). Accordingly, the movement of these Gram-negative cells is winding or snake-like. A single polar flagellum was observed when cells were grown in liquid medium. Ageing cells contained intracellular granules (probably poly-β-hydroxybutyrate). Cells were not encapsulated at the conditions tested. On Nfb agar plates supplemented with 20 mg yeast extract and triple-strength bromothymol blue, the colonies were 0.5 mm in diameter and appeared grey and dull. On half-strength DYGS agar plates, the bacteria formed small adhesive colonies. Colonies grown on Congo red medium were scarlet. In semi-solid nitrogen-free malate medium, they formed a veil-like pellicle that first developed several millimetres below the medium surface and later moved close to the surface.

#### Phenotypic features

The new *Azospirillum* sp. isolates were chemoorganoheterotrophic and exhibited a respiratory type of metabolism with oxygen as the terminal electron acceptor. Nitrate could be used for denitrification. N<sub>2</sub> could be fixed at microaerobic conditions. Oxidase and urease tests were positive. Biotin was not required for growth. The optimum temperature for growth was tested at 25, 30, 37 and 42 °C. The best growth was observed at 30 °C and no growth occurred at 37 or 42 °C. Of the range of pH values tested (3.0–10.5), the best growth occurred at pH 6.0–7.0. Growth did not occur in the presence of 3% NaCl. Aesculin was hydrolysed.

The API20 NE system showed that arabinose, gluconate, malate and mannitol were utilized in 1–3 d by 90–100% of the tested strains. The tests for β-glucosidase, urease and β-galactosidase were positive. The API50 CHE test performed under aerobic cultivation showed that glycerol, D-mannitol, D-sorbitol, D-fructose and aesculin were positive for more than 90% of the tested strains within 1–6 d incubation. When tested with the remaining substrates in the API20 NE and API50 CHE tests, after 6 d more than 90% of the strains tested failed to utilize the following as sole sources of carbon and energy: adonitol, N-acetylglucosamine, starch, amygdalin, D-arabinose, D-arabitol, L-arabitol, arbutin, caprate, cellobiose, 5-keto-gluconate, 2-keto-gluconate, citrate, dulcitol, erythritol, L-fructose, β-gentiobiose, glycogen, inositol, inulin, lactose, D-lyxose, maltose, mannose, D-mannose, melezitose, melibiose, methyl α-D-glucoside, methyl α-D-mannoside, methyl β-xyloside, phenylacetate, D-raffinose, rhamnose, ribose, saccharose, salicine, L-sorbose, D-tagatose, trehalose, D-turanose, xylitol, D-xylose and L-xylose. Occasional and varying growth occurred after 6 d on adipate, galactose, D-glucose and gluconate. Microaerobic N<sub>2</sub>-fixing-dependent growth was observed with the carbon sources malate, sorbitol, mannitol and glycerol. No growth occurred with maltose, sucrose, ribose or inositol. Growth with glucose in semi-solid nitrogen-free media was not uniform. With fructose as the substrate, the pellicles were very weak. A summary of the results of carbon-source utilization suitable for the differentiation of *A. doebereinae* sp. nov. from the known *Azospirillum* species is presented in Table 2.

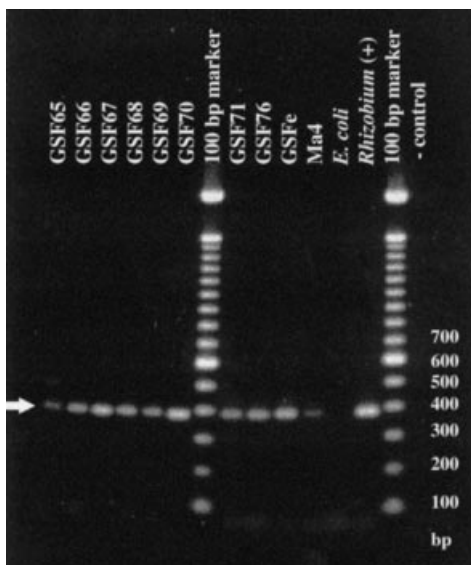
#### Nitrogen-fixing ability

All *Azospirillum* sp. strains were isolated from the roots of *Miscanthus* by using semi-solid nitrogen-free media. They did not lose the capacity to grow with N<sub>2</sub> under microaerobic conditions, even after repeated transfers on complex media. The N<sub>2</sub>-fixation ability could be proved by a multidisciplinary approach. All strains were subjected to a *nifD*-specific PCR amplification. The expected 390 bp amplification product (Stoltzfus *et al.*, 1997) was observed with all strains

**Table 2.** Physiological differences between *A. doebereinae* sp. nov. isolates and the other *Azospirillum* species

Data were compiled from Magalhães *et al.* (1983), Falk *et al.* (1986), Reinhold *et al.* (1987), Khammas *et al.* (1989), Ben Dekhil *et al.* (1997) and this study. –, Present in 10% or less of the strains; +, present in 90% or more of the strains; d, 11–89% of all strains positive; ND, not defined.

Phenotypic feature	<i>A. doebereinae</i> sp. nov.	<i>A. lipoferum</i>	<i>A. largimobile</i>	<i>A. brasilense</i>	<i>A. halopraeferens</i>	<i>A. irakense</i>	<i>A. amazonense</i>
Carbon utilization with API50							
CHE and API20 NE (aerobic):							
N-Acetylglucosamine	–	+	+	–	ND	+	d
D-Glucose	d	+	+	d	–	+	+
Glycerol	+	+	+	+	+	–	–
D-Mannitol	+	+	+	–	+	–	–
D-Ribose	–	+	+	–	+	d	+
D-Sorbitol	+	+	+	–	–	–	–
Sucrose	–	–	ND	–	–	+	+
Acid from (API50, anaerobic):							
D-Glucose	d	+	+	–	–	–	–
D-Fructose	+	+	+	–	+	–	–
Biotin requirement	–	+	–	–	+	–	–
Optimal temperature for growth (°C)	30	37	28	37	41	33	35
Polymorphic cells	+	+	+	–	+	+	+



**Fig. 3.** PCR products derived from specific amplification of the *nifD* gene.

tested (Fig. 3). In the case of strain GSF71<sup>T</sup>, this PCR product was purified and sequenced. The comparison of the resulting sequence with the EMBL database revealed a similarity with the *nifD* gene of *A. brasilense* of 90% (Passaglia *et al.*, 1991). The resulting amino acid sequence had 89% similarity to the nitrogenase molybdenum–iron protein  $\alpha$  chain (nitrogenase component I, dinitrogenase, *nifD* protein) of *A. brasilense*.

The expression of nitrogenase was proved by immunodetection of blotted cell extracts of strains GSF71<sup>T</sup>, GSF65, Ma4 and *A. brasilense* Wa3 (positive control) grown under nitrogen-fixing conditions, using an antiserum against the dinitrogenase reductase of *R. rubrum* and *A. vinelandii*. When 1.5  $\mu$ g total protein of

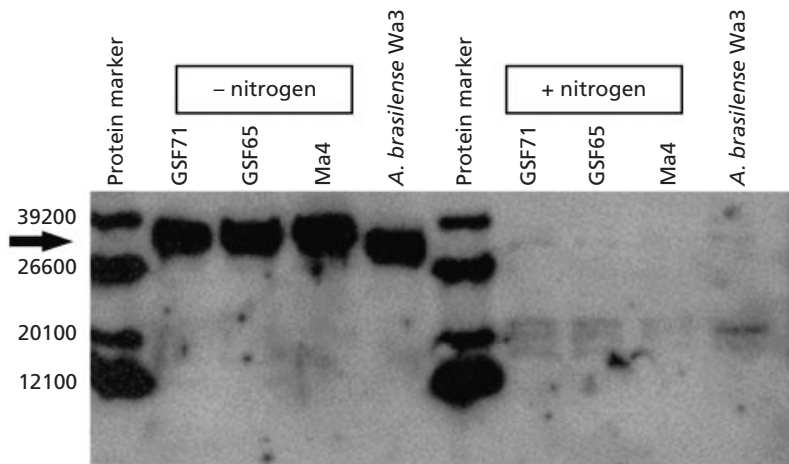
the cell extract was used, the protein A-purified antiserum reacted with a double band of approximately 31–35 kDa, if the bacteria were grown under dinitrogen-dependent conditions (Fig. 4). The dinitrogenase reductase of strains GSF71<sup>T</sup>, GSF65 and Ma4 was slightly bigger in size than that of *A. brasilense* Wa3 (Hartmann *et al.*, 1986). In the case of half-strength DYGS medium supplemented with 10 mM NH<sub>4</sub>Cl, no protein bands appeared at the 31–35 kDa position after Western blotting analyses, though three very faint bands appeared at the 21–15 kDa position.

The activity of the nitrogenase was investigated by using an acetylene-reduction assay in nitrogen-free semi-solid NFb medium. Strain GSF71<sup>T</sup> was able to reduce acetylene to ethylene with a mean ratio of approximately 100 nmol ethylene per hour per 10<sup>8</sup> cells at 30 °C without any addition of yeast extract. This amount is comparable with the values of other *Azospirillum* species (Reinhold *et al.*, 1987; Magalhães *et al.*, 1983).

All these results prove the diazotrophic character of the new *A. doebereinae* isolates.

### Comparison with species belonging to the genus *Azospirillum*

The above-mentioned properties of *A. doebereinae* isolates from *Miscanthus* matched with the general characteristics of the genus *Azospirillum*. However, they differed from their nearest relatives in some physiological properties. In contrast to *A. largimobile* and *A. lipoferum*, they were unable to grow with N-acetylglucosamine and D-ribose. *A. doebereinae* strains did not need biotin supplement as compared to *A. lipoferum* and *A. halopraeferens* and they grew with D-mannitol and D-sorbitol as sole carbon sources unlike *A. brasilense*. Unlike *A. halopraeferens*, *A. doebereinae* was able to use D-sorbitol but was unable to use D-ribose (Table 2). The analysis of the 16S



**Fig. 4.** Immunodetection of the nitrogenase reductase of *A. doebereinae* sp. nov. strains GSF71<sup>T</sup>, GSF65 and Ma4 and *A. brasilense* strain Wa3 with a specific polyclonal antiserum against nitrogenase reductase (a gift from Professor P.W. Ludden, Madison, WI, USA). The bacterial cells were grown under nitrogen-fixing conditions in semi-solid NFb medium (– nitrogen) and (as the non-nitrogen-fixing control) in media with 10 mM ammonium (+ nitrogen).

rDNA sequence showed that strain GSF71<sup>T</sup> is phylogenetically related to *A. lipoferum*, *A. largimobile* and *A. brasilense* (95.9–96.6% 16S rDNA sequence homology). Sequence differences of more than 3% are sufficient to allow the proposal of a new species (Stackebrandt & Goebel, 1994). The group of new isolates from *Miscanthus* roots appeared homogeneous in their physiological properties and oligonucleotide probing. The application of discriminatory physiological tests and oligonucleotide probes Adoeb94 and Adoeb587 to whole-cell hybridization is appropriate for differentiating and identifying *A. doebereinae* isolates.

#### Description of *Azospirillum doebereinae* sp. nov.

*Azospirillum doebereinae* (doeb'ér.ein.er.ae. N.L. gen. fem. n. *doebereinae* of Döbereiner, in honour of Johanna Döbereiner, who isolated and characterized many *Azospirillum* spp. and other diazotrophic plant-associated bacteria and greatly stimulated the whole field of rhizosphere microbiology, plant-associated nitrogen fixation and plant growth promotion by root-colonizing diazotrophs).

The bacteria are Gram-negative curved rods or S-shaped, 1.0–1.5 µm in width, 2.0–30 µm in length (longer cells occur especially in alkaline semi-solid NFb or JNFb media) and motile with a winding or snake-like movement. A single polar flagellum is observed when the cells are grown in liquid medium. Ageing cells contain intracellular granules. Cells are not encapsulated. The optimum growth temperature is 30 °C and no growth occurs at 37 °C. The best growth is observed at pH values between 6.0 and 7.0. The metabolism is of a typical respiratory nature; arabinose, D-fructose, gluconate, glycerol, malate, mannitol and sorbitol can be used as carbon sources. Dinitrogen fixation occurs in microaerobic nitrogen-limited conditions. Microaerobic dinitrogen-fixing-dependent growth occurs with malate, sorbitol, mannitol, fructose and glycerol. Catalase, oxidase and urease activities are positive and gelatin is not hydrolysed. NO<sub>3</sub><sup>-</sup> is reduced to NO<sub>2</sub><sup>-</sup>. The G+C content of

the DNA is 69.6 ± 3 mol%. The most closely related species, according to 16S rRNA sequence data, are *Azospirillum lipoferum*, *Azospirillum largimobile* and *Azospirillum brasilense*. The type strain is GSF71<sup>T</sup> (= DSM 13131<sup>T</sup>) and its G+C content is 70.7 mol%. The bacterium is associated – as far as is known – with the roots of *Miscanthus sinensis* cv. 'Giganteus' and *Miscanthus sacchariflorus* and occurs also in the rhizosphere soil of these plants.

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