Herbaspirillum hiltneri sp. nov., isolated from surface-sterilized wheat roots

Michael Rothballer,[1] Michael Schmid,[1] Ilona Klein,[1]† Andreas Gattinger,[2] Sabine Grundmann[2] and Anton Hartmann[1]

[1,2]Department of Rhizosphere Biology[1], Institute of Soil Ecology[1,2], GSF – National Research Centre for Environment and Health, Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany

Correspondence
Anton Hartmann
anton.hartmann@gsf.de

The genus *Herbaspirillum* of the *Betaproteobacteria* mainly comprises diazotrophic bacteria with a potential for endophytic and systemic colonization of a variety of plants. The plant-associated bacterial isolates N3^T, N5 and N9 were derived from surface-sterilized wheat roots. After phylogenetic analysis of 16S rRNA gene sequence data the isolates could be allocated to the genus *Herbaspirillum*, and 99·9 % similarity to the sequence of *Herbaspirillum lusitanum* P6-12^T was found. A set of 16S rRNA gene-targeted oligonucleotide probes was developed for the identification of the three novel isolates and *H. lusitanum* (Hhilu446), and for the specific detection of several other *Herbaspirillum* species described recently. For higher phylogenetic resolution, the 23S rRNA gene sequences of all members of the genus was sequenced and used to construct a phylogenetic tree. Isolates N3^T, N5 and N9 formed a group that was distinct from all other *Herbaspirillum* species. In addition, isolate N3^T and *H. lusitanum* P6-12^T exhibited a DNA–DNA hybridization value of only 25 %. The value for DNA–DNA hybridization between N3^T and other members of the genus *Herbaspirillum* was between 14 and 32 %; DNA–DNA hybridization between strain N3^T and isolates N5 and N9 produced values above 95 %. This places the three isolates as representatives of a novel species within the genus *Herbaspirillum*. A Biolog GN2 assay supported this conclusion. The major fatty acids were $C_{16:1}\omega7c$, $C_{16:0}$ and $C_{18:1}\omega7c$, and the DNA G+C content ranged from 60·9 to 61·5 mol%. Therefore these three isolates should be classified within a novel species, for which the name *Herbaspirillum hiltneri* sp. nov. is proposed. The type strain is N3^T (=DSM 17495^T=LMG 23131^T).

On the basis of comparative sequence analysis of rRNA-encoding genes, the genus *Herbaspirillum* is affiliated phylogenetically to the *Betaproteobacteria* (Schmid *et al.*, 2005). The genus was first described with the species *Herbaspirillum seropedicae* (Baldani *et al.*, 1986), which has been isolated from rice, maize and sorghum plants. After detailed taxonomic studies, the mildly plant-pathogenic [*Pseudomonas*] *rubrisubalbicans* was reclassified to the genus *Herbaspirillum* as *Herbaspirillum rubrisubalbicans* (Baldani *et al.*, 1996). Another species of this genus, *Herbaspirillum frisingense*, was isolated from the C4-fibre plants *Miscanthus* spp., *Spartina spectinata* and *Pennisetum purpureum* by Kirchhof *et al.* (2001). From root nodules of garden beans (*Phaseolus vulgaris*), several strains representing one distinct species were isolated and described as *Herbaspirillum lusitanum* (Valverde *et al.*, 2003). These root-colonizing *Herbaspirillum* species were detected not only on the surface of the root, but also as intra- and intercellular colonizers in the root interior (Olivares *et al.*, 1997). These bacteria seem to prefer plants of the family Gramineae as hosts (Kirchhof *et al.*, 2001), but they are also found on other plant species (Baldani *et al.*, 1996). According to Döbereiner *et al.* (1993), the close association of endophytically colonizing herbaspirilli results in an additional supply of bacterially fixed nitrogen for the host plant. Experimental evidence for this thesis was provided by Elbeltagy *et al.* (2001) using rice (*Oryza officinalis*) inoculated with *Herbaspirillum* sp. strain B501.

The organisms listed so far all display the ability to fix atmospheric nitrogen, but, according to the latest taxonomic data, this can no longer be considered a common feature

†Present address: Institute of Biochemical Plant Pathology, GSF – National Research Centre for Environment and Health, Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany.

Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains N3^T, N5 and N9 are DQ150563–DQ150565, respectively, and those for the 23S rRNA gene sequences of strains N3^T, N5 and N9 are DQ150552–DQ150554.

A similarity matrix based on 16S and 23S rRNA gene sequences of *Herbaspirillum* species is available as supplementary material in IJSEM Online.

within the genus *Herbaspirillum*. In Korea, a 4-chlorophenol-degrading strain was isolated from river sediments near an industrial plant (Bae *et al.*, 1996). Because of its morphological and physiological attributes, this isolate was first thought to belong to the species *Comamonas testosteroni* (Bae *et al.*, 1996), but further phylogenetic analyses based on 16S rRNA gene sequences, as well as DNA–DNA hybridization experiments, indicated that this classification was incorrect; the name *Herbaspirillum chlorophenolicum* was proposed by Im *et al.* (2004) for this non-plant-associated strain. Ding & Yokota (2004) discovered a novel species in well water, and described it as *Herbaspirillum putei*; in the same study, the authors proposed the transfer of [*Aquaspirillum*] *autotrophicum* and [*Pseudomonas*] *huttiensis* to the genus *Herbaspirillum* as *Herbaspirillum autotrophicum* and *Herbaspirillum huttiense*. All three isolates were unable to fix nitrogen under laboratory conditions; only in *H. putei* was the *nifH* gene required for nitrogen fixation detected by PCR-based methods.

In this publication, we characterize the three isolates N3^T, N5 and N9, which originated from surface-sterilized wheat roots (*Triticum aestivum*). On the basis of 16S and 23S rRNA gene sequence data, as well as DNA–DNA hybridization results and phenotypic features, these isolates belong to a novel species within the genus *Herbaspirillum*.

Isolates N3^T, N5 and N9 were derived from 4- to 8-week-old wheat plants (*T. aestivum* var. Naxos) grown in agricultural soil from Neumarkt (Oberpfalz, Germany). Surface sterilization was carried out with chloramine T (1 % w/v) for 10 min. After surface sterilization, roots were washed three times with 1 × PBS, crushed and then plated on NB agar [nutrient broth no. 4 (Fluka) solidified with 16 g agar l^{-1}] at appropriate dilutions to obtain single colonies. Colonies were picked and singled out on new plates. With the exception of *H. lusitanum* P6-12^T (a gift from J. Igual, Instituto de Recursos Naturales y Agrobiología, Salamanca, Spain), reference strains of *Herbaspirillum* were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany) or the IAM Culture Collection (Institute of Applied Microbiology, The University of Tokyo, Japan). All *Herbaspirillum* strains were grown at 30 °C on NB medium. For nitrogen-free microaerobic growth conditions, semi-solid JNFb medium was used as described by Döbereiner (1995).

Oxidase activity was determined from the oxidation of 1 % *N*,*N*-dimethyl-*p*-phenylenediamine hemioxalate. The presence of catalase activity was demonstrated by bubble formation in a 3 % (v/v) H_2O_2 solution. Metabolism of various substrates was tested in at least three replicates with Biolog GN2 Microplates according to the protocol provided by the manufacturer. Temperature and pH growth optima were determined in NB liquid medium (nutrient broth no. 4) after 16 h growth at 30 °C. Culture medium (5 ml) was inoculated with 10 µl preculture grown overnight. After incubation, the cell density was measured photometrically at 436 nm. The pH was adjusted by adding 0·05 M MES or MOPS buffer (Sigma-Aldrich).

Chromosomal DNA was isolated with a NucleoSpinTissue kit (Macherey Nagel). A PCR was performed in a Thermocycler Primus 25 (MWG-Biotech) in a total volume of 50 µl per reaction, according to standard protocols. The annealing temperature was 50 °C for all primers. The universal 16S primer 616-V (5′-AGAGTTTGATYMTGGCTCAG-3′) and 630-R (5′-CAKAAAGGAGGTGATCC-3′), as well as the universal 23S primers 118-V (5′-CCGAATGGGGRAACCC-3′) and 985-R (5′-CCGGTCCTCTCGTACT-3′) were used for the PCR and sequencing. Additionally, the internal 23S primer 1019-V (5′-TAGCTGGTTCTYYCCGAA-3′) was used for sequencing only. Amplified DNA fragments were cloned with the TOPO TA cloning kit (Invitrogen). Clones were sequenced using the Big Dye Terminator labelling kit (Applera) with an ABI PRISM 3730 DNA analyser (Applied Biosystems). For phylogenetic analyses, the 16S and 23S rRNA gene sequences obtained were aligned with the FastAligner version 1.03 tool implemented in the ARB software package (Strunk & Ludwig, 1997; Ludwig *et al.*, 2004). Phylogenetic tree construction was performed by using the maximum-likelihood (Olsen *et al.*, 1994), neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Felsenstein, 1993) methods.

For fluorescence *in-situ* hybridization (FISH), 1 ml pure overnight culture was fixed with 4 % paraformaldehyde for at least 1 h at 4 °C (Amann *et al.*, 1990). Hybridization with fluorochrome (fluorescein, Cy3)-labelled oligonucleotide probes was performed according to the protocols described by Manz *et al.* (1992) and Amann *et al.* (1992). New genus- and species-specific oligonucleotide probes based on 16S rRNA gene sequence data were created using the probe-design and probe-match tools of the ARB software package (Strunk & Ludwig, 1997; Ludwig *et al.*, 2004). Hybridization conditions for the *in-situ* application of the newly developed probes had to be optimized by gradually increasing the formamide concentration in the hybridization buffer, as described by Manz *et al.* (1992). To determine the correct stringency conditions for hybridization, non-target bacterial species with only one or two mismatches in the target sequence were included in each hybridization.

Light-microscopic observation of cell morphology and size, as well as wavelength-specific detection of FISH-labelled cells, was performed with an Axioplan 2 epifluorescence microscope (Zeiss) equipped with filter sets 01, 09 and 15.

For scanning electron microscopy, cells were grown overnight in NB medium and then harvested from 1 ml cell suspension by centrifugation at 5000 ***g*** for 5 min. The cells were washed twice with 1 × PBS and fixed with 2 ml 1 % glutaraldehyde in PBS (pH 7·4) overnight at 4 °C. The fixed cells were dehydrated through a series of ethanol solutions at increasing concentrations (50, 70, 80, 95 and 100 % ethanol). Ethanol was replaced with liquid CO_2, and the samples were dried in a critical-point dryer. Cells were

sputter-coated with platinum and examined in a scanning electron microscope (JSM 6300F; JEOL).

PCR amplification of the *nifH* and *nifD* genes was carried out by using bacterial genomic DNA as a template. Various primers and reaction conditions were used as described by Zehr & McReynolds (1989), Kloos *et al.* (1995), Stoltzfus *et al.* (1997) and Kirchhof *et al.* (2001).

For DNA–DNA hybridizations, about 3 g cell material was produced for each isolate by centrifuging a 2 l overnight culture for 25 min at 5000 ***g***. After two washing steps with 1 × PBS, cells were resuspended in 10 ml 50 % 2-propanol. All further steps were carried out by the DSMZ according to the protocols of Cashion *et al.* (1977) and De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980). Renaturation rates were calculated with the program TRANSFER.BAS of Jahnke (1992).

The DNA G+C contents of the isolates were determined by the method described by Mesbah & Whitman (1989) with an HPLC system (LaChrom; Merck/Hitachi) using an L7400 UV detector.

The analysis of cellular fatty acids was performed according to a slightly modified procedure described by Gattinger *et al.* (2002). Briefly, cells grown overnight on NB medium were washed twice with 1 × PBS. Lipids were extracted from these fresh cells, according to the Bligh–Dyer method, using phosphate buffer (0·05 M, pH 7·4), methanol and chloroform as extraction solvents. The resulting lipid material was subjected to mild alkaline methanolysis (Zelles & Bai, 1993) to yield fatty acid methyl esters (FAME). One aliquot of the FAME sample was derivatized with 1-trimethylsilyl imidazole (TMSI) and the other was derivatized with dimethyl disulfide (DMDS) (Zelles & Bai, 1993) to generate analytes suitable for GC and the localization of functional groups in the FAME molecule. GC/MS was performed with a Hewlett Packard 5971A mass selective detector combined with a 5890 series II GC system, equipped with an HP 5 capillary column (50 m length, 0·25 mm internal diameter; coated with a cross-linked 5 % phenylmethyl rubber phase with a film thickness of 0·3 μm), with helium as the carrier gas, at operating conditions as described elsewhere (Zelles & Bai, 1993). The identification and quantification of individual components were achieved using chromatography software (HP ChemStation; SOLVIT) and comparison with a FAME standard mixture. Under these GC conditions FAMEs containing 10 or more carbon atoms could be determined.

The three isolates N3^T, N5 and N9 were Gram-negative, motile, slightly curved rods that formed circular, smooth, opaque and convex colonies after overnight growth on NB agar at 30 °C. They were positive for oxidase and catalase and showed optimal growth at temperatures between 26 and 34 °C and at pH values between 6 and 8. For examination of cell size and flagella, scanning electron micrographs were prepared (Fig. 1a, b). Up to three unipolar flagella could be detected, but most cells had two flagella. The size of single cells ranged between 1·6 and 2·0 μm in length and between 0·5 and 0·6 μm in diameter. The isolates were unable to grow on semi-solid, nitrogen-free JNFb medium (Kirchhof *et al.*, 2001) and detection of *nifD* and *nifH* genes was not possible with PCR-based methods using various specific primers. According to these results, the isolates do not possess the ability to fix atmospheric nitrogen. As the isolates were derived from surface-sterilized wheat roots, they seem to be very closely associated with the plant host and perhaps even possess the potential for endophytic colonization.

All three isolates showed the same characteristic carbon-source utilization in the Biolog GN2 test, but differed clearly from the other *Herbaspirillum* species. Numerous sugars, as well as fatty acids and some amino acids and alcohols, were readily metabolized. Disaccharides and trisaccharides could not be used as carbon sources. The ability to use L-phenylalanine as a carbon source was characteristic of isolates N3^T, N5 and N9; the other *Herbaspirillum* species did not have this capacity. Further details are listed in Table 1. The major fatty acids detected were $C_{16:1}\omega7c$, $C_{16:0}$ and $C_{18:1}\omega7c$; smaller proportions of $C_{17:0}$ cyclo and $C_{18:0}$ were also found. All of the other fatty acids detected were present at <1 mol% (Table 2). Similar results were obtained for other *Herbaspirillum* species, which showed the same dominant fatty acids with only slight differences in mole ratios (Ding & Yokota, 2004).

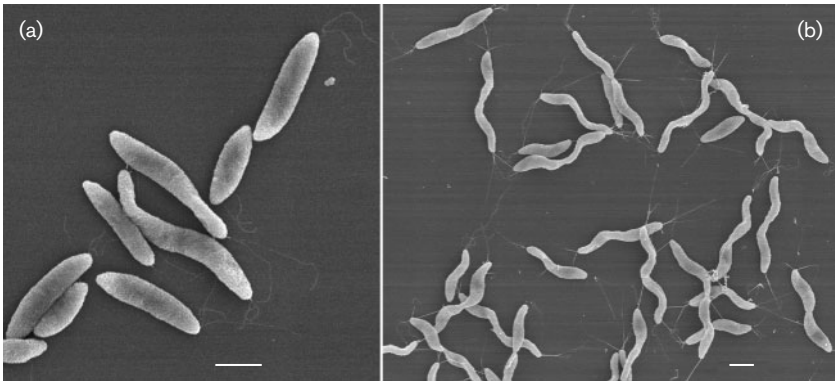

Fig. 1. Scanning electron micrographs of isolate N3^T. In (a), several single cells with polar flagella can be seen. The two elongated cells in the middle of the picture were just about to divide. In (b), virtually all cells shown are dividing. One, two or three polar flagella are clearly visible on every cell. Bars, 1 μm.

Table 1. Utilization of different carbon sources in Biolog GN2 microplates by members of the genus *Herbaspirillum*

Strains: 1, *H. putei* IAM 15032^T; 2, *H. huttiense* DSM 10281^T; 3, *H. autotrophicum* DSM 732^T; 4, *H. chlorophenolicum* IAM 15024^T; 5, *H. frisingense* GSF30^T; 6, *H. rubrisubalbicans* DSM 9440^T; 7, *H. seropedicae* DSM 6445^T; 8, *H. lusitanum* P6-12^T; 9, strains N3^T, N5 and N9 (identical results were obtained for the three strains). +, Positive; (+), ambiguous; −, negative. All strains were positive for utilization of malic acid, methyl pyruvate, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-gluconic acid, *β*-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, D-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline and L-pyroglutamic acid. All strains were negative for water (negative control), α-cyclodextrin, *N*-acetyl-D-galactosamine, D-cellobiose, gentiobiose, α-D-lactose, lactulose, maltose, D-melibiose, D-raffinose, sucrose, turanose, α-ketovaleric acid, sebacic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-ornithine, DL-carnitine, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2,3-butanediol and glucose 1-phosphate.

Carbon source	1	2	3	4	5	6	7	8	9
Dextrin	−	−	−	−	−	+	−	−	−
Glycogen	−	+	+	+	+	+	+	+	+
Tween 40	−	+	+	+	+	+	+	+	+
Tween 80	−	+	+	+	+	+	+	+	+
N-Acetyl-D-glucosamine	−	−	−	+	+	−	+	+	+
Adonitol	+	+	−	−	+	+	+	+	−
L-Arabinose	+	+	−	+	+	+	+	+	+
D-Arabitol	+	+	−	+	+	+	+	+	+
i-Erythritol	(+)	+	−	−	−	+	−	−	−
D-Fructose	−	−	−	−	+	+	+	+	−
L-Fucose	(+)	+	−	+	+	+	+	+	+
D-Galactose	+	+	−	+	+	+	+	+	+
α-D-Glucose	+	+	−	+	+	+	+	+	+
myo-Inositol	−	−	−	−	−	−	+	−	−
D-Mannitol	+	+	−	+	+	+	+	+	+
D-Mannose	−	−	−	−	+	+	+	−	−
Methyl *β*-D-glucoside	−	−	−	−	−	+	−	−	−
D-Psicose	−	−	−	+	+	−	+	+	+
L-Rhamnose	−	−	−	−	−	−	+	+	−
D-Sorbitol	+	+	−	+	+	+	+	−	+
D-Trehalose	−	−	−	−	−	+	−	−	−
Xylitol	+	+	−	−	+	+	+	−	+
D-Galactonic acid lactone	−	−	+	+	+	+	+	+	+
D-Galacturonic acid	−	+	−	−	+	+	+	+	+
D-Glucosaminic acid	−	−	−	+	+	−	−	+	+
D-Glucuronic acid	−	+	−	−	−	+	+	+	+
α-Hydroxybutyric acid	−	+	+	+	+	+	+	+	+
γ-Hydroxybutyric acid	−	−	+	−	−	−	−	−	−
p-Hydroxyphenylacetic acid	+	−	+	−	+	+	+	−	−
Itaconic acid	(+)	−	+	+	+	+	+	+	+
Malonic acid	−	−	+	−	+	−	−	+	+
Quinic acid	+	+	−	−	−	+	+	−	−
D-Saccharic acid	+	+	−	+	+	+	+	+	+
Glucuronamide	−	−	−	−	−	+	+	+	+
L-Alaninamide	−	−	−	+	−	+	−	−	+
L-Alanine	−	−	+	+	+	+	−	+	+
L-Alanyl glycine	−	+	−	−	+	−	−	−	−
L-Leucine	−	−	−	−	+	+	−	−	−
L-Phenylalanine	−	−	−	−	−	−	−	−	+
D-Serine	−	−	−	−	+	−	−	−	−
L-Serine	−	−	−	−	+	−	−	−	−
L-Threonine	−	+	−	+	+	+	−	−	+
γ-Aminobutyric acid	(+)	+	−	+	+	+	+	−	−
2-Aminoethanol	+	+	−	−	+	+	+	−	−
Glycerol	+	+	−	−	+	+	+	+	+
DL-α-Glycerol phosphate	−	−	−	−	+	−	+	+	−
Glucose 6-phosphate	−	−	−	−	−	−	+	−	−

Table 2. Fatty acid profiles of isolates N3^T, N5 and N9

Mean values of two measurements ± range of values are given in mol%. FAMEs $C_{12:0}$, $C_{15:0}$ iso, $C_{24:0}$, $C_{10:0}$ 2-OH, $C_{10:0}$ 3-OH, $C_{12:0}$ 2-OH, $C_{12:0}$ 3-OH, $C_{12:0}$ 11-OH, $C_{13:1}$ 12-OH and $C_{17:1}$ 17-OH could not be detected in any of the isolates. ND, Not detectable; tr, trace amount (<0·1 %).

FAME	N3^T	N5	N9
$C_{14:0}$	0·5±0·3	0·3±0·1	0·2±0·0
$C_{15:0}$ anteiso	0·1±0·0	ND	ND
$C_{15:0}$	0·2±0·1	0·1±0·0	0·1±0·0
$C_{16:1}\omega7c$	44·5±1·8	44·9±0·6	46·8±2·8
$C_{16:1}\omega7t$	0·2±0·1	0·3±0·0	ND
$C_{16:1}\omega5$	0·1±0·0	0·1±0·0	0·1±0·0
$C_{16:0}$	26·4±2·5	24·6±0·4	26·3±0·7
$C_{17:1}\omega7$	0·1±0·0	0·1±0·0	0·1±0·0
$C_{17:0}$ cyclo	4·0±0·9	6·0±0·9	3·8±5·0
$C_{17:0}$	0·1±0·1	0·1±0·0	0·1±0·1
$C_{18:1}\omega9$	0·4±0·1	0·2±0·1	0·2±0·0
$C_{18:1}\omega7c$	18·2±4·9	20·3±0·4	19·4±1·1
$C_{18:1}\omega7t$	0·1±0·0	0·1±0·0	0·1±0·0
$C_{18:0}$	2·7±0·3	1·6±0·1	2·1±0·6
$C_{19:0}$ cyclo	0·1±0·1	0·1±0·0	0·1±0·0
$C_{20:0}$	0·1±0·1	0·1±0·0	0·1±0·0
$C_{22:0}$	0·1±0·1	0·1±0·0	0·1±0·0
$C_{10:1}$ 9-OH	0·1±0·0	ND	ND
$C_{11:1}$ 11-OH	0·1±0·0	ND	ND
$C_{12:1}$ 11-OH	0·1±0·0	0·1±0·0	ND
$C_{14:0}$ 3-OH	tr	tr	
$C_{14:0}$ 2-OH	tr	tr	tr
$C_{13:1}$ 13-OH	0·1±0·0	ND	tr
$C_{16:0}$ methoxy*	0·8±0·4	0·3±0·0	0·2±0·1
$C_{16:0}$ 3-OH	tr	tr	tr
$C_{16:0}$ 2-OH	tr	tr	tr
$C_{16:1}$ 15-OH	0·7±0·3	0·1±0·0	ND

*Binding position could not be determined with this method.

After sequencing of the almost complete 16S rRNA genes, the three isolates were taxonomically allocated by means of phylogenetic analysis. They had identical 16S rRNA gene sequences and showed 97 % or greater sequence similarity to the known *Herbaspirillum* species (see Supplementary Table S1 available in IJSEM Online). As a value of 95 % or more is considered to be a criterion for affiliation to the same genus (Ludwig *et al.*, 1998), these results provided evidence that the three isolates belong to the genus *Herbaspirillum*. The highest sequence similarity (99·9 %) was detected with *H. lusitanum* P6-12^T, which was an indication that N3^T, N5 and N9 were representatives of this species. However, all species of the genus *Herbaspirillum* exhibit a high degree of 16S rRNA gene sequence similarity, which ranges between 97·0 and 99·9 % (Supplementary Table S1). This is characteristic of species whose divergence has begun relatively recently and which are therefore hard to differentiate by 16S rRNA gene sequence-based methods (Fox *et al.*, 1992). In this case, analysis of 16S rRNA gene sequences cannot provide sufficient evidence for species affiliation (Rossello-Mora & Amann, 2001).

In order to clarify the species affiliation of the novel strains, 2·5 kb fragments of the 23S rRNA genes of all the *Herbaspirillum* species were sequenced and phylogenetically analysed. The three isolates showed similarities of 99·8–99·9 % with respect to each other (see Supplementary Table S1). The sequence similarities between *H. lusitanum* P6-12^T and the three isolates were 98·9–99·0 %, while the similarities within the genus ranged from 96·3 to 99·7 %. Since there are not as many 23S rRNA gene sequences available as there are for the 16S rRNA gene, the threshold values for species and genus level are not so clearly defined using the former (Rossello-Mora & Amann, 2001). However, as the type strains of *H. huttiense* and *H. putei* show 23S rRNA gene sequence similarity of 99·7 %, which is higher than the similarity between our isolates and *H. lusitanum* P6-12^T, these results provide a first indication that N3^T, N5 and N9 represent a distinct species within the genus *Herbaspirillum*. With the higher resolution of the 23S rRNA gene sequence data, the construction of a phylogenetic tree became possible (Fig. 2): it showed two distinct clusters of *Herbaspirillum* species. One cluster consisted of the type strains of *H. seropedicae*, *H. rubrisubalbicans*, *H. huttiense*, *H. putei*, *H. frisingense* and *H. chlorophenolicum*, while the other contained the type strains of *H. autotrophicum* and *H. lusitanum* and isolates N3^T, N5 and N9. The closely related genus *Collimonas* is difficult to separate from the genus *Herbaspirillum* by phylogenetic analysis based on 16S rRNA or 23S rRNA gene sequence data, but it is clearly distinct when biochemical and physiological characteristics are considered (de Boer *et al.*, 2004).

DNA–DNA hybridization was used to compare isolate N3^T with the type strains of all other *Herbaspirillum* species and with isolates N5 and N9. With this technique, the values for hybridization of isolate N3^T with N5 and with N9 were found to be 95 and 100 %, respectively, which clearly indicates the affiliation of the three isolates to one species. For N3^T and *H. lusitanum* P6-12^T, values of only 25 % could be detected. This is far below the threshold of 70 % that is typically reached in DNA–DNA hybridizations between strains of the same species (Wayne *et al.*, 1987). Although this guideline value cannot be considered as absolute and is extended to 50 % in some exceptional cases (Stackebrandt & Goebel, 1994), the affiliation of isolate N3^T to the species *H. lusitanum* could be ruled out according to these results. A DNA–DNA hybridization value as low as this is unusual in the context of a 16S rRNA gene sequence similarity of almost 100 %, but there are several comparable examples in the literature. Fox *et al.* (1992) detected similarly low DNA–DNA hybridization values when comparing several *Bacillus globisporus* and *Bacillus psychrophilus* strains, although a 99·5 % 16S rRNA gene sequence similarity, as well as various phenotypic traits, pointed towards an affiliation of the strains to the same species. Jaspers & Overmann (2004)

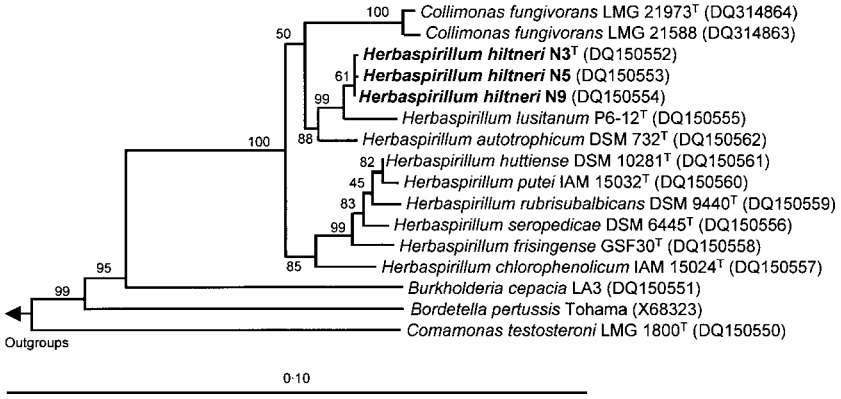

Fig. 2. Phylogenetic tree calculated with the neighbour-joining method based on 23S rRNA gene sequence data. Bootstrap values (percentages of 1000 resamplings) are given at branching points. NCBI accession numbers for sequence data are given in parentheses. The tree topology did not change significantly using maximum-likelihood and maximum-parsimony methods. The tree includes all known members of the genus *Herbaspirillum*. Bar, 10 % sequence divergence.

isolated 11 strains from a bacterial freshwater community whose members had identical 16S rRNA gene sequences but which exhibited great genetic diversity and specific utilization of different carbon sources. Further examples exist in species of the genus *Aeromonas* (Martinez-Murcia *et al.*, 1992) and *Halobacillus* (Amoozegar *et al.*, 2003). There is no exact threshold for genus definition based on DNA–DNA hybridization values, but if the values between the individual *Herbaspirillum* species (10–45 %; Ding & Yokota, 2004) are considered, the data presented unambiguously show that the isolates should be allocated to the genus *Herbaspirillum*. The values for DNA–DNA hybridization between isolate N3^T and the other *Herbaspirillum* species were as follows: *H. autotrophicum* DSM 732^T, 32 %; *H. rubrisubalbicans* DSM 9440^T, 30 %; *H. huttiense* DSM 10281^T, 28 %; *H. chlorophenolicum* IAM 15024^T, 25 %; *H. seropedicae* DSM 6445^T, 20 %; *H. frisingense* GSF30^T, 17 %; and *H. putei* IAM 15032^T, 14 %. Results were obtained from three independent measurements, with a standard deviation of 5 %.

Kirchhof *et al.* (2001) designed specific 16S rRNA gene-targeted probes for the detection of all members of the genus *Herbaspirillum* that had validly published names at that time. The highly variable region at *Escherichia coli* positions 446–463 made differentiation possible down to the species level. Using the latest 16S rRNA gene sequence data available from the NCBI database (National Center for Biotechnology Information, US National Library of Medicine, Bethesda, USA) and sequences obtained within the scope of this work, the same binding position could be used for the development of oligonucleotide probes that were specific for the newly described *Herbaspirillum* species. At the appropriate formamide concentration (given in Table 3), all probes allowed specific detection of their respective target organisms. In the case of probe Hhilu446, differentiation of *H. lusitanum* and the three isolates (N3^T, N5 and N9) was not possible, because of their high levels of 16S rRNA gene sequence similarity. The same is true for the pair *H. huttiense* and *H. putei*, which are both detected by

Table 3. Oligonucleotide probes for the specific detection of *Herbaspirillum* species by FISH

Binding positions in the 16S rRNA gene sequence of *E. coli* are according to Brosius *et al.* (1981). FA (%) indicates the percentage of formamide in the hybridization buffer.

Probe	Specificity	Binding position	Sequence (5′–3′)	FA (%)	Reference
HERB69	*H. seropedicae*, *H. rubrisubalbicans*, *H. frisingense*, *H. chlorophenolicum*, *H. huttiense*, *H. putei*	69–88	AGCAAGCTCCTATGCTGC	20	Kirchhof *et al.* (2001)
Hahl998	*H. autotrophicum*, ***H. hiltneri* sp. nov.**, *H. lusitanum*	998–1015	CTCTTCAGGATTCCGTAC	20	This work
Hfris446	*H. frisingense*	446–463	TCCAGAACCGTTTCTTCC	50	Kirchhof *et al.* (2001)
Hsero446	*H. seropedicae*	446–463	GCCAAAACCGTTTCTTCC	35	Kirchhof *et al.* (2001)
Hrubri446	*H. rubrisubalbicans* (competitor for Hhupu446)	446–463	GCTACCACCGTTTCTTCC	60	Kirchhof *et al.* (2001)
Hhupu446	*H. huttiense*, *H. putei* (with Hrubri446 as competitor)	446–463	GCTACTACCGTTTCTTCC	35	This work
Hchloro446	*H. chlorophenolicum*	446–463	GCTGCAACCGTTTCTTCC	50	This work
Haut446	*H. autotrophicum*	446–463	GCCCTCACCGTTTCTTCC	35	This work
Hhilu446	***H. hiltneri* sp. nov.**, *H. lusitanum*	446–463	ACCAAGACCGTTTCTTCC	35	This work

Hhupu446. In addition, this probe has only one weakly destabilizing mismatch from the respective *H. rubrisubalbicans* target region. Therefore, in order to discriminate *H. rubrisubalbicans*, the application of an unlabelled oligonucleotide of Hrubri446 as a competitor is necessary when Hhupu446 is used.

The probe HERB69, which had been designed for the detection of the whole genus *Herbaspirillum*, did not hybridize with the species *H. lusitanum*, *H. autotrophicum* and the isolates N3^T, N5 and N9. Thus, with the help of the probe-design tool of the ARB software package, the probe Hahl998 was designed, which binds to a 16S rRNA gene sequence region specific for these species. This probe can be used in an equimolar mixture together with HERB69 at the same formamide concentration to detect all members of the genus *Herbaspirillum*.

Description of *Herbaspirillum hiltneri* sp. nov.

Herbaspirillum hiltneri (hilt′ne.ri. N.L. gen. masc. n. *hiltneri* of Hiltner, in honour of Professor Lorenz Hiltner, who coined the rhizosphere concept in 1904).

Cells are Gram-negative, slightly curved rods that are motile due to (generally) two unipolar flagella. Circular, smooth, opaque and convex colonies are formed on NB agar after overnight growth at 30 °C. They are oxidase- and catalase-positive and the optimal growth temperature is 26–34 °C. The pH optimum lies between 6 and 8. Single cells are 1·6–2·0 μm in length and 0·5–0·6 μm in diameter. A wide variety of sugars and alcohols, as well as fatty acids and some amino acids, are metabolized. In contrast to all other *Herbaspirillum* species, *H. hiltneri* sp. nov. is able to use L-phenylalanine as a carbon source. Disaccharides and trisaccharides are not metabolized. Shows 98·9 % 23S rRNA gene sequence similarity to its closest relative, *H. lusitanum*, but is not distinguishable from *H. lusitanum* on the basis of 16S rRNA gene sequence data. Values for DNA–DNA hybridization to the other *Herbaspirillum* species range between 32 and 14 %; values for hybridization to isolates N5 and N9 exceed 95 %. DNA G+C content is 60·9–61·5 mol%. Major fatty acids are $C_{16:1}\omega7c$, $C_{16:0}$ and $C_{18:1}\omega7c$.

The type strain, N3^T (=DSM 17495^T=LMG 23131^T), was isolated in close association with roots of *Triticum aestivum*. Its DNA G+C content is 60·9 mol%.

Acknowledgements

We appreciate the skilful assistance of Daniela Weber and Angelika Schulz throughout this work, as well as the help of Helga Wehnes (GSF – Institute of Pathology) with the preparation of the electron micrographs. We are also indebted to Professor J. Igual for helpful advice and for providing *H. lusitanum* strains.

References

Amann, R. I., Krumholz, L. & Stahl, D. A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative and environmental studies in microbiology. *J Bacteriol* **172**, 762–770.

Amann, R. I., Zarda, B., Stahl, D. A. & Schleifer, K. H. (1992). Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* **58**, 3007–3011.

Amoozegar, M. A., Malekzadeh, F., Malik, K. A., Schumann, P. & Sproer, C. (2003). *Halobacillus karajensis* sp. nov., a novel moderate halophile. *Int J Syst Evol Microbiol* **53**, 1059–1063.

Bae, H. S., Lee, J. M., Kim, Y. B. & Lee, S. T. (1996). Biodegradation of the mixtures of 4-chlorophenol and phenol by *Comamonas testosteroni* CPW301. *Biodegradation* **7**, 463–469.

Baldani, J. I., Baldani, V. L. D., Seldin, L. & Döbereiner, J. (1986). Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. *Int J Syst Bacteriol* **30**, 86–93.

Baldani, J. I., Pot, B., Kirchhof, G. & 8 other authors (1996). Emended description of *Herbaspirillum*; inclusion of [*Pseudomonas*] *rubrisubalbicans*, a mild plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and classification of a group of clinical isolates (EF group 1) as *Herbaspirillum* species 3. *Int J Syst Bacteriol* **46**, 802–810.

Brosius, J., Dull, T. L., Sleeter, D. D. & Noller, H. F. (1981). Gene organization of primary structure of a ribosomal operon from *Escherichia coli*. *J Mol Biol* **148**, 107–127.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

de Boer, W., Leveau, J. H., Kowalchuk, G. A., Klein Gunnewiek, P. J., Abeln, E. C., Figge, M. J., Sjollema, K., Janse, J. D. & van Veen, J. A. (2004). *Collimonas fungivorans* gen. nov., sp. nov., a chitinolytic soil bacterium with the ability to grow on living fungal hyphae. *Int J Syst Evol Microbiol* **54**, 857–864.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Ding, L. & Yokota, A. (2004). Proposals of *Curvibacter gracilis* gen. nov., sp. nov. and *Herbaspirillum putei* sp. nov. for bacterial strains isolated from well water and reclassification of [*Pseudomonas*] *huttiensis*, [*Pseudomonas*] *lanceolata*, [*Aquaspirillum*] *delicatum* and [*Aquaspirillum*] *autotrophicum* as *Herbaspirillum huttiense* comb. nov., *Curvibacter lanceolatus* comb. nov., *Curvibacter delicatus* comb. nov. and *Herbaspirillum autotrophicum* comb. nov. *Int J Syst Evol Microbiol* **54**, 2223–2230.

Döbereiner, J. (1995). Isolation and identification of aerobic nitrogen-fixing bacteria from soil and plants. In *Methods in Applied Soil Microbiology and Biochemistry*, pp. 134–141. Edited by K. Alef & P. Nannipieri. London: Academic Press.

Döbereiner, J., Reis, V. M., Paula, M. A. & Olivares, F. L. (1993). Endophytic diazotrophs in sugar cane, tuber plants and cereals. In *New Horizons in Nitrogen Fixation*, pp. 671–676. Edited by R. Palacios, J. Mora & W. E. Newton. Dordrecht: Kluwer.

Elbeltagy, A., Nishioka, K., Sato, T., Suzuki, H., Ye, B., Hamada, T., Isawa, T., Mitsui, H. & Minamisawa, K. (2001). Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. *Appl Environ Microbiol* **67**, 5285–5293.

Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethyl sulfoxide solutions: acceleration of the renaturation rate. *Biopolymers* **19**, 1315–1327.

Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.

Gattinger, A., Schloter, M. & Munch, J. C. (2002). Phospholipid etherlipid and phospholipid fatty acid fingerprints in selected euryarchaeotal monocultures for taxonomic profiling. *FEMS Microbiol Lett* **213**, 133–139.

Huß, V. A. R., Festl, H. & Schleifer, K.-H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Im, W. T., Bae, H. S., Yokota, A. & Lee, S. T. (2004). *Herbaspirillum chlorophenolicum* sp. nov., a 4-chlorophenol-degrading bacterium. *Int J Syst Evol Microbiol* **54**, 851–855.

Jahnke, K. D. (1992). Basic computer program for evaluation of spectroscopic DNA renaturation data from GILFORD System 2600 spectrometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15**, 61–73.

Jaspers, E. & Overmann, J. (2004). Ecological significance of microdiversity: identical 16S rRNA gene sequences can be found in bacteria with highly divergent genomes and ecophysiologies. *Appl Environ Microbiol* **70**, 4831–4839.

Kirchhof, G., Eckert, B., Stoffels, M., Baldani, J. I., Reis, V. M. & Hartmann, A. (2001). *Herbaspirillum frisingense* sp. nov., a new nitrogen-fixing bacterial species that occurs in C4-fibre plants. *Int J Syst Evol Microbiol* **51**, 157–168.

Kloos, K., Fesefeldt, A., Gliesche, C. G. & Bothe, H. (1995). DNA-probing indicates the occurrence of denitrification and nitrogen fixation genes in *Hyphomicrobium*. Distribution of denitrifying and nitrogen fixing isolates of *Hyphomicrobium* in a sewage treatment plant. *FEMS Microbiol Ecol* **18**, 205–213.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K. H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554–568.

Ludwig, W., Strunk, O., Westram, R. & 29 other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

Manz, W., Amann, R., Ludwig, W., Wagner, M. & Schleifer, K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclass of *Proteobacteria*: problems and solutions. *Syst Appl Microbiol* **15**, 593–600.

Martinez-Murcia, A. J., Benlloch, S. & Collins, M. D. (1992). Phylogenetic interrelationships of members of the genera *Aeromonas* and *Plesiomonas* as determined by 16S ribosomal DNA sequencing: lack of congruence with results of DNA–DNA hybridizations. *Int J Syst Bacteriol* **42**, 412–421.

Mesbah, M. & Whitman, W. B. (1989). Measurement of deoxyguanosine/thymidine ratios in complex mixtures by high-performance liquid chromatography for determination of the mole percentage guanine + cytosine of DNA. *J Chromatogr* **479**, 297–306.

Olivares, F. L., James, E. K., Baldani, J. I. & Döbereiner, J. (1997). Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirillum*. *New Phytol* **135**, 723–737.

Olsen, G. J., Matsuda, H., Hagström, R. & Overbeek, R. (1994). fastDNAmL: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **10**, 41–48.

Rossello-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schmid, M., Baldani, J. I. & Hartmann, A. (2005). The Genus *Herbaspirillum*. In *The Prokaryotes: an Evolving Electronic Resource for the Microbiological Community*, 3rd edn, release 3.19. Edited by M. Dworkin. New York: Springer. http://link.springer-ny.com/link/service/books/10125/

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Stoltzfus, J. R., So, R., Malarvithi, P. P., Ladha, J. K. & de Bruijn, F. J. (1997). Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen. *Plant Soil* **194**, 25–36.

Strunk, O. & Ludwig, W. (1997). ARB: a software environment for sequence data. http://www.arb-home.de/

Valverde, A., Velázquez, E., Gutiérrez, C., Cervantes, E., Ventosa, A. & Igual, J.-M. (2003). *Herbaspirillum lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*. *Int J Syst Evol Microbiol* **53**, 1979–1983.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Zehr, J. P. & McReynolds, L. A. (1989). Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Appl Environ Microbiol* **55**, 2522–2526.

Zelles, L. & Bai, Q. Y. (1993). Fractionation of fatty acids derived from soil lipids by soil phase extraction and their quantitative analysis by GC-MS. *Soil Biol Biochem* **25**, 130–134.