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Herbaspirillum frisingense sp. nov., a new nitrogen-fixing bacterial species that occurs in C4-fibre plants

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The enrichment of nitrogen-fixing bacteria from the C4-fibre plants, *Spartina pectinata*, *Miscanthus sinensis*, *Miscanthus sacchariflorus* and *Pennisetum purpureum*, with nitrogen-free semi-solid media led to the isolation of *Herbaspirillum*-like strains among other diazotrophic bacteria. On the basis of physiological properties, phylogenetic analysis comparing 16S rDNA sequences and DNA–DNA hybridization experiments of chromosomal DNA the new isolates could be grouped together in a new species with the proposed name *Herbaspirillum frisingense* sp. nov. Morphological characteristics, such as cell size and shape, colony appearance, motility and flagellation are largely identical to the known species *Herbaspirillum rubrisubalbicans* and *Herbaspirillum seropedicae*. On the basis of utilization of adipate (−), *N*-acetyl-D-glucosamine (+), *meso*-erythritol (−), L-rhamnose (−) and *meso*-inositol (−) *Herbaspirillum frisingense* sp. nov. can be distinguished from other known *Herbaspirillum* spp. Nitrogen-fixing capability was examined by PCR amplification of the *nifD* gene and an acetylene reduction assay, and was found with all isolates tested. 16S rDNA sequence similarity to the other *Herbaspirillum* spp. is 98·5–99·1%. In genomic DNA–DNA hybridization experiments *Herbaspirillum frisingense* sp. nov. forms a homogeneous group with 70–100±10% similarity, clearly distinct from *Herbaspirillum seropedicae* and *Herbaspirillum rubrisubalbicans* with 1–34% similarity. 16S rRNA-targeted oligonucleotide probes, specific for the whole genus *Herbaspirillum* and for three *Herbaspirillum* species were designed and are suitable for fluorescence *in situ* hybridization. The DNA G+C content of *Herbaspirillum frisingense* sp. nov. is 63±2 mol%, in agreement with the values of 61–65% for the genus. PCR fingerprinting exhibits a consistent pattern for groups of strains isolated from the same plant, suggesting a low genomic diversity among bacteria inhabiting C4-gramineous plant tissues. Low genetic DNA diversity seems to be common between probable endophytic bacterial isolates of the same taxon. The type strain of *Herbaspirillum frisingense* sp. nov. is GSF30^T (= DSM 13128^T).

Keywords: *Herbaspirillum* spp., *Miscanthus* spp., *Pennisetum purpureum*, *Spartina pectinata*, diazotrophic bacteria

Abbreviations: LINEs, long interspersed elements; TEM, transmission electron microscopy.

The EMBL accession numbers for the 16S rDNA sequences of *Herbaspirillum rubrisubalbicans* LMG 2286^T, *Herbaspirillum seropedicae* LMG 6513^T, *Herbaspirillum frisingense* sp. nov. 75B, *Herbaspirillum frisingense* sp. nov. GSF30^T and *Herbaspirillum frisingense* sp. nov. Mb11 are AJ238356, AJ238361, AJ238357, AJ238358 and AJ238359, respectively.

INTRODUCTION

The genus *Herbaspirillum* was first reported by Baldani *et al.* (1986) as a nitrogen-fixing bacterium associated with the roots of rice (*Oryza sativa*), maize (*Zea mays*) and sorghum (*Sorghum bicolor*). At this stage of investigation the genus only consisted of one species, *Herbaspirillum seropedicae*. Later on, the mild plant pathogen in sugarcane, formerly named *Pseudomonas rubrisubalbicans*, was reclassified as *Herbaspirillum rubrisubalbicans* (Baldani *et al.*, 1996). Both species were demonstrated to occur in roots and stems of sugarcane, sorghum and rice, and found to be able to fix nitrogen (Pimentel *et al.*, 1991; Ureta *et al.*, 1995). *Herbaspirillum seropedicae* was also found in other gramineous plants (Baldani *et al.*, 1992; Olivares *et al.*, 1996). A third species, *Herbaspirillum* species 3, was classified on the basis of DNA–rRNA and DNA–DNA hybridization experiments (Gillis *et al.*, 1990; Baldani *et al.*, 1996). These isolates are of very different, partly clinical origin and unable to fix nitrogen. *Herbaspirillum rubrisubalbicans* is capable of colonizing the vascular system of sorghum and sugarcane, causing light disease symptoms depending on the host plant variety (Olivares *et al.*, 1997; James *et al.*, 1997). The observation that both species are not able to survive well in uncropped soils (Baldani *et al.*, 1992), but found in surface sterilized plant tissues in substantial numbers, led to the conclusion that these *Herbaspirillum* spp. are endophytes (James & Olivares, 1997).

Some varieties of sugarcane exhibit a nitrogen contribution of 40–60 % from biological nitrogen fixation (Urquiaga *et al.*, 1992; Boddey *et al.*, 1995; Yoneyama *et al.*, 1997). For other crops, like sorghum (Giller *et al.*, 1986), maize (Boddey, 1987) and rice (Baldani *et al.*, 1995), a biological fixed nitrogen input of 20–30 % was shown. It is still not proven how nitrogen-fixing competence of bacterial endophytes contributes this essential nutrient to plant metabolism and therefore benefits plant growth (James & Olivares, 1997). Also, other mechanisms, probably phytohormone production (Bastian *et al.*, 1998), could be responsible for the plant growth promotion, resulting in higher yields of plant material (Baldani *et al.*, 1995). The enormous biomass production of C4-grasses like *Miscanthus* spp., *Pennisetum purpureum* and *Spartina pectinata*, which are cultivated for fibre production and for renewable energy resources (Hotz, 1993; Klug & Orth, 1997), combined with relatively low nitrogen requirements (Hotz *et al.*, 1993) encouraged us to investigate these plants for associations with nitrogen-fixing bacteria. The application of nitrogen-free semi-solid media (Döbereiner, 1995) led to the isolation of various nitrogen-fixing bacteria. One group exhibited characteristics of *Herbaspirillum* (Kirchhof *et al.*, 1997). These isolates were subjected to further taxonomical analyses and turned out to represent a new *Herbaspirillum* species, distinct from other already described species of *Herbaspirillum*.

METHODS

Strain isolation. Washed roots and aerial parts (stems, leaves) of *Miscanthus sinensis* cv. Giganteus, *Miscanthus sacchariflorus*, *Spartina pectinata* grown in Freising, Germany, and *Pennisetum purpureum* cv. Cameroon, Cana d'Africa, Gramafante, Guaçu, Merker, Merker × 239, Mineiro, Mott, Piracicaba, Roxo, s/pelo and Taiwan grown in Brazil, were macerated. Serial dilutions in 4 % (w/v) sucrose solution down to 10^{-6} were inoculated into vials containing NFb or JNFb semi-solid nitrogen-free media, according to Döbereiner (1995). After 4–6 d incubation at 30 °C pellicle-forming bacteria were subjected to further purification steps by streaking on NFb and JNFb agar plates containing an additional 20 mg yeast extract l^{-1} and single colonies were again transferred for cultivation in the appropriate semi-solid nitrogen-free medium. Strain designation of the isolates and plant tissue sources are listed in Table 1.

Phenotypic characterization. Morphological characteristics were investigated by phase-contrast and transmission electron microscopy (TEM). The type of flagellation was determined by electron microscopy using preparations negatively stained with uranyl acetate. Colony morphology, pigmentation and motility were tested on solid yeast-extract-supplemented (0·05 %) NFb medium, containing 6 ml 0·5 % bromthymolblue indicator solution l^{-1} (Baldani *et al.*, 1986). Swarming behaviour was observed in NB soft agar (0·8 % agar) by measuring the halo diameter of a centred loopful of inoculum from an NFb liquid overnight culture after 5 d at 35 °C. The pH growth optimum was tested using semi-solid JNFb medium with the pH adjusted to the required value after autoclaving. An inoculum of 20 µl of a cell culture pregrown in modified DYGS medium (Rodrigues Neto *et al.*, 1986), named 1/2 DYGS (g l^{-1}: dextrose, 1·0; malate, 1·0; peptone, 1·5; yeast extract, 2·0; $MgSO_4 . 7H_2O$, 0·5; L-glutamic acid, 1·5; pH 6·0), was added to each flask and acetylene reduction tests were performed after 72 h incubation at 30 °C. The optimum growth temperature was evaluated in liquid 1/2 DYGS medium.

Physiological properties were screened with the API20NE and API50CHE galleries (bioMérieux) and the BIOLOG-GN MicroPlate carbon source metabolization pattern plates, according to the manufacturers' instructions.

Pellicle-forming capability (microaerophilic, dinitrogen-fixation-dependent growth) with different carbon sources was analysed for selected strains. Therefore, the malate component in semi-solid JNFb medium was replaced by *N*-acetyl-D-glucosamine, L-arabinose, D-fructose, D-glucose, *meso*-inositol, L-rhamnose, malate, *meso*-erythritol and L-tartrate, respectively. Three replicates of each medium were inoculated with 10 µl of a washed overnight culture in 1/2 DYGS medium and incubated at 30 °C over a period of 3–4 d.

Evidence of nitrogen-fixing capacity estimated by acetylene reduction and *nifD* amplification. The capability of the isolated *Herbaspirillum* strains to fix atmospheric nitrogen was tested with the classical acetylene reduction assay (Burris, 1972). For this purpose strain Mb11 was grown in five parallels in a 15 ml volume of semi-solid nitrogen-free NFb medium. The 23 ml vials were closed by rubber septa and 0·1 vols (air volume) acetylene was added 36 h after the inoculation. The amount of ethylene was measured every 30 min over a period 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

Table 1. Origin of the bacterial strains

Spartina pectinata and *Miscanthus* spp. plants were grown in Freising, Germany, while *Pennisetum purpureum* was cultivated in Seropédica, Brazil. DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Isolate	Plant	Tissue
Herbaspirillum frisingense sp. nov.		
GSF3	*S. pectinata*	Washed roots
GSF36	*S. pectinata*	Washed stems
GSF20	*M. sinensis* cv. Giganteus	Washed stems
GSF28	*M. sacchariflorus*	Washed roots
GSF22, 24, 27, 35	*M. sacchariflorus*	Washed stems
GSF23, 25, 30^T (= DSM 13128^T), 34	*M. sacchariflorus*	Washed leaves
Mb1, 9, 10, 11 (= DSM 13130), 13, 14, 16, 19, 20, 22	*M. sacchariflorus*	Washed roots
70B, 74B, 96B	*P. purpureum* cv. Cana Africa	Washed roots
37	*P. purpureum* cv. Gramafante	Washed stems
61B	*P. purpureum* cv. Guaçu	Washed stems
114B	*P. purpureum* cv. Guaçu	Washed roots
72B	*P. purpureum* cv. Merker	Washed stems
65H, 73B, 90B, 101H, 121B	*P. purpureum* cv. Merker × 239	Washed roots
11B	*P. purpureum* cv. Mineiro	Washed stems
75B, 118B	*P. purpureum* cv. Mineiro	Washed roots
112B, 115B	*P. purpureum* cv. Mott	Washed stems
106B	*P. purpureum* cv. Mott	Washed roots
60B, 69B, 122B	*P. purpureum* cv. Piracicaba	Washed stems
78B	*P. purpureum* cv. Piracicaba	Washed roots
36B, 41B	*P. purpureum* cv. Roxo	Washed stems
48H	*P. purpureum* cv. s/pelo	Washed stems
95B	*P. purpureum* cv. s/pelo	Washed roots
84B (= DSM 13129), 103H, 113B	*P. purpureum* cv. Taiwan	Washed stems
67B, 117B	*P. purpureum* cv. Taiwan	Washed roots
Herbaspirillum seropedicae		
16B	*P. purpureum* cv. Merker	Washed roots
80B	*P. purpureum* cv. Cameroon	Washed roots
81B	*P. purpureum* cv. Roxo	Washed roots
91B	*P. purpureum* cv. Piracicaba	Washed roots

chromatograph equipped with a flame-ionization detector and a prepacked column (HayeSep N; Supelco). Calculations were based on peak area and the software CHEMSTATION (Hewlett Packard) was used for data analysis. For the determination of cell number, serial dilutions of the homogenized semi-solid culture were stroked on 1/2 DYGS media and counted after incubation at 30 °C for 5 d.

Additionally, a selection of isolates was inspected for the presence of the *nifD* gene by PCR with primers and reaction conditions according to 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 a 100 bp ladder standard (Gibco-BRL).

Genomic DNA–DNA hybridization. High-molecular-mass DNA was prepared following the method of Mayer & Schleifer (1978), omitting the CTAB precipitation step.

Duplicate samples of 1 and 10 μg high-molecular-mass DNA of each investigated strain were denatured by alkaline incubation (200 μl 0·4 M NaOH, 20 min, room temperature) and immobilized on an uncharged nylon membrane (Quiabrane Nylon; Diagen) using a dot-blot vacuum unit (Schleicher & Schuell). After drying the filters they were baked for 20 min at 80 °C. For DNA–DNA hybridization, the method of Tjernberg *et al.* (1989) was followed with modifications. The respective probe DNA (0·3 μg) was labelled with [α^{-32}P]dCTP by nick translation, according to the kit manufacturer's instructions (Gibco-BRL). The filters were preincubated in 10 ml of a solution consisting of 35% (v/v) formamide, 2× SSC, 5× Denhardt's solution, 1% glycine, 1·5 mg calf thymus DNA ml^{-1} (3 × 20 s sonicated) for 6 h at the hybridization temperature. The temperature was chosen at 25 °C below the thermal denaturation midpoint ($T_{M(2\times SSC)}$) of the probe DNA. For herbaspirilla with a minimum 61 mol% G+C content the $T_{M(2\times SSC)}$ is 98·4 °C. Therefore, in our experiments a standard hybridization temperature of 53 °C was chosen and the required stringent reaction conditions corrected with 30% (v/v) formamide (−0·7 °C/1% formamide). For the hybridization solution 0·1 μg labelled probe DNA and 700 μg calf thymus DNA were mixed with 1·5 ml formamide and denatured for 15 min at room temperature. Then, the following components were added to the required final concentration: 2× SSC, 1× Denhardt's solution and 0·3% SDS, made up to a final volume of 5 ml with H_2O. The preincubation solution was replaced by 5 ml hybridization solution. After hybridization overnight at 53 °C the membranes were washed 2–3 times

for 10–15 min with 15 ml of a solution containing 2 × SSC/0·1 % SDS and 3–4 times for 10–30 min with another solution containing 0·1 × SSC/0·1 % SDS at the hybridization temperature. Finally, the liquid was drained off the filters, they were sealed in a plastic wrap and exposed to X-ray film in the presence of intensifying screens for 3–72 h at −100 °C.

Autoradiographic signals were quantified densitometrically from flat bed scans with interpretative software (RFLP-Scan; Scanalytics). The percentage binding of heterologous DNA was correlated with 100 % binding of homologous DNA after consideration of the amount of filter-bound DNA [calculated densitometrically after hybridization with an oligonucleotide mix directed to universal, eubacterial rDNA sequences (Lee *et al*., 1993)]. In this case hybridization was carried out at 50 °C, according to the procedure described by Kirchhof & Hartmann (1992). The values were background-corrected and the means of the duplicates were calculated.

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

Phylogenetic analysis based on 16S rDNA sequences. 16S rDNA sequences were determined commercially by DSMZ, Braunschweig, Germany. Sequences of 1465–1485 bp of the selected strains and the type strains *Herbaspirillum seropedicae* (LMG 6513^T) and *Herbaspirillum rubrisubalbicans* (LMG 2286^T) were amplified by PCR (Rainey *et al*., 1996) and the purified products were subjected to direct sequencing using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The resulting sequences were added to an alignment of about 6000 published homologous sequences from bacteria using the alignment tool of the software package ARB (http://www.mikro.biologie.tu-muenchen.de). Phylogenetic analysis was performed by applying distance matrix (ARB, Felsenstein, Jukes–Cantor), maximum-parsimony (ARB) and maximum-likelihood methods (fastDNAml; Maidak *et al*., 1996) as described by Wittke *et al*. (1997) on different data sets.

Oligonucleotide probes. To design diagnostic genus- and species-specific oligonucleotide probes, 16S rDNA sequence data was used. The aligned sequences were screened for unique base compositions specific for each species and the genus by using the PROBE DESIGN tool of the ARB software package. Additionally, the strains were analysed in hybridization experiments with 23S rDNA domain III targeting probes (Baldani *et al*., 1996) following the method described by Kirchhof & Hartmann (1992). Stringent hybridization conditions for the 23S rDNA targeting probes were tested in dot-blot hybridization experiments by utilizing various hybridization temperatures. *In situ* hybridization conditions for the new 16S rDNA targeting oligonucleotide probes were optimized by gradually increasing the formamide concentration in the hybridization buffer as described by Manz *et al*. (1992), including closely related reference strains of *β-Proteobacteria* in the tests.

Strain analysis by PCR fingerprinting. The genomic diversity of the isolates was examined in arbitrary PCR fingerprint analysis, applying a repetitive mammalian sequence (LINEs, *l*ong *in*terspersed *e*lements; Smida *et al*., 1996) derived primer GRK (5′-GAG TTT GGC AAA GAC CC-3′) (Kirchhof *et al*., 1997). The resulting banding patterns were displayed on a 1·5 % agarose gel, visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

Morphological characterization

During the isolation of nitrogen-fixing bacteria from *Miscanthus* spp. and *Spartina pectinata* grown in Germany, and *Pennisetum purpureum* cultivars grown in Brazil, 57 bacterial strains forming veil-like pellicles in semi-solid NFb and JNFb media, characteristic for nitrogen-fixing bacteria, were found. Four isolates could be identified as *Herbaspirillum seropedicae* (Kirchhof *et al*., 1997). The other isolates exhibited a white colony morphology with blue-green centres on NFb agar plates (with 6 ml 0·5 % bromthymolblue solution l^{-1} added). This coloured centre was not highly marked compared to known members of the genus *Herbaspirillum*. Light microscopy inspection revealed the bacterial cells to be Gram-negative, motile, thin rods. TEM investigations demonstrated a spiral cell shape with one to three, mostly two, unipolar flagella (Fig. 1). The cells were 0·5–0·7 μm in diameter and 1·4–1·8 μm long. Swarming could be observed on soft NB agar plates.

Physiological properties

In accordance with the genus description (Baldani *et al*., 1986), all *Herbaspirillum*-like isolates investigated showed catalase, oxidase and urease activity. Gelatin was not hydrolysed. NO_3^- was reduced to NO_2^- but not to N_2 by all (selection of 10) isolates tested. Using the BIOLOG-GN plate system (22 selected strains), the strains exhibited a typical respiratory metabolism with a broad spectrum of sugars and alcohols: adonitol, L-arabinose, D-fructose, L-fucose, galactose, D-glucose, glycerol, mannitol, mannose, ribose, sorbitol, D-xylose and xylitol were oxidized. Di- and trisaccharides, e.g. cellobiose, lactose, maltose, sucrose, melibiose, trehalose and raffinose, were not metabolized. Organic acids such as acetate, *cis*-aconitate, caprate, citrate, formate, galacturonate, gluconate, α-hydroxybutyrate, *p*-hydroxyphenylacetate, itaconate, α-ketobutyrate, α-ketoglutarate, DL-lactate, malate, malonate, propionate, phenylacetate, D-saccharate, succinate and succinamic acid were metabolized by the majority (> 80 %) of all tested strains. Fatty acid oxidation, tested with Tween 40 and 80, was positive. The amino acids D- and L-alanine, L-asparagine, L-aspartate, L-glutamate, L-leucine, L-proline and L-pyroglutamate were also metabolized.

The reaction pattern concerning species-differentiating substrates (Baldani *et al*., 1996) as tested by API20NE (10 strains) and API50CHE galleries (four strains) are listed in Table 2. With respect to *meso*-erythritol, L-rhamnose, *meso*-inositol, *N*-acetyl-D-glucosamine and adipate the new species showed a distinct pattern compared to the other *Herbaspirillum* species.

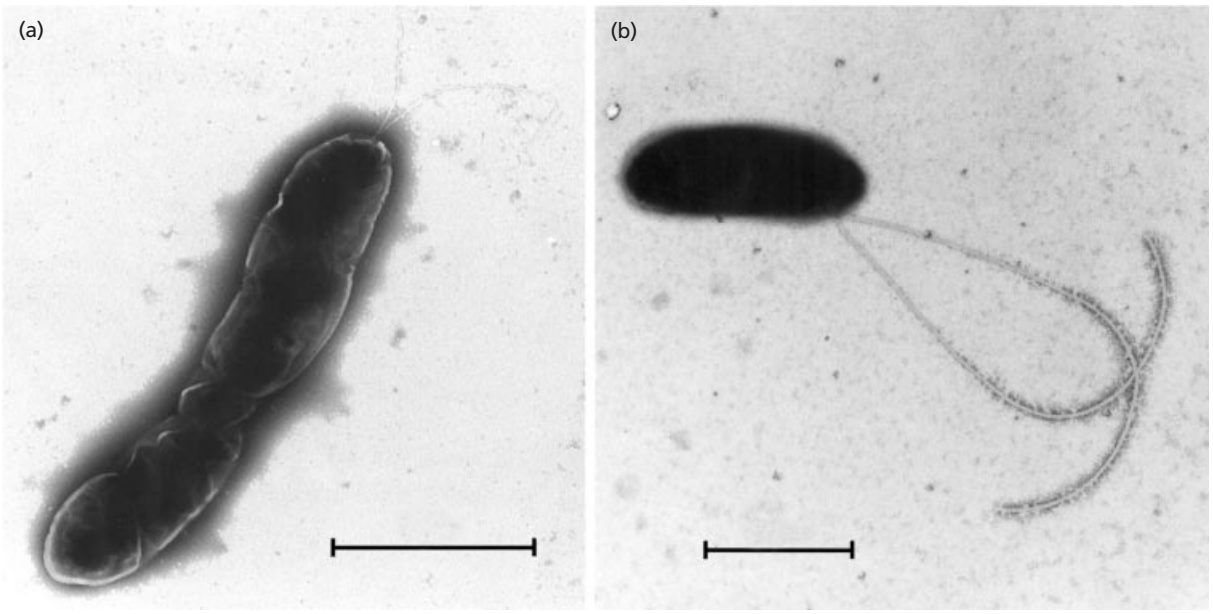

Fig. 1. Transmission electron micrographs of *Herbaspirillum frisingense* sp. nov. Mb11 cells grown in 1/2 DYGS medium. (a) Dividing cell; (b) cell with two unipolar flagella. Bars, 1 μm.

Table 2. Characteristics useful for the identification of bacteria belonging to the genus *Herbaspirillum* and for differentiating *Herbaspirillum frisingense* sp. nov. from the other known species

Specifications for *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans* and *Herbaspirillum* sp. 3 are adapted from Baldani *et al*. (1986, 1996) and Gillis *et al*. (1990). All species were positive for oxidase, catalase and metabolism of sugars, alcohols, carbon acids, amino acids. All species were Gram-negative and negative for metabolism of di- and trisaccharides. ND, Not determined.

Characteristic	*Herbaspirillum seropedicae*	*Herbaspirillum rubrisubalbicans*	*Herbaspirillum frisingense*	*Herbaspirillum* sp.	
				3a	3b
Morphology/mobility					
Cell dimensions (μm)	1·5–5·0 × 0·6–0·7	1·5–5·0 × 0·6–0·7	1·4–1·8 × 0·5–0·7	ND	ND
Flagellation	1–3; bipolar	Several; bipolar	1–3; unipolar	ND	ND
Swarming on soft NB agar	+	+	+	ND	ND
Physiological properties					
Optimal growth temperature (°C)	34	30	30–37	ND	ND
Optimal growth pH	5·3–8·0	5·7–6·8	6·0–7·0	ND	ND
N_2 fixation	+	+	+	−	−
Utilization of carbon sources					
N-Acetyl-D-glucosamine	+	−	+	−	+
meso-Inositol	+	−	−	−	−
L-Rhamnose	+	−	−	−	−
meso-Erythritol	−	+	−	−	−
Adipate	−	−	−	+	+
DNA G+C content (mol%)	64–65	62–63	61–65	ND	61

Microaerophilic growth without a nitrogen component in the medium (except in the case of *N*-acetyl-D-glucosamine as nutrient) with different carbon sources resulted in a unique utility pattern (Table 3). All tested isolates were able to form a pellicle with L-arabinose, *N*-acetyl-D-glucosamine, D-fructose, D-glucose, malate, mannitol and L-tartrate (except strain 84B). They lacked this property if *meso*-inositol or L-rhamnose was used as carbon source. In this test, differences in carbon source metabolism compared to

Table 3. Pellicle formation in semi-solid media by various *Herbaspirillum* strains with different carbon sources and without nitrogen source except where specified

+++, Thick pellicle coming to the medium surface; ++, pellicle formation some millimetres below the medium surface; +, very fine pellicle below the surface; −, no growth; +N, addition of NH_4Cl (1 g l^{-1}).

Carbon source	*Herbaspirillum seropedicae* Z67^T	*Herbaspirillum rubrisubalbicans* LMG 2286^T	*Herbaspirillum frisingense* GSF30^T	*Herbaspirillum frisingense* 84B
Malate	+++*	+++*	+++*	+++*
D-Glucose	+++	+++	++	++
N-Acetyl-D-glucosamine	+++*	−	+++*	+++*
L-Arabinose	+++	+++	+++	+++
Mannitol	+++	+++	+++	+++
D-Fructose	+	+	+	+
D-Fructose+N	+++	+++	+++	+++
meso-Inositol	++	−	−	−
L-Tartrate	+	+	++*	−
L-Rhamnose	+++	−	−	−
meso-Erythritol+N	−	+++	−	−

* Alkalinization of the medium.

the type strains of *Herbaspirillum seropedicae* (LMG 6513^T) and *Herbaspirillum rubrisubalbicans* (LMG 2286^T) could be observed. In contrast to the fibre plant isolates and *Herbaspirillum rubrisubalbicans* LMG 2286^T, *Herbaspirillum seropedicae* LMG 6513^T exhibited pellicular growth with *meso*-inositol and L-rhamnose. *Herbaspirillum rubrisubalbicans* LMG 2286^T lacked this property, if *N*-acetyl-D-glucosamine was offered as carbon source (Table 3). Growth with *meso*-erythritol and with the addition of NH_4Cl was found only with *Herbaspirillum rubrisubalbicans* LMG 2286^T.

The optimum growth temperature was tested at 25, 30, 37 and 42 °C. Within this range, the investigated strains showed typical exponential growth with an optimum growth rate between 30 and 37 °C and only slight growth at 42 °C, which is in agreement with other species of the genus (Baldani *et al*., 1996). In the pH optimum experiment, the analysed *Herbaspirillum frisingense* sp. nov. isolates GSF3, GSF30^T, 75B and 84B, as well as *Herbaspirillum seropedicae* LMG 6513^T and *Herbaspirillum rubrisubalbicans* LMG 2286^T, showed optimal acetylene reduction between pH 6·0 and 7·0.

Nitrogen-fixing capacity

Herbaspirillum frisingense sp. nov. strain Mb11 was able to reduce acetylene to ethylene with a mean ratio of 130 nmol ethylene h^{-1} per 10^8 cells at an incubation temperature of 30 °C. These data are in good agreement with the acetylene reduction ability of *Herbaspirillum seropedicae* at 37 °C (Baldani *et al*., 1986).

A subset of eight *Herbaspirillum* isolates (Mb1, 11, 13, 14, 16, 19, 20, 22) and the type strains *Herbaspirillum rubrisubalbicans* LMG 2286^T and *Herbaspirillum seropedicae* LMG 6513^T were subjected to *nifD*-specific PCR amplification. After electrophoretic separation of the products in 1 % agarose gels a distinct band of 390 bp was observed, as expected (Stoltzfus *et al*., 1997) (data not shown).

Phylogenetic position analysed by 16S rDNA sequence data

The 16S rRNA sequence analysis revealed that *Herbaspirillum seropedicae* (LMG 6513^T), *Herbaspirillum rubrisubalbicans* (LMG 2286^T) and the isolates Mb11, GSF30^T and 75B cluster as a close group within the β-subclass of the *Proteobacteria* (together with *Oxalobacter formigenes* as demonstrated by Sievers *et al*., 1998). The phylogenetic tree presented in Fig. 2 illustrates the position of these strains relative to the major lines of descent of the β-subclass of the *Proteobacteria*. The tree was constructed by a maximum-likelihood analysis of 70 16S rRNA sequences from members of the β-subclass. The topology of the maximum-likelihood tree was confirmed by using a distance and maximum-parsimony analysis. It has to be noted that the position of *Oxalobacter formigenes* was not affected by these different treeing methods.

The mutual overall sequence similarity values for the herbaspirilla group are between 98·5 and 99·4 % and were clearly distinct from the next nearest relatives analysed, 'Ultramicrobacterium' strains D-6 and ND5 (Iizuka *et al*., 1998) with 95·8–97·3 % sequence similarity. The more distantly related species, *Janthinobacterium lividum* and *Oxalobacter formigenes*, showed 16S rDNA similarities of 95·4–96·2 % and 94·6–95·4 %, respectively, with the *Herbaspirillum* spp. sequences studied. Within the herbaspirilla the 16S rDNA sequences of strains GSF30^T, Mb11 and 75B, isolated

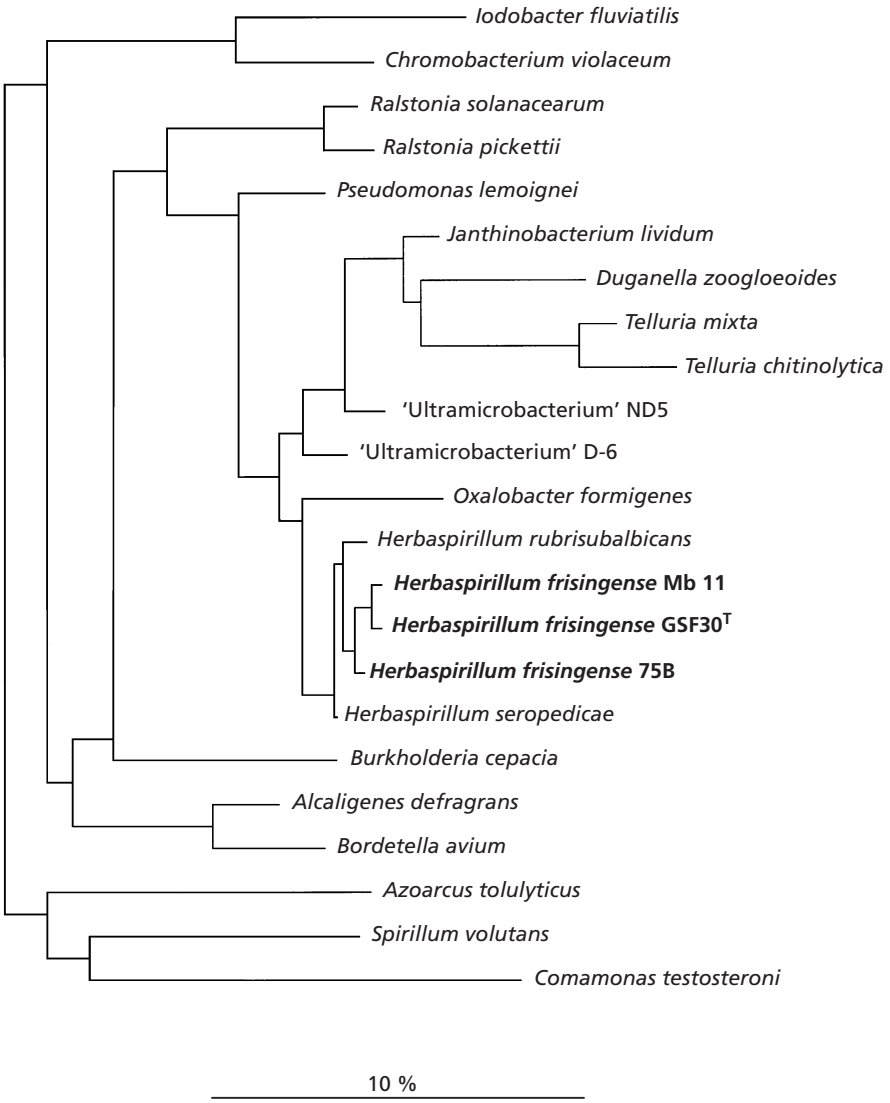

Fig. 2. Phylogenetic tree reflecting the relationships of *Herbaspirillum frisingense* sp. nov. to other members of the β-subclass of the *Proteobacteria*. The tree was constructed by maximum-likelihood analysis based on 70 16S rDNA sequences of the β-subclass. Only sequence positions which share common residues in at least 50 of the members of the β-subclass of the *Proteobacteria* were included for the calculation of this tree. For the sake of clarity, only a selection of reference sequences is shown. The bar indicates 10% estimated base changes. Strain and accession numbers of the species shown in this tree are: *Iodobacter fluviatilis* ATCC 3305^T, M2251; *Chromobacterium violaceum* ATCC 12472^T, M22510; *Ralstonia solanacearum* CIP 238, U28226; *Ralstonia pickettii* PKO1, L37367; *Pseudomonas lemoignei* LMG 2207^T, X92555; *Janthinobacterium lividum* DSM 1522^T, Y08846; *Duganella zoogloeoides* IAM 12670, D14256; *Telluria mixta* ACM 17, X65589; *Telluria chitinolytica* ACM 3522^T, X65590; 'Ultramicrobacterium' ND5, AB008506; 'Ultramicrobacterium' D-6, AB008504; *Oxalobacter formigenes* ATCC 35274^T, V49757; *Herbaspirillum rubrisubalbicans* LMG 2286^T, AJ238356; *H. frisingense* Mb11, AJ238359; *Herbaspirillum frisingense* GSF30^T, AJ238358; *Herbaspirillum frisingense* 75B, AJ238357; *Herbaspirillum seropedicae* Z67^T, AJ238361; *Burkholderia cepacia* ATCC 25416^T, M22518; *Alcaligenes defragrans* 54Pin, AJ005447; *Bordetella avium* ATCC 35086^T, U04947; *Azoarcus tolulyticus* Td15, L33688; *Spirillum volutans* ATCC 19544^T, M34131; *Comamonas testosteroni* ATCC 11996^T, M11224.

from fibre plant tissues, form a tight cluster with similarities of 98·9 and 99·4%. These strains also have sequence similarities of 98·7–99·1% compared to *Herbaspirillum seropedicae* (LMG 6513^T). This degree of sequence similarity does not conclusively imply a new species of *Herbaspirillum* (Stackebrandt & Goebel, 1994) but DNA–DNA hybridization experiments and physiological tests showed that the strains GSF30^T, Mb11 and 75B constitute a distinct new species, proposed as *Herbaspirillum frisingense* sp. nov.

DNA–DNA hybridization and DNA base composition

To demonstrate conclusively the intergeneric relationship within the nitrogen-fixing species of *Herbaspirillum*, DNA–DNA hybridization experiments with a selection of 10 strains isolated from *Miscanthus* and *Pennisetum*, the type strains of *Herbaspirillum seropedicae* (LMG 6513^T) and *Herbaspirillum rubrisubalbicans* (LMG 2286^T) and *Escherichia coli* JM83 as control were performed. The overall variation of the calculated genomic hybridization values estimated with the method described above was $\pm$10%. Therefore, organisms with DNA similarities from 60 to 110% were considered as belonging to one species. Consequently, three clear clusters (besides *E. coli* with overall 0% hybridization) could be recognized (Table 4). The chromosomal DNA of *Herbaspirillum seropedicae* LMG 6513^T shows 11–34% hybridization with the new plant isolates and with *Herbaspirillum rubrisubalbicans* LMG 2286^T. The genomic hybridization values of chromosomal *Herbaspirillum rubrisubalbicans* LMG 2286^T DNA with the DNA samples of all other strains ranged between 1 and 25%. Strains GSF23, 24, 25, 28, 30^T and 35 isolated from *Miscanthus* spp. and 60B, 75B, 84B and 118B isolated from *Pennisetum purpureum* formed a clear third cluster with hybridization values from 60 to 110% and therefore could be grouped in the new species.

The DNA G+C content of *Herbaspirillum frisingense* sp. nov. was calculated from the thermal DNA denaturation results to be 63$\pm$2 mol%, which is in

Table 4. DNA–DNA hybridization (percentage genomic hybridization)

ND, Not determined.

	GSF23	GSF24	GSF25	GSF28	GSF30^T	GSF35	60B	75B	84B	118B	*H. ser.* LMG 6513^T	*H. rub.* LMG 2286^T	*E. coli*
GSF23	100	91	96	100	96	70	ND	73	ND	60	21	15	0
GSF24		99	100	93	93	95	94	82	66	65	31	25	0
GSF25			105	110	76	93	73	ND	ND	72	11	6	0
GSF28				100	94	110	80	83	ND	63	29	18	0
GSF30^T					100	ND	86	76	81	102	19	10	0
GSF35						100	84	ND	62	66	34	21	0
60B							100	ND	91	100	ND	13	0
75B								100	100	88	21	8	0
84B									110	100	17	8	0
118B										100	14	1	0
***H. ser.* LMG 6513^T**											100	9	0
***H. rub.* LMG 2286^T**												100	0
E. coli													100

Table 5. Oligonucleotide probes, their sequences, target positions, specificities and formamide concentrations in the hybridization buffer required for specific *in situ* hybridizations

Probe	Sequence 5′→3′	Target position (16S rRNA positions)	Specificity	Formamide (%)
HERB 68	AGCAAGCTCCTATGCTGC	68–85	Genus *Herbaspirillum*	35
HERB 1432	CGGTTAGGCTACCCACTT	1432–1449	Genus *Herbaspirillum*	35
Hrubri 445	GCTACCACCGTTTCTTCG	445–462	*Herbaspirillum rubrisubalbicans*	60
Hsero 445	GCCAAAACCGTTTCTTCC	445–462	*Herbaspirillum seropedicae*	35
Hfris 445	TCCAGAACCGTTTCTTCC	445–462	*Herbaspirillum frisingense*	50

accordance with the values of 60–65% for the genus *Herbaspirillum* (Baldani *et al.*, 1996).

Design and optimization of diagnostic oligonucleotide probes

The 23S rRNA-targeting oligonucleotide probes specific for *Herbaspirillum rubrisubalbicans* (HR) and *Herbaspirillum seropedicae* (HS) (Baldani *et al.*, 1996) did not allow a clear identification and differentiation of the new isolates. In RNA dot-blot hybridization experiments, only a minor part of the *Pennisetum* isolates could be assigned to the known species *Herbaspirillum seropedicae* (Kirchhof *et al.*, 1997). The major portion of isolates from *Miscanthus sacchariflorus* roots (Mb1, 9, 11, 13, 14, 16, 19, 20, 22) showed positive signals with probe HR (Eckert *et al.*, 1998). Sequencing the 23S rDNA region of strain Mb11, which serves as probe target, showed the identical complementary base composition as the HR probe. A new 23S rRNA targeting probe, beta 20, reacting with the rRNA of *Herbaspirillum*-like isolates which did not belong to one of the known species, was designed by Kirchhof *et al.* (1997). Further investigations, including physiological properties and 16S rDNA sequence comparison (see above) indicated a close relationship of the *Miscanthus* isolates, whose rRNA hybridized with the *Herbaspirillum rubrisubalbicans* probe HR, and the strains isolated from *Miscanthus* and *Pennisetum* whose rRNA bound to probe beta 20. Therefore, the HR probe targeting the hypervariable stretch within domain III of the 23S rDNA is not specific for *Herbaspirillum rubrisubalbicans* and varies within the *Herbaspirillum frisingense* sp. nov. isolates.

Based on the PROBE DESIGN tool of the ARB software package the 16S rDNA sequences were inspected for unique stretches in base composition specific for the genus *Herbaspirillum*, the two known species *Herbaspirillum rubrisubalbicans* and *Herbaspirillum seropedicae* and for the new species *Herbaspirillum frisingense*. Two genus- and three species-specific oligonucleotide probes for the two known species, *Herbaspirillum rubrisubalbicans* and *Herbaspirillum seropedicae*, as well as for all isolates of the new species, *Herbaspirillum frisingense*, were designed and optimized for *in situ* hybridization experiments (Amann *et al.*, 1995; Table 5). The specificity and sensitivity of the probes were evaluated by hybridization with mixtures

Table 6. Results of whole-cell *in situ* hybridizations with 16S rRNA targeted oligonucleotide probes under stringent hybridization conditions

Results with all probes were negative for the following species: *Agrobacterium rhizogenes* DSM 30148^T, *Alcaligenes faecalis* LMG 1229^T, *Alcaligenes ruhlandii* DSM 653^T, *Azospirillum amazonense* DSM 2784, *Brevundimonas diminuta* DSM 1635, *Burkholderia andropogonis* LMG 2129^T, '*Burkholderia brasilense*' M130, *Burkholderia caryophylli* LMG 2155^T, *Burkholderia cepacia* LMG 1222^T, *Burkholderia glumae* LMG 2196^T, *Burkholderia plantarii* LMG 9035^T, *Janthinobacterium lividum* DSM 1522^T, *Neisseria elongata* LMG 5124^T and *Ralstonia eutropha* LMG 1199^T.

Bacterium*	**Probe†**				
	HERB 1432 (35% FA)	**HERB 68 (35% FA)**	**Hrubri 445 (60% FA)**	**Hsero 445 (35% FA)**	**Hfris 445 (50% FA)**
Herbaspirillum frisingense isolates from *Miscanthus* spp.					
Mb1	++	++	−	−	++
Mb10	++	++	−	−	++
Mb11	++	++	−	−	++
Mb13	++	++	−	−	++
Mb14	++	++	−	−	++
Mb19	++	++	−	−	++
Mb20	++	++	−	−	++
Mb22	++	++	−	−	++
GSF23	++	++	−	−	++
GSF24	++	++	−	−	++
GSF25	++	++	−	−	++
GSF28	++	++	−	−	++
GSF30^T	++	++	−	−	++
GSF34	++	++	−	−	++
Herbaspirillum frisingense isolates from *Pennisetum purpureum*					
13B	++	++	−	−	++
60B	++	++	−	−	++
75B	++	++	−	−	++
84B	++	++	−	−	++
103B	++	++	−	−	++
Herbaspirillum rubrisubalbicans					
LMG 2286^T	++	++	++	−	−
LMG 1278	++	++	++	−	−
LMG 6415	++	++	++	−	−
LMG 6416	++	++	++	−	−
LMG 6420	++	++	++	−	−
Herbaspirillum seropedicae					
LMG 6513^T	++	++	−	++	−
LMG 2284	++	++	−	++	−
16B (isolated from *P. purpureum*)	++	++	−	++	−
80B (isolated from *P. purpureum*)	++	++	−	++	−
Herbaspirillum species 3					
LMG 5523	++	++	−	−	−
LMG 5321	++	++	−	−	−
LMG 2285	++	++	−	−	−
Azospirillum brasilense DSM 1690^T	−	−	ND	−	ND
Burkholderia vietnamiensis LMG 10929^T	−	−	ND	−	ND
Chromobacterium violaceum LMG 1267^T	−	−	−	−	ND

* LMG, Laboratorium voor Microbiologie, Universiteit Gent, Belgium; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

† FA, Formamide; ND, not determined.

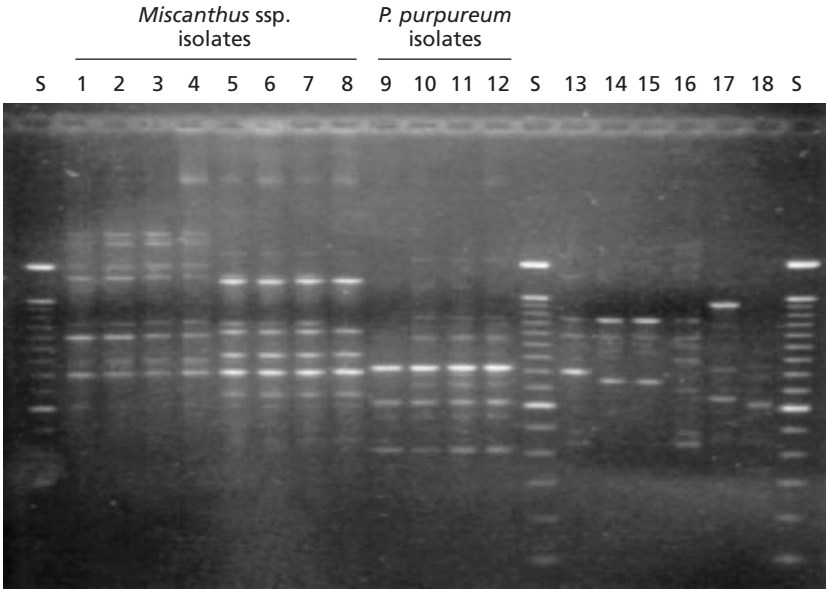

Fig. 3. Inter-LINE PCR fingerprints of *Herbaspirillum* spp. strains. Lanes: S, 123 bp standard; 1–12, *Herbaspirillum frisingense* strains Mb1, Mb11, Mb16, Mb20, GSF24, GSF28, GSF30^T, GSF35, 60B, 75B, 84B and 118B, respectively; 13–16, *Herbaspirillum rubrisubalbicans* LMG 2286^T, LMG 6415, LMG 6420 and LMG 1278, respectively; 17–18, *Herbaspirillum seropedicae* LMG 6513^T and LMG 2284, respectively.

of whole paraformaldehyde-fixed cells of herbaspirilla and reference organisms of phylogenetically related taxa. Stringent hybridization conditions were adjusted by gradually increasing the formamide concentration in the hybridization buffer while keeping the ionic strength (0·9 M NaCl) and hybridization temperature (46 °C) constant. The two genus-specific probes, HERB 68 and HERB 1432, are complementary to different regions of the 16S rRNA and hybridize under stringent hybridization conditions with all members of the genus *Herbaspirillum* (Table 6). The species-specific probes, Hsero 443, Hrubri 443 and Hfris 443, are targeted to the same diagnostic region within the 16S rRNA (*E. coli* position 445–462). They can be used as diagnostic tools to distinguish *Herbaspirillum rubrisubalbicans*, *Herbaspirillum seropedicae* and *Herbaspirillum frisingense* at species level. Non nitrogen-fixing group 3 herbaspirilla (Baldani *et al*., 1996) could be identified with the genus-specific probes HERB 1432 and HERB 68, but not with any of the species-specific probes (Table 6). These findings further support the assumption that members of this group form a distinct species, as proposed by Gillis *et al*. (1990) and Baldani *et al*. (1996). The application of the new *Herbaspirillum* probe set allowed a rapid and highly reliable nested identification of the reference strains and isolates belonging to this genus.

PCR fingerprinting

PCR fingerprinting is a common tool to permit the differentiation of DNA at the level of strains (Rademaker & De Bruijn, 1997). With this method the relative clonal relationship of a set of isolates can be determined and the genomic diversity can be described (Versalovic *et al*., 1994). The clonal diversity of a variety of isolates was analysed by a PCR fingerprinting approach using an oligonucleotide directed to sequences derived from eukaryotic LINEs supposedly conserved in all cells (Smida *et al*., 1996; Kirchhof *et al*., 1997). With this technique highly similar band patterns of the PCR products of *Herbaspirillum* isolates from *Miscanthus* or *Pennisetum*, respectively, could be found (Kirchhof *et al*., 1997). When further *Miscanthus* isolates (Mb1, 11, 16, 20) as well as several *Herbaspirillum rubrisubalbicans* and *Herbaspirillum seropedicae* strains were included, band patterns with some variability could be observed (Fig. 3). The bacterial strains formed a unique banding pattern in relation to their date of isolation and plant origin. Both series of isolates from *Miscanthus* (Mb1, 11, 16, 20 and GSF24, 28, 30^T, 35) had three bands of the same size out of seven or eight respective clear recognizable amplification products. The *Herbaspirillum frisingense* isolates from *Pennisetum* showed no obvious pattern variation, but their common pattern was different from the ones of the *Miscanthus* isolates (Fig. 3). The fingerprints of the *Herbaspirillum rubrisubalbicans* and *Herbaspirillum seropedicae* reference strains were clearly different from the *Herbaspirillum frisingense* isolates. These findings further indicate that the level of genetic diversity of bacterial strains may be correlated with their plant origin (McArthur *et al*., 1988). The selective conditions of the endophytic habitat as well as fibre plant reproduction via rhizomes may influence the distribution of the plant-associated bacteria and possibly cause the limited genomic variability observed among the investigated strains of *Herbaspirillum frisingense*.

Description of *Herbaspirillum frisingense* sp. nov.

Herbaspirillum frisingense (fri.sin.gen′se. L. gen. n. *frisingense* of Frisinga, now known as Freising, a town in Germany where the *Spartina pectinata* and *Miscanthus* spp. plants were grown from which the organisms were first isolated).

Characteristics useful for the identification of the genus *Herbaspirillum* as well as differentiating the new species *Herbaspirillum frisingense* sp. nov. are summarized in Table 2. Cells are slightly curved, spiral rods and motile. The cells have mostly two, occasionally one or three unipolar flagella and are able to swarm on soft nutrient agar. The cells are 0·5–0·7 μm in diameter and 1·4–1·8 μm long. The strains are able to fix atmospheric nitrogen and grow well with N_2 as sole nitrogen source under microaerobic conditions. The optimum growth temperature in the presence of nitrogen is between 30 and 37 °C. The pH optimum is between 6·0 and 7·0. Catalase, oxidase and urease activities are positive and gelatin is not hydrolysed. NO_3^- is reduced to NO_2^- but not to N_2. The metabolism is of typical respiratory nature, a broad spectrum of sugars and alcohols are oxidized. Di- and trisaccharides are not metabolized. Organic acids are preferably utilized as carbon sources. Fatty acid oxidation is positive. Amino acids are also utilized. Microaerophilic growth was observed with L-arabinose, *N*-acetyl-D-glucosamine, D-fructose, D-glucose, malate, mannitol and L-tartrate and does not occur with *meso*-erythritol, *meso*-inositol and L-rhamnose. 16S rDNA sequence similarity within the species is 98·8–99·4%. The G+C content of the DNA is 63 ± 2 mol%. The type strain is GSF30^T (= DSM 13128^T); DNA G+C content is 64 mol%. The organisms are associated with roots, stems and leaves of the C4-gramineous plants *Spartina pectinata*, *Miscanthus sinensis*, *Miscanthus sacchariflorus* and *Pennisetum purpureum*.

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