Hansschlegelia zhihuaiae sp. nov., isolated from a polluted farmland soil

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A novel Gram-negative, aerobic, coccoid-shaped strain designated S 113^T was isolated from a polluted-soil sample collected in Jiangsu Province, China. A polyphasic taxonomic study including phylogenetic analysis based on the 16S rRNA gene sequence and determination of phenotypic characteristics was performed on the new isolate. The highest 16S rRNA gene sequence similarity was 96.8%, with *Hansschlegelia plantiphila* S₁^T. The predominant respiratory quinone was ubiquinone 10 (Q-10). The major fatty acids were C_{18:1ω7c} and C_{16:0}. The G + C content of the DNA was about 65.7 mol%. DNA–DNA hybridization experiments showed 44.9% relatedness for strain S 113^T with its closest relative, *H. plantiphila* NCIMB 14035^T. The dominant phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and phosphatidylcholine. The results of our polyphasic taxonomic analysis indicate that strain S 113^T represents a novel species within the genus *Hansschlegelia*, for which the name *Hansschlegelia zhihuaiae* sp. nov. is proposed. The type strain is S 113^T (=DSM 18984^T =CCTCC AB 206143^T =KCTC 12880^T).

The family *Methylocystaceae* was proposed by Bowman (2005, 2006) to harbour the group II methanotrophs, and the type genus is *Methylocystis* (Bowman *et al.*, 1993). At the time of writing, the family contains six established genera: *Albibacter*, *Methylocystis*, *Methylopila*, *Methylosinus*, *Pleomorphomonas* and *Terasakiella*. Most members of the family have been isolated from soil, groundwater or other complex environmental samples (Bowman *et al.*, 1993; Doronina *et al.*, 1998, 2001; Satomi *et al.*, 2002; Dedysh *et al.*, 2007). The genus *Hansschlegelia*, which was described by Ivanova *et al.* (2007), is also a member of family *Methylocystaceae*; the name has recently been validly published (Ivanova *et al.*, 2010). Many methanotrophic bacteria represent critical links in the global carbon cycle, act as N₂ fixers, degrade a wide array of organic contaminants and display many enzymic functions (Gulledge *et al.*, 2001). 16S rRNA gene sequence analysis has shown that members of the family *Methylocystaceae* are able to utilize methanol as a carbon source and exhibit the serine or ribulose-bisphosphate pathway for formaldehyde assimilation (Gallego *et al.*, 2005).

In the course of an investigation of the bacterial community in a farmland soil that was seriously contaminated by sulfonyleurea herbicides in Jiangsu province, China, strain S 113^T was isolated and subjected to a polyphasic taxonomic analysis. We propose here that it represents a novel species in the genus *Hansschlegelia*.

Strain S 113^T was isolated using the medium and methods described by Huang *et al.* (2007) from a sample of polluted farmland soil. The pure culture was preserved in a 25% (v/v) glycerol solution in distilled water at –80 °C.

The morphological characteristics of the strain were observed by transmission electron microscopy (model H-7650; Hitachi) after incubation for 3 days at 30 °C on YTC agar (0.3% yeast, 0.5% tryptone and 0.001% CaCl₂). Electron microscopy preparations were performed as described by Yoon *et al.* (2003). Growth was tested in liquid mineral medium K in which methanol or methylamine was added at a concentration of 0.5% (v/v) and formaldehyde was added at 0.05% (v/v), as described by Doronina *et al.* (1998). The method of Yamaguchi & Yokoe (2000) was followed to determine acid production from carbohydrates. Utilization of sole carbon sources was performed as described by Zhou *et al.* (2007). Growth was tested at 5, 10, 25, 30, 35, 37 and 40 °C in YTC broth and pH tolerance was tested using modified YTC broth adjusted to pH 3.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0. Antibiotic susceptibility was examined by placing antibiotic

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S 113^T is DQ916067.

A supplementary table and a supplementary figure are available with the online version of this paper.

discs on YTC plates as described by Zhou *et al.* (2007). Gram-staining and other physiological characteristics were assessed according to the methods of Doronina *et al.* (1998) and Gerhardt *et al.* (1994). API 20NE strips (bioMérieux) were used to investigate additional biochemical features.

Biomass for molecular systematic and chemotaxonomic tests was obtained using YTC broth or methanol medium K by incubation at 30 °C for 10 days in shaking flasks (about 180 r.p.m.). Respiratory quinones were extracted from lyophilized cells and the sample was purified and analysed by HPLC using the procedures reported by Hu *et al.* (2001). Cellular fatty acid composition was determined as described by Sasser (1990) using the Microbial Identification System (MIDI, Inc.). Polar lipid analyses were carried out as described by Minnikin *et al.* (1984). The predominant respiratory quinone of strain S 113^T was ubiquinone 10 (Q-10). The major fatty acids were C_{18:1}ω7c (77 %) and C_{16:0} (14.3 %); the detailed fatty acid profile is displayed in Supplementary Table S1, available in IJSEM Online. The dominant phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and phosphatidylcholine.

Genomic DNA for base composition analysis was prepared following the procedure of Marmur (1961). DNA G+C content was determined by reversed-phase HPLC according to Tamaoka & Komagata (1984); the DNA G+C content of S 113^T was 65.7 mol%. DNA–DNA relatedness was determined by the initial renaturation rate method in 2 × SSC (De Ley, 1970). Genomic DNA–DNA relatedness between S 113^T and *Hansschlegelia plantiphila* NCIMB 14035^T was 44.9 %, which is below the value of 70 % recommended for species definition (Wayne *et al.*, 1987).

PCR amplification of the 16S rRNA gene was performed as described by Xu *et al.* (2003). The 16S rRNA gene sequence was aligned manually with reference sequences retrieved from the GenBank database following BLAST searches. Phylogenetic trees were reconstructed using the software package MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to Kimura's two-parameter model; Kimura, 1980, 1983) and clustering were based on the neighbour-joining and maximum-parsimony methods (Saitou & Nei, 1987). Bootstrap analysis was applied to evaluate the topology of the neighbour-joining tree by performing 1000 resamplings (Felsenstein, 1985).

The almost-complete 16S rRNA gene sequence (1444 bp) of strain S 113^T was determined. The neighbour-joining phylogenetic tree (Fig. 1) reconstructed from 16S rRNA gene sequences showed clearly that strain S 113^T belongs to the genus *Hansschlegelia*; the strain showed 96.8 % 16S rRNA gene sequence similarity value to its closest neighbour, *H. plantiphila* S₁^T.

Phylogenetic analysis, genomic DNA–DNA hybridization and chemotaxonomy support the inclusion of the new isolate as a member of genus *Hansschlegelia*. Strain S 113^T can be differentiated easily from the only species currently classified in this genus, *H. plantiphila*, by some important phenotypic properties such as motility, catalase activity, H₂S production and NaCl tolerance (Table 1). The type strains of the two species also displayed significant differences in fatty acid and polar lipid compositions (Table 1 and Supplementary Table S1). On the basis of the phylogenetic and chemotaxonomic evidence together with the phenotypic characteristics presented in this study, the newly isolated strain S 113^T is assigned to a novel species

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain S 113^T compared with members of representative genera of the family *Methylocystaceae* and some non-methylotrophs in the phylum *Proteobacteria*. Numbers at nodes indicate percentage bootstrap values from 1000 resamplings; values ≥ 50 % are shown. Bar, 0.02 substitutions per nucleotide position. The maximum-parsimony tree showed essentially the same topology (not shown).

Table 1. Phenotypic characteristics that differentiate strain S 113^T from its phylogenetically closest neighbour, *H. plantiphila* NCIMB 14035^T

+, Positive; –, negative; (+), weakly positive. Data for major fatty acids, phospholipids, DNA G + C content and NaCl tolerance of *H. plantiphila* S₁^T were taken from Ivanova *et al.* (2007); other data were obtained in this study.

Characteristic	Strain S 113 ^T	<i>H. plantiphila</i> NCIMB 14035 ^T
Cell morphology	Coccoid	Short rod
Flagella	+	–
NaCl tolerance	<0.5 %	<2 %
H ₂ S production	–	+
Catalase	–	+
Oxidase	(+)	+
Urease	(+)	+
Carbon source utilization		
Succinate	–	(+)
Formaldehyde	–	+
Methylamine	–	+
Starch	+	–
Pyruvate	+	–
Major fatty acids	C _{18:1ω7c} , C _{16:0}	C _{16:0} , C _{19:0} cyclo, C _{18:1ω7c}
Dominant phospholipids*	DPG, PC, PE, PME, PG	DPG, PC, PE
DNA G + C content (mol%)	65.7	68.5

*DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidylmonomethylethanolamine.

in the genus *Hansschlegelia*, for which the name *Hansschlegelia zhihuaiae* sp. nov. is proposed.

Description of *Hansschlegelia zhihuaiae* sp. nov.

Hansschlegelia zhihuaiae [zhi.hua.i'ae. N.L. fem. gen. n. *zhihuaiae* named after Zhihua Wu (1935–2001), a Chinese pedologist who devoted herself to the study of soil biology].

Cells are Gram-negative, non-spore-forming, aerobic coccoids (0.6–0.9 × 1.0–1.3 µm) (Supplementary Fig. S1). Colonies are circular and white on YTC agar, 1.0–2.0 mm in diameter after 3 days of cultivation at 30 °C. Moderately sensitive to NaCl (<0.5 %). Shows good growth in liquid mineral medium K with 0.5 % (v/v) methanol but not with formaldehyde or methylamine. Good growth occurs at 25–30 °C and at pH 6.5–7.5, but not above pH 9.0 or below pH 6.0. Starch is hydrolysed; casein, cellulose, DNA, gelatin and Tween 80 are not hydrolysed. Acid is produced from adonitol, melezitose and sucrose, but not from D-arabinose, cellobiose, dulcitol, D-fructose, L-fucose, D-galactose, D-glucose, inositol, lactose, maltose, D-mannitol, melibiose, raffinose, L-rhamnose, D-ribose, L-sorbose, D-sorbitol, trehalose or D-xylose. D-Arabinose, glucosamine, pyruvate and starch are utilized. N-Acetylglucosamine, acetate, aesculin, cellobiose, dulcitol, dextrin, D-erythrose, D-fructose, D-galactose, gelatin, D-glucose, inulin, inositol, lactose, D-mannose, maltose, melibiose, D-mannitol, mucic acid, malonate, raffinose, D-ribose, ribulose, D-sorbitol, D-sorbose, salicin, trehalose, Tween 80, turanose, tartrate, xylitol and D-xylose are not utilized. Weak oxidase and

urease activities are present. Arginine decarboxylase, arginine dihydrolase, catalase, DNase, β-galactosidase, lysine decarboxylase, methyl α-D-glucosidase, ornithine decarboxylase and phenylalanine decarboxylase activities are absent. H₂S is not produced (triple sugar-iron reaction). Reduces nitrates to nitrites. Methyl red and Voges–Proskauer tests are negative. KCN (0.0075 %) is not tolerated. Susceptible to (µg per disc unless indicated) chloramphenicol (30), doxycycline (30), gentamicin (10), kanamycin (30), neomycin (30), polymyxin B (100 U), rifampicin (5), streptomycin (10) and tetracycline (30); resistant to ampicillin (10), erythromycin (15), lincomycin (15), penicillin G (10 U) and vancomycin (30). The major fatty acids are C_{18:1ω7c} and C_{16:0}. The dominant phospholipids are diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and phosphatidylglycerol. The DNA G + C content of the type strain is 65.7 mol% (HPLC).

The type strain, S 113^T (=DSM 18984^T =CCTCC AB 206143^T =KCTC 12880^T), was isolated from the surface layer of a polluted farmland soil from Jiangsu province, China.

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