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Azospirillum agricola sp. nov., a nitrogen-fixing species isolated from cultivated soil

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A polyphasic approach was used to characterize a novel nitrogen-fixing bacterial strain, designated CC-HIH038^T, isolated from cultivated soil in Taiwan. Cells of strain CC-HIH038^T were Gram-stain-negative, facultatively aerobic and spiral-shaped, with motility provided by a single polar flagellum. The 16S rRNA gene sequence analysis of strain CC-HIH038^T showed highest sequence similarity to *Azospirillum doebereinerae* (98.0 %), *Azospirillum thiophilum* (97.5 %), *Azospirillum rugosum* (97.4 %) and *Azospirillum zeae* (97.2 %) and lower sequence similarity (<97.0 %) to all other species of the genus *Azospirillum*. According to DNA–DNA association, the relatedness values of strain CC-HIH038^T with *A. doebereinerae*, *A. thiophilum*, *A. rugosum* and *A. zeae* were 51.8 %, 41.2 %, 56.5 % and 37.5 %, respectively. Strain CC-HIH038^T was able to grow at 20–37 °C and pH 7.0–8.0. Strain CC-HIH038^T gave positive amplification for dinitrogen reductase (*nifH* gene); the activity was recorded as 8.4 nmol ethylene h^{-1}. The predominant quinone system was ubiquinone Q-10 and the DNA G + C content was 68.8 mol%. The major fatty acids found in strain CC-HIH038^T were $C_{16:0}$, iso-$C_{18:0}$, $C_{16:0}$ 3-OH, $C_{14:0}$ 3-OH/iso-$C_{16:1}$ and $C_{18:1}\omega7c$/$C_{18:1}\omega6c$. Based on the distinct phylogenetic, phenotypic and chemotaxonomic traits together with results of comparative 16S rRNA gene sequence analysis, strain CC-HIH038^T is considered to represent a novel species in the genus *Azospirillum*, for which the name *Azospirillum agricola* sp. nov. is proposed. The type strain is CC-HIH038^T (=BCRC 80909^T=JCM 30827^T).

The genus *Azospirillum* is a member of the class *Alphaproteobacteria* and includes a species previously named '*Spirillum lipoferum*' (Beijerinck, 1925). The genus *Azospirillum*, which originally comprised two species, namely *Azospirillum lipoferum* and *Azospirillum brasilense*, was first described from the reclassification of '*Spirillum lipoferum*', the root-associated nitrogen fixer appearing as vibrioid cells with single polar flagella (Tarrand *et al.*, 1978). Members of the genus *Azospirillum* have been considered important among rhizobacteria promoting plant growth and recognized as biofertilizers, owing to their plant-growth-promoting activities. Typically, members of the genus *Azospirillum* are Gram-stain-negative, rod- or spiral-shaped and non-spore-forming diazotrophs. Poly-β-hydroxybutyrate granules are observed after few days' incubation. The major fatty acids are $C_{16:0}$, $C_{16:0}$ 3-OH, $C_{18:1}$ 2-OH, $C_{14:0}$ 3-OH/iso-$C_{16:1}$, $C_{16:1}\omega7c$/$C_{16:1}\omega6c$ and $C_{18:1}\omega7c$/$C_{18:1}\omega6c$; the common major respiratory quinone is ubiquinone Q-10.

The members of the nitrogen-fixing genus *Azospirillum* are mainly distributed in soils and frequently associated with grasses, cereals and crops (Döbereiner & Day 1976; Ladha *et al.*, 1987; Lin *et al.*, 2015; Kirchhof *et al.*, 1997). *Azospirillum rugosum* (Young *et al.*, 2008) and *Azospirillum picis* (Lin *et al.*, 2009) were isolated from oil-contaminated soil and discarded road tar. *Azospirillum thiophilum* was isolated from a sulfide spring (Lavrinenko *et al.*, 2010). *Azospirillum humicireducens* (Zhou *et al.*, 2013) was isolated from a microbial fuel cell. Currently, species belonging to the genus *Azospirillum* with validly published names are recorded in the LPSN website (Euzeby's nomenclature list, http://www.bacterio.net/azospirillum.html). After the recent reclassification of *Azospirillum irakense* to '*Niveispirillum irakense*' and *Azospirillum amazonense* to *Nitrospirillum amazonense*, at present this genus encompasses 17 species with validly published names (Lin *et al.*, 2015).

While investigating bacterial diversity, a cultivated soil sample was collected from Wufeng District, Taichung City, Taiwan

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nifH* gene sequences of strain CC-HIH038^T are KR296799 and KT634300, respectively.

A figure is available with the online Supplementary Material.

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(24° 02.9032′ N 120° 688.178′ E) and used to isolate bacterial strains by the standard serial dilution-plating technique on nutrient agar (NA; HiMedia) and R2A agar (Difco) at 30 °C for 3 days. A presumably novel strain (designated strain CC-HIH038^T) was selected for further phenotypic and phylogenetic characterization. Strain CC-HIH038^T was routinely grown aerobically on NA at 30 °C for 2 days and stored at −80 °C in nutrient broth (HiMedia) supplemented with 30 % (v/v) glycerol for long-term preservation.

The present work was undertaken to study the taxonomic status of a newly isolated diazotrophic bacterium. For taxonomic purposes, type strains of *Azospirillum doebereinerae* BCRC 17732^T (Eckert *et al.*, 2001), *A. thiophilum* DSM 21654^T (Lavrinenko *et al.*, 2010), *A. rugosum* DSM 19657^T (Young *et al.*, 2008) and *Azospirillum zeae* LMG 23989^T (Mehnaz *et al.*, 2007b) were analysed under the conditions described by Lin *et al.* (2011, 2012, 2013, 2014, 2015) and Young *et al.* (2015). For direct comparative analysis, all these strains were grown on NA at 30 °C for 2 days, unless specified otherwise.

A commercial DNA extraction kit (MO BIO UltraClean) was used to extract the genomic DNA of CC-HIH038^T for gene amplification (16S rRNA gene and *nifH* gene) and DNA G+C content. To obtain the almost complete 16S rRNA gene sequence of strain CC-HIH038^T, PCR was performed with bacterial universal primers pA and pH (Edwards *et al.*, 1989). Gene cycle sequencing was performed using the Bigdye terminator kit (Heiner *et al.*, 1998) and an ABI 3730 Genetic Analyzer (Applied Biosystems). The *nifH* gene was amplified by PCR using the primer set PolF and PolR, and conditions described previously by Poly *et al.* (2001). A genus-specific PCR primer pair was used for rapid identification of the genus *Azospirillum*. The forward primer Azo494-F and reverse primer Azo756-R were used to amplify a genus-specific fragment (Lin *et al.*, 2011). For the analysis of DNA G+C content, DNA samples were prepared and degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated via HPLC [Hitachi L-2130 chromatograph equipped with a Hitachi L-2200 autosampler, Hitachi L-2455 diode array detector, and a reverse-phase C18 column (Phenomenex Synergi 4 μ Fusion-RP80 250 × 4.60 mm)].

The DNA fragments encoding the 16S rRNA gene were assembled using the Vector NTI 9.0 software (IBI) and deposited in GenBank using sequin software (NCBI). The almost complete 16S rRNA gene sequence (1457 nt) of strain CC-HIH038^T was compared with those of all recognized type strains using the 16S rRNA gene sequence database (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). Phylogenetic analysis was performed with MEGA6 software (version 6.0; Tamura *et al.*, 2013). Closely related 16S rRNA gene sequences were retrieved from EzTaxon-e and GenBank and aligned by using the CLUSTAL_X (1.83) program (Thompson *et al.*, 1997). Phylogenetic trees were reconstructed using 16S rRNA gene sequences with the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods and evaluated by bootstrap analysis (Felsenstein, 1985) after 1000 replications.

DNA–DNA association was determined between strain CC-HIH038^T and *A. doebereinerae* BCRC 17732^T, *A. thiophilum* DSM 21654^T, *A. rugosum* DSM 19657^T and *A. zeae* LMG 23989^T. DNA samples were loaded onto positively charged membranes as described by Seldin & Dubnau (1985) and used to construct hybridization probes by labelling with digoxigenin–11-dUTP. Experiments were carried out in triplicate for each sample.

To investigate chemotaxonomic characteristics, strain CC-HIH038^T and the reference strains were harvested at a similar physiological age with similar growth kinetics. Isoprenoid quinones were purified by the methods of Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). Fatty acid methyl esters were prepared, separated and identified according to the standard protocol (Paisley, 1996) of the Microbial Identification System (MIDI) (Sasser, 1990) by gas chromatography (Agilent 7890A) using a flame-ionization detector. To extract fatty acid methyl esters, strain CC-HIH038^T was cultured simultaneously on NA for 48 h at 30 °C (the same conditions as our previous studies). Grown culture was harvested from the plate and subjected to saponification, methylation and extraction (Miller, 1982). Identification and comparison were done by using the Aerobe (RTSBA6) database of the MIDI System (Sherlock version 6.0).

The Gram-staining reaction was performed as described by Murray *et al.* (1994), and bacterial motility was observed by light microscopy (Zeiss A3000). Cells of strain CC-HIH038^T were stained with 0.2 % uranyl acetate and their morphology studied by transmission electron microscopy (JEOL JEM-1400). Catalase activity was determined by assessing the bubble production by cells in 3 % (v/v) H_2O_2 and oxidase activity was determined using 1 % (w/v) *N*,*N*,*N*′,*N*′,-tetramethyl-1,4-phenylenediamine reagent (bioMérieux). The following properties were tested in parallel under the same conditions. Carbon source utilization patterns were determined using Biolog GN2 MicroPlate (bioMérieux, France) after incubation for 2 days. Nitrate reduction, indole production, activities of β-galactosidase, urease, hydrolysis of aesculin and gelatin and assimilation of 12 substrates were tested with API 20 NE strips at 30 °C for 2 days. The activities of various enzymes were determined using the API ZYM system (bioMérieux) according to the manufacturer's instructions.

Colony morphology, presence of flagella and morphology of the cells of strain CC-HIH038^T were investigated using colonies or cells grown on NA. Growth of strain CC-HIH038^T was also tested on tryptic soy agar (Difco), marine agar (HiMedia) and nitrogen-free agar (Reinhold *et al.*, 1987) to determine its morphological characteristics. Anaerobic growth was tested on NA supplemented with potassium nitrate (0.1 %, w/v) using an anaerobic chamber (Coy) filled with $H_2/CO_2/N_2$ (5 : 19 : 76) gas. Growth of strain

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CC-HIH038^T was tested using nutrient broth at different temperatures (4, 15, 20, 25, 30, 37, 40, 45 and 50 °C), NaCl concentrations (1–10 % in 1 % increments) and pH values (pH 4.0–10.0, at intervals of 1.0 pH unit) using the following buffer systems: pH 4.0–5.0: 0.1 M citric acid/0.1 M trisodium citrate; pH 6.0–8.0: 0.2 M Na_2HPO_4/0.2 M NaH_2PO_4; pH 9.0–10.0: 0.1 M $NaHCO_3$/0.1 M Na_2CO_3.

The acetylene-reduction assay described by Hardy *et al.* (1973) was used to assay nitrogen-fixing capability. Vials (30 ml) containing 10 ml nitrogen-free semisolid agar were inoculated with strain CC-HIH038^T, sealed with rubber septa and incubated at 30 °C in a dark incubator. After 72 h, 10 % (v/v) of the air phase was replaced with acetylene (Koch & Evans, 1966) and the vials were reincubated. The amount of ethylene was measured for a total of 24 h using a gas chromatograph (FID Gas Chromatograph, HITACHI model 163) equipped with a flame-ionization detector and a packed column (1.0 m × 2.0 mm i.d., steel column packed with Porapak-T 80–100). Conditions of analysis were: carrier gas, nitrogen; flow rate, 35 ml h^{-1}; temperature of the flame-ionization detector, 110 °C; column temperature, 65 °C. The almost complete (1457 nt) sequence of the 16S rRNA gene was compiled. The 16S rRNA gene sequence analyses of strain CC-HIH038^T indicated that the strain was affiliated to the genus *Azospirillum* with high similarity to *A. doebereinerae* (98.0 %), *A. thiophilum* (97.5 %), *A. rugosum* (97.4 %) and *A. zeae* (97.2 %), and other species showed lower levels of similarity (<97.0 %).

Based on the similarity of 16S rRNA gene sequence, strains *A. doebereinerae* BCRC 17732^T, *A. thiophilum* DSM 21654^T, *A. rugosum* DSM 19657^T and *A. zeae* LMG 23989^T were selected as reference strains for DNA–DNA hybridization studies. The DNA–DNA relatedness values of strain CC-HIH038^T with *A. doebereinerae*, *A. thiophilum*, *A. rugosum* and *A. zeae* were 51.8 %, 41.2 %, 56.5 % and 37.5 %, respectively, which were lower than the threshold value (70 %) recommended for distinguishing novel species (Wayne *et al.*, 1987). Using the established molecular criteria for species-level relatedness, strain CC-HIH038^T shows less DNA–DNA relatedness with the most closely related species, which supports its genomic distinction as a representative of a separate species within the genus *Azospirillum*.

Phylogenetic trees were reconstructed using 16S rRNA gene sequences (total 29 nt were collected from GenBank) with neighbour-joining, maximum-likelihood and maximum-parsimony methods. Regardless of different evolutional comparisons, a similar topology was obtained in all phylogenetic trees, which indicates that novel strain CC-HIH038^T forms an individual cluster from other recognized species of the genus *Azospirillum* (Fig. 1). In this

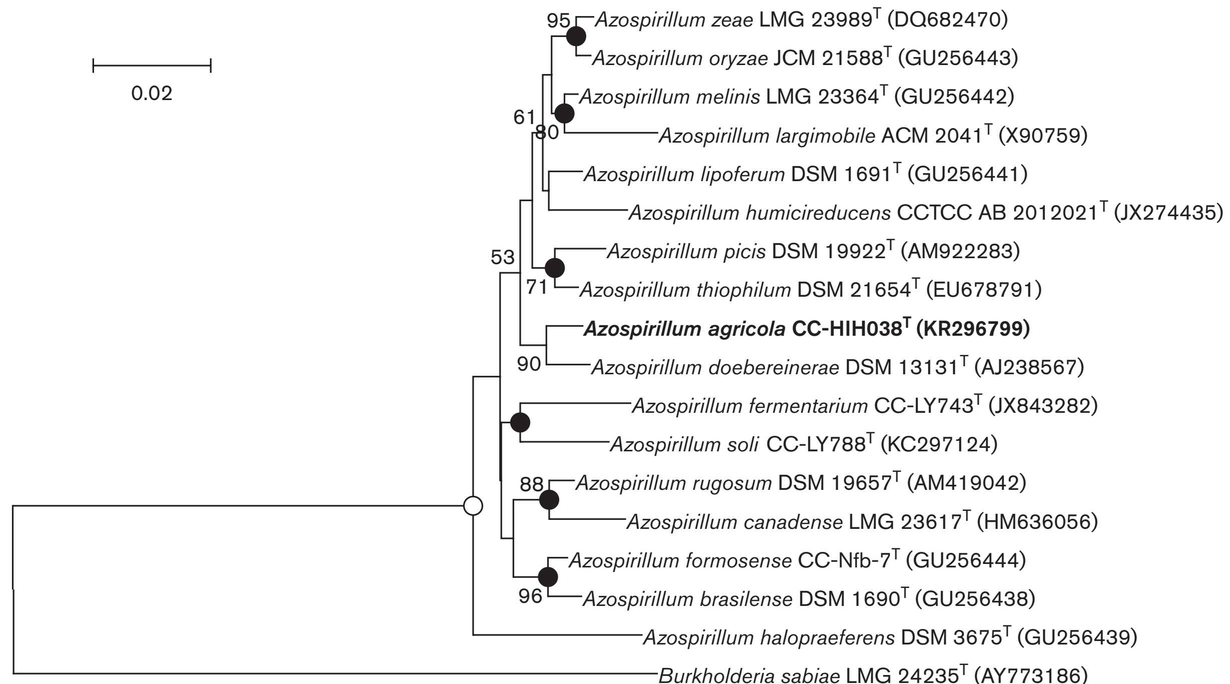

Fig. 1. Phylogenetic analysis of species of the genus *Azospirillum* based on 16S rRNA gene sequences. Distances and clustering were determined using the neighbour-joining method with the software package MEGA version 6. Open circles indicate that the corresponding nodes were recovered in the tree reconstructed based on the maximum-likelihood algorithm; filled circles indicate that the corresponding nodes were also recovered based on the maximum-likelihood and maximum-parsimony algorithms. Bootstrap values (>50 %) based on 1000 replications are listed as percentages at the branching points. Bar, 0.02 substitutions per nucleotide position.

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study, dinitrogen reductase (*nifH* gene) was amplified and sequenced; the genus-specific primer pair amplified the expected fragment with the template DNA isolated from strain CC-HIH038^{T}, which also demonstrated that strain CC-HIH038^{T} belongs to the genus *Azospirillum*.

Colonies were pink, circular and raised after 2 days of incubation on NA. Poly-β-hydroxybutyrate granules were observed by transmission electron microscopy (Figure S1, available in the online Supplementary Material). Strain CC-HIH038^{T} was facultatively aerobic, able to grow at 25–37 °C, pH 7.0–8.0, and tolerate less than 1 % NaCl (w/v) in nutrient broth. Additionally, strain CC-HIH038^{T} showed several distinct physiological and biochemical characteristics; a comparison of the phenotypic properties between strain CC-HIH038^{T} and the type strains of recognized species in the genus *Azospirillum* is given in Table 1. Strain CC-HIH038^{T} was able to reduce acetylene to ethylene with a mean value of 8.4 nmol ethylene h^{-1} at 30 °C. Free-living nitrogen-fixing activity was also compared with that of previously described bacteria, namely, *A. rugosum* IMMIB AFH-6^{T} (Young *et al.*, 2008), *A. picis* IMMIB TAR-3^{T} (Lin *et al.*, 2009), *Azospirillum formosense* CC-Nfb-7^{T} (Lin *et al.*, 2012), *Azospirillum fermentarium* CC-LY743^{T} (Lin *et al.*, 2013), *Azospirillum soli* CC-LY788^{T} (Lin *et al.*, 2015); the free-living nitrogen-fixing activities of these species were 18, 93, 25, 10.6, 6.5 nmol ethylene h^{-1}, respectively. Detailed phenotypic characteristics of strain CC-HIH038^{T} are given in the species description. The DNA G+C content analysis of strain CC-HIH038^{T}

Table 1. Differential characteristics within the genus *Azospirillum*

Strains: 1, strain CC-HIH038^{T}; 2, *A. doebereinerae* BCRC 17732^{T}; 3, *A. thiophilum* DSM 21654^{T}; 4, *A. rugosum* DSM 19657^{T}; 5, *A. zeae* LMG 23989^{T}; 6, *A. brasilense* BCRC 12270^{T}; 7, *A. formosense* BCRC 80273^{T}. All test strains were positive for the utilization of L-arabinose, D-fructose, pyruvic acid methyl ester, β-hydroxybutyric acid, DL-lactic acid and succinic acid as carbon sources (Biolog GN2 system); gave positive reactions for acid and alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase (API ZYM system); assimilated L-arabinose and malic acid; and gave positive reactions for β-glucosidase and β-galactosidase (API 20NE system). +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3	4	5	6	7
Nitrate reduction	+	+	+	+	+	−	+
Urease	+	+	+	+	+	+	−
Gelatin hydrolysis	+	−	−	−	−	−	−
Carbon source utilization							
Tween 40	+	−	+	−	−	+	+
Tween 80	−	−	+	−	−	+	+
D-Mannitol	−	+	+	−	+	−	−
D-Sorbitol	−	+	+	−	+	−	−
cis-Aconitic acid	+	−	−	−	−	+	+
Citric acid	+	+	−	−	−	−	−
α-Hydroxybutyric acid	+	−	−	−	−	−	+
Quinic acid	+	−	−	−	−	−	−
L-Alaninamide	+	−	−	−	−	−	+
L-Ornithine	+	−	+	+	−	−	−
L-Threonine	+	+	−	−	−	−	−
Glycerol	+	−	+	−	+	+	+
D-Glucose 6-phosphate	+	+	−	−	−	−	−
Enzyme activities							
Valine arylamidase	+	+	+	+	+	+	−
Cystine arylamidase	+	+	−	+	+	+	−
Trypsin	−	W	−	−	+	−	−
β-Galactosidase	−	+	−	−	+	−	−
β-Glucosidase	+	+	−	+	+	+	+
Assimilation (API 20NE)							
D-Glucose	−	−	+	+	−	+	−
D-Mannose	+	−	−	−	−	−	−
D-Mannitol	−	+	+	−	+	−	−
Potassium gluconate	−	−	+	+	W	+	+
DNA G+C content	69.8±0.2	70.7*	70.4±0.1*	70.0±0.2	67.6*	69–71*	64–66

*DNA G+C content data from Lavrinenko *et al.* (2010) and Mehnaz *et al.* (2007a, b).

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Table 2. Comparison of the cellular fatty acid contents (%) of strain CC-HIH038^T and closely related species

Strains: 1, strain CC-HIH038^T; 2, *A. doebereinerae* BCRC 17732^T; 3, *A. thiophilum* DSM 21654^T; 4, *A. rugosum* DSM 19657^T; 5, *A. zeae* LMG 23989^T; 6, *A. brasilense* BCRC 12270^T; 7, *A. formosense* BCRC 80273^T. TR, Trace (less than 1 %); −, not detected.

Fatty acid	1	2	3	4	5	6	7
Saturated							
$C_{14:0}$	TR	TR	TR	1.1	−	−	−
$C_{16:0}$	9.5	7.4	6.2	5.7	6.2	4.1	4.4
$C_{18:0}$	1.1	TR	TR	−	−	−	−
Unsaturated							
$C_{17:1}\omega 6c$	TR	TR	1.5	−	−	−	−
iso-$C_{18:0}$	6.1	2.8	−	−	−	−	−
$C_{19:0}$ cyclo $\omega 8c$	−	TR	7.4	−	−	−	−
Hydroxy							
iso-$C_{15:0}$ 3-OH	−	TR	4.8	4.8	3.8	2.7	3.4
$C_{16:0}$ 3-OH	5.0	7.2	5.4	6.6	4.7	3.7	4.2
$C_{18:1}$ 2-OH	2.8	6.7	1.3	7.9	7.0	5.9	6.9
Summed features*							
2	7.1	8.9	4.9	8.3	5.6	4.8	5.8
3	2.9	8.0	21.5	11.4	16.4	13.1	15.1
5	TR	−	−	2.7	−	−	−
8	62.0	51.0	42.3	45.3	53.4	60.2	56.4

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2: $C_{14:0}$ 3-OH/iso-$C_{16:1}$; summed feature 3: $C_{16:1}\omega 7c$/$C_{16:1}\omega 6c$; summed feature 5: anteiso-$C_{18:0}$/$C_{18:2}\omega 6,9c$; summed feature 8: $C_{18:1}\omega 7c$/$C_{18:1}\omega 6c$.

showed that the genomic DNA G+C content was 68.8±0.1 mol%, which is within the range for similarity with the genus *Azospirillum*. The predominant respiratory quinone was ubiquinone Q-10. The major fatty acids (>5 %) in strain CC-HIH038^T were $C_{16:0}$ (9.5 %), iso-$C_{18:0}$ (6.1 %), $C_{16:0}$ 3-OH (5.0 %), $C_{14:0}$ 3-OH/iso-$C_{16:1}$ (7.1 %) and $C_{18:1}\omega 7c$/$C_{18:1}\omega 6c$ (62.0 %). The fatty acid profile was similar to those for recognized species of the genus *Azospirillum* (Table 2).

Based on the distinct phylogenetic, phenotypic, biochemical and chemotaxonomic data provided, strain CC-HIH038^T is a member of the genus *Azospirillum*; the novel strain CC-HIH038^T is proposed to represent a novel species.

Description of *Azospirillum agricola* sp. nov.

Azospirillum agricola [a.gri′co.la L. n. *ager* field; L. suff. *-cola* (from L. n. *incola*) a dweller, inhabitant; L. masc. n. *agricola* field dwelling].

Cells are Gram-stain-negative, facultatively aerobic, spiral-shaped diazotrophs (3.2–3.5 μm in length and 1.3–1.7 μm in diameter); motile with monotrichous flagellum, catalase- and oxidase-positive. Colonies are pink, circular and raised after 2 days of incubation on NA; colony size is about 1–2 mm. Grows at temperatures ranging from 20 to 37 °C, pH 7.0–8.0; tolerates less than 1% (w/v) NaCl. Cells are able to grow on nitrogen-free medium, nutrient broth and R2A agar; accumulates poly-β-hydroxybutyrate granules in cell bodies. Utilizes numerous compounds as sole carbon source, including Tween 40, L-arabinose, D-fructose, D-galactose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, citric acid, D-gluconic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, DL-lactic acid, succinic acid, glucuronamide, DL-alanine, L-asparagine, L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-proline, L-serine, urocanic acid, glycerol, α-D-glucose 1-phosphate and D-glucose 6-phosphate. Acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and β-glucosidase are positive in the API ZYM system. The major fatty acids are $C_{16:0}$, iso-$C_{18:0}$, $C_{16:0}$ 3-OH, $C_{14:0}$ 3-OH/iso-$C_{16:1}$, and $C_{18:1}\omega 7c$/$C_{18:1}\omega 6c$. The predominant quinone system is ubiquinone Q-10.

The type strain, CC-HIH038^T (=BCRC 80909^T=JCM 30827^T), was isolated from a cultivated soil sample in Taiwan. The DNA G+C content of the type strain is 69.8±0.1 mol%.

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