Azospirillum formosense sp. nov., a diazotroph from agricultural soil

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A Gram-negative, spiral or rod-shaped, non-spore-forming diazotrophic bacterium, designated CC-Nfb-7^T, was isolated from agricultural soil in Yunlin County, Taiwan. 16S rRNA gene sequence analysis showed that strain CC-Nfb-7^T was most closely related to *Azospirillum brasilense* DSM 1690^T (97.4 % 16S rRNA gene sequence similarity), *Azospirillum rugosum* IMMIB AFH-6^T (96.8 %) and *Azospirillum oryzae* JCM 21588^T (96.6 %); <96.5 % 16S rRNA gene sequence similarity was found with all other members of the genus *Azospirillum*. DNA–DNA relatedness between strain CC-Nfb-7^T and *A. brasilense* DSM 1690^T, *A. rugosum* DSM 19657^T and *A. oryzae* JCM 21588^T was 38.9, 30.1 and 31.8 %, respectively. The respiratory quinone was ubiquinone Q-10. The major fatty acids were summed feature 8 (consisting of C_{18:1ω7c} and/or C_{18:1ω6c}), summed feature 3 (consisting of C_{16:1ω7c} and/or C_{16:1ω6c}), summed feature 2 (consisting of C_{14:0} 3-OH and/or iso-C_{16:1}), C_{16:0}, C_{18:0} 2-OH and C_{16:0} 3-OH. The polar lipids consisted mainly of phosphatidylglycerol, phosphatidylcholine and one unidentified phospholipid. Furthermore, moderate amounts of phosphatidylethanolamine, phosphatidyl-dimethylethanolamine and one unidentified aminophospholipid were also detected. Strain CC-Nfb-7^T could be distinguished from members of phylogenetically related species by differences in phenotypic properties. On the basis of morphological, chemotaxonomic and phylogenetic data, strain CC-Nfb-7^T represents a novel species within the genus *Azospirillum*, for which we propose the name *Azospirillum formosense* sp. nov. The type strain is CC-Nfb-7^T (=BCRC 80273^T=JCM 17639^T=DSM 24137^T).

The genus *Azospirillum* originally comprised two species, namely *Azospirillum lipoferum* and *Azospirillum brasilense*, when it was first created with the reclassification of ‘*Spirillum lipoferum*’, a root-associated nitrogen-fixing vibrioid bacterium with single polar flagella (Tarrand *et al.*, 1978). Until now, a total of 14 species has been described: *A. lipoferum* (Tarrand *et al.*, 1978), *A. brasilense* (Helsel *et al.*, 2006), *A. amazonense* (Falk *et al.*, 1985), *A. canadense* (Mehnaz *et al.*, 2007a), *A. doebereineriae* (Eckert *et al.*, 2001), *A. halopraeferens* (Reinhold *et al.*, 1987), *A. irakense* (Khammas *et al.*, 1989), *A. largimobile* (Ben Dekhil *et al.*, 1997), *A. melinis* (Peng *et al.*, 2006), *A. oryzae* (Xie &

Yokota, 2005), *A. picis* (Lin *et al.*, 2009), *A. rugosum* (Young *et al.*, 2008), *A. thiophilum* (Lavrinenko *et al.*, 2010) and *A. zae* (Mehnaz *et al.*, 2007b). Members of the genus are widely distributed in environments such as soils of tropical, subtropical and temperate regions, where they are frequently associated with grasses, cereals and crops (Bally *et al.*, 1983; Döbereiner & Day, 1976; Kirchhof *et al.*, 1997; Ladha *et al.*, 1987; Patriquin *et al.*, 1983). *A. picis* and *A. rugosum* were isolated from oil-contaminated soil and discarded road tar, respectively, and *A. thiophilum* was isolated from a sulfide spring.

The genus *Azospirillum* is widely known as containing free-living nitrogen-fixing plant-growth-promoting rhizobacteria (Okon & Vanderleyden, 1997; Okon & Itzigsohn, 1992), and belongs to the Gram-negative alphaproteobacteria. The versatile C- and N-metabolism in this genus makes it well adapted to the rhizosphere and successfully competitive

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nifH* gene sequences of strain CC-Nfb-7^T are GU256444 and HM193519, respectively.

Two supplementary figures and a supplementary table are available with the online version of this paper.

in this environment (Steenhoudt & Vanderleyden, 2000). Members of the versatile genus *Azospirillum* are known as biofertilizers and are commonly used in field tests (Steenhoudt & Vanderleyden, 2000) because of their ability to produce plant hormones such as abscisic acid, ethylene, gibberellic acid, indole 3-acetic acid (Bashan *et al.*, 2004) and zeatin (Tien *et al.*, 1979) and plant growth regulatory substances such as polyamines (Thuler *et al.*, 2003) and siderophores (Saxena *et al.*, 1986), and to generate osmotic stress responses in plants (Aziz *et al.*, 1997) and solubilize phosphate (Seshadri *et al.*, 2000).

Strain CC-Nfb-7^T was isolated from agricultural soil in Yunlin County, Taiwan, on nitrogen-free broth (NFB) agar (Reinhold *et al.*, 1987). The isolate was subsequently cultivated on nutrient agar (NA; Hi-Media) and R2A agar (Oxoid) to determine its morphological and phenotypic characteristics. Strain CC-Nfb-7^T was able to grow at 20–37 °C and at pH 5.0–9.0 on these media. Growth with 0–5 % NaCl was determined in nutrient broth (NB). Gram-staining was performed as described by Murray *et al.* (1994). The phenotypic and biochemical characteristics were investigated using the API ZYM, API 20 E and API 20 NE systems (bioMérieux) and carbon source utilization was investigated using the Biolog GN II and API 50 CH systems. Physiological tests were performed according to Lin *et al.* (2009). The morphology of negatively stained cells was determined by transmission electron microscopy. For thin sections, cells were prefixed with 2 % (v/v) glutaraldehyde and 3 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at room temperature and then washed three times in the same buffer. Strain CC-Nfb-7^T grew aerobically on NA and R2A agar and formed rough, light-pink (NA) or white (R2A agar) colonies after 24 h at 37 °C. Cells were Gram-negative, spiral or slightly curved rods, which were motile by means of a single polar flagellum. Cells contained poly- β -hydroxybutyrate granules after 5 days of growth (Fig. 1).

To assay nitrogen-fixing capability, the acetylene-reduction assay was used, as described by Hardy *et al.* (1973). Vials

(30 ml) containing 10 ml semi-solid NFB medium were inoculated with CC-Nfb-7^T, sealed with rubber septa and incubated at 30 °C in the dark. After 72 h, 10 % (v/v) of the air phase was replaced with acetylene (Koch & Evans, 1966) and the vials were incubated. The amount of ethylene produced in 24 h was measured using a FID GC (model 163; Hitachi) equipped with a flame-ionization detector and a packed column (1.0 m $\times$ 2.0 mm i.d., steel column packed with Porapak-T 80-100). The conditions of analysis were as follows: carrier gas, nitrogen; flow rate, 35 ml h⁻¹; temperature of the flame-ionization detector, 110 °C; column temperature, 80 °C. The isolate (10⁸ cells) was able to reduce acetylene to ethylene at a mean rate of 25 nmol ethylene h⁻¹ at 30 °C, which is similar to the nitrogen-fixing activities of *A. picis* IMMIB TAR-3^T (Lin *et al.*, 2009) and *A. rugosum* IMMIB AFH-6^T (Young *et al.*, 2008) (93 and 18 nmol ethylene h⁻¹, respectively).

Bacterial genomic DNA was isolated using an UltraClean Microbial Genomic DNA Isolation kit (MO BIO), according to the manufacturer's instructions. The 16S rRNA gene was amplified using the bacterial universal primers 1F (5'-GAGTTTGATCATGGCTCAGA-3') and 9R (5'-AAGGAGGTGATCCAACCGCA-3'). The amplification products were about 1500 bp long (Hung *et al.*, 2005). Sequencing reactions were performed using a BigDye Terminator kit (Heiner *et al.*, 1998) and the sequence was determined using an automatic DNA sequencer (ABI PRISM 310; Applied Biosystems) (Watts & MacBeath, 2001). 16S rRNA gene sequences of members of the genus *Azospirillum* were obtained from GenBank and recorded in FASTA format using ReadSeq software (<http://searchlauncher.bcm.tmc.edu/seq-util/readseq.html>). Sequence comparison and analysis was carried out using CLUSTAL X version 1.83 (Thompson *et al.*, 1997) and phylogenetic analysis was performed using MEGA4 (Tamura *et al.*, 2007). Sequence similarities and phylogenetic distances (K_{nuc} values) were determined according to Kimura (1980) and evolutionary trees were inferred using maximum parsimony (Fitch, 1971), neighbour joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981). An evolutionary distance matrix was calculated using the correction of Jukes & Cantor (1969). The topology of the neighbour-joining tree was evaluated by bootstrap analysis (Felsenstein, 1985) with 1000 resamplings. Strain CC-Nfb-7^T exhibited highest 16S rRNA gene sequence similarity with *A. brasilense* DSM 1690^T (97.4 %), *A. rugosum* IMMIB AFH-6^T (96.8 %) and *A. oryzae* JCM 21588^T (96.6 %); all other members of the genus *Azospirillum* showed lower levels of similarity (<96.5 %). Sequence divergence of ≥ 3 % is considered to be strong evidence that bacteria are not related at the species level (Stackebrandt & Goebel, 1994). The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences is shown in Fig. 2.

The *nifH* sequence was obtained by PCR using the primer set PolF (5'-TGCGAYCCSAARGCBGACTC-3') and PolR (5'-ATSGCCATCATYNTCRCCGGA-3') and the conditions described by Poly *et al.* (2001). The expected size of the product was about 360 bp. Strain CC-Nfb-7^T exhibited

Fig. 1. Transmission electron micrograph of a cell of strain CC-Nfb-7^T. Bar, 0.5 μ m.

Fig. 2. Neighbour-joining phylogenetic analysis based on 16S rRNA gene sequences, showing the position of strain CC-Nfb-7^T in the genus *Azospirillum*. Bootstrap values >50% based on 1000 replications are shown at branch nodes. Bar, 0.02 substitutions per nucleotide position.

highest *nifH* sequence similarities with *A. lipoferum* DSM 1691^T (95.4%), *A. brasilense* sp. 7^T (94.8%) and *A. oryzae* JCM 21588^T (93.9%). The neighbour-joining phylogenetic analysis based on *nifH* sequences (Fig. S1, available in IJSEM Online) also revealed that strain CC-Nfb-7^T belonged to the genus *Azospirillum*.

DNA–DNA hybridization was performed using reagents from Roche Diagnostics, according to the manufacturer's instructions. Briefly, chromosomal DNA of *A. brasilense* DSM 1690^T was used to construct hybridization probes by labelling with digoxigenin-11-dUTP. DNA samples from strain CC-Nfb-7^T, *A. rugosum* DSM 19657^T and *A. oryzae* JCM 21588^T were loaded onto positively charged membranes as described by Seldin & Dubnau (1985). Pre-hybridization and hybridization conditions for the labelled probes were used according to manufacturer's instructions for the DIG DNA Labeling and Detection kit. After hybridization, the blots were subjected to stringent washing and the chemiluminescence detection kit based on CPD STAR was used. DNA–DNA relatedness was determined by scanning the X-ray membranes and determining the intensities using the UVP Vision Acquisition and Analysis Software version 6.8 (BioSpectrumR imaging system). Self-hybridization values were considered to represent 100% of the maximum achievable signal and values obtained with the other strains were compared with this standard. Hybridization was carried out in triplicate for each sample. DNA–DNA relatedness between strain

CC-Nfb-7^T and *A. brasilense* DSM 1690^T, *A. rugosum* DSM 19657^T and *A. oryzae* JCM 21588^T was 38.9, 30.1 and 31.8%, respectively.

For analysis of DNA G+C content, DNA was prepared and degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture was then separated by HPLC. The DNA G+C content of strain CC-Nfb-7^T was 65.5 ± 0.6 mol%. For analysis of the fatty acids, strain CC-Nfb-7^T and its closest phylogenetic neighbours were grown on NA for 72 h at 30 °C and harvested by scraping. Fatty acid methyl esters were prepared, separated and identified according to the standard protocol of the Microbial Identification system (MIDI) (Sasser, 1990) using an Agilent 7890A GC fitted with a flame-ionization detector and the Aerobe (RTSBA6) database. The fatty acid composition of strain CC-Nfb-7^T was similar to those of recognized members of the genus *Azospirillum*. The major fatty acids in strain CC-Nfb-7^T were summed feature 8 (consisting of C_{18:1}ω7c and/or C_{18:1}ω6c; 56.4%), summed feature 3 (consisting of C_{16:1}ω7c and/or C_{16:1}ω6c; 15.1%), summed feature 2 (consisting of C_{14:0} 3-OH and/or iso-C_{16:1} I; 5.8%), C_{18:1} 2-OH (6.9%), C_{16:0} (4.4%) and C_{16:0} 3-OH (4.2%) (Table S1). For strain CC-Nfb-7^T, polar lipids were extracted and analysed by two-dimensional TLC according to Minnikin *et al.* (1984) and isoprenoid quinones were purified by the methods outlined in Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). The predominant quinone system was

ubiquinone (Q-10). The major lipids in strain CC-Nfb-7^T were phosphatidylcholine, phosphatidylglycerol and one unidentified phospholipid. Furthermore, moderate amounts of phosphatidylmethylethanolamine, phosphatidylethanolamine and one unidentified aminophospholipid were also determined (Fig. S2).

The physiological characteristics that differentiate strain CC-Nfb-7^T from other members of the genus *Azospirillum* are given in Table 1. The carbon source utilization pattern of strain CC-Nfb-7^T is given in the species description. Most members of the genus *Azospirillum* were positive for utilization of L-arabinose, glycerol and D-fructose, but negative for utilization of sucrose, L-rhamnose, maltose and lactose. Strain CC-Nfb-7^T utilized D-galactose and D-glucose, but *A. brasilense* DSM 1690^T and *A. rugosum* DSM 19657^T did not. With API 20 NE, strain CC-Nfb-7^T, *A. brasilense* DSM 1690^T, *A. rugosum* DSM 19657^T and *A. oryzae* JCM 21588^T reduced nitrate and nitrite, but strain CC-Nfb-7^T did not produce urease. Strain CC-Nfb-7^T, *A. brasilense* DSM 1690^T and *A. oryzae* JCM 21588^T assimilated L-arabinose, but *A. rugosum* DSM 19657^T did not. Strain CC-Nfb-7^T, *A. brasilense* DSM 1690^T and *A. oryzae* JCM 21588^T produced β -glucosidase and β -galactosidase and assimilated potassium gluconate and malic acid.

On the basis of morphological, chemotaxonomic and physiological data, strain CC-Nfb-7^T represents a novel species in the genus *Azospirillum*, for which the name *Azospirillum formosense* sp. nov. is proposed.

Description of *Azospirillum formosense* sp. nov.

Azospirillum formosense (for.mo.sen'se. N.L. neut. adj. *formosense* pertaining to Formosa, Taiwan).

Cells are Gram-negative, spiral or slightly curved rods (2.5 × 0.8 µm). Motile via a single polar flagellum. Oxidase- and catalase-positive. Pink colonies form after 48–72 h, which become wrinkled and dry with time. Cells can grow on nitrogen-free medium, NB and R2A agar. Grows at 20–37 °C (optimum 30 °C), at pH 5.0–9.0 (optimum pH 7.0) and with 0–2 % (w/v) NaCl. Positive for nitrogen fixation (25 nmol ethylene h⁻¹). Biotin is not necessary for growth. With Biolog GN II, the following carbon sources are utilized: Tweens 40 and 80, L-arabinose, D-fructose, D-galactose, α -D-glucose, D-psicose, pyruvic acid methyl ester, *cis*-aconitic acid, D-galacturonic acid, D-gluconic acid, α - and β -hydroxybutyric acids, α -ketoglutaric acid, DL-lactic acid, succinic acid, bromosuccinic acid, glucuronamide, L-alaninamide, D-alanine, L-glutamic acid, L-leucine, L-proline, L-serine and glycerol. With API 50 CH, positive for assimilation of glycerol, L-arabinose, D-xylose, D-fructose,

Table 1. Physiological differences between strain CC-Nfb-7^T and its relatives within the genus *Azospirillum*

Strains: 1, *Azospirillum formosense* sp. nov. CC-Nfb-7^T; 2, *A. brasilense* DSM 1690^T (Mehnaz *et al.*, 2007a); 3, *A. canadense* LMG 23617^T (Mehnaz *et al.*, 2007a,b); 4, *A. doebereineriae* DSM 13131^T (Eckert *et al.*, 2001); 5, *A. halopraeferens* DSM 3675^T (Reinhold *et al.*, 1987; Mehnaz *et al.*, 2007a); 6, *A. largimobile* DSM 9441^T (Ben Dekhil *et al.*, 1997); 7, *A. lipoferum* DSM 1691^T (Lavrinenko *et al.*, 2010); 8, *A. melinis* LMG 23364^T (Peng *et al.*, 2006); 9, *A. oryzae* JCM 21588^T (Mehnaz *et al.*, 2007b); 10, *A. picis* DSM 19922^T (Lin *et al.*, 2009); 11, *A. rugosum* DSM 19657^T (Young *et al.*, 2008); 12, *A. thiophilum* DSM 21654^T (Lavrinenko *et al.*, 2010); 13, *A. zeae* LMG 23989^T (Mehnaz *et al.*, 2007b; Mehnaz & Lazarovits, 2006). Data in parentheses were taken from this study. None of the strains utilized sucrose. +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13			
Polymorphic cells	—	—	ND	+	+	+	+	ND	ND	ND	ND (+)	+	ND			
Growth with 3 % NaCl	—	—	—	—	+	—	—	+	—	—	—	+	—			
pH range	5.0–9.0	6.0–8.0	5.0–7.0	6.0–7.0	6.8–8.0	ND	5.7–6.8	4.0–8.0	6.0–7.0	6.5–9.0	ND	6.5–8.5	5.0–7.0			
Temperature optimum (°C)	30	37	25–30	30	41	28	37	20–33	30	37	22–37	37	30			
Nitrate reduction	+	+	(+)	+	—	—	+	+	+	(+)	+	+	+			
Urease	—	+	(+)	—	+	+	ND	—	ND	+	(+)	+	—			
Carbon sources																
L-Arabinose	+	+	(+)	—	+	+	+	+	+	(+)	—	—	(—)	+	+	
D-Galactose	+	—	(—)	—	+	—	+	+	+	+	(+)	+	—	(—)	+	+
D-Glucose	+	—	(—)	—	—	—	+	+	+	+	(+)	+	—	(—)	+	+
Glycerol	+	+	(+)	—	+	+	+	+	+	+	(+)	+	+	(—)	+	+
D-Mannitol	—	—	(—)	—	+	+	+	+	+	—	(—)	+	—	(—)	+	+
D-Sorbitol	—	—	(—)	—	+	—	+	+	+	—	(—)	+	—	(—)	+	+
D-Fructose	+	+	(+)	—	+	+	+	+	+	+	(+)	+	+	(+)	+	+
L-Rhamnose	—	—	(—)	—	—	ND	—	—	+	—	(—)	—	—	(—)	—	—
Maltose	—	—	(—)	—	—	ND	—	—	+	—	(—)	+	—	(—)	+	—
Lactose	—	—	(—)	—	—	ND	—	—	+	—	(—)	—	—	(—)	—	—
Biotin requirement	—	—	(—)	—	—	+	(—)	—	+	(—)	—	ND	(—)	—	—	—
DNA G + C content (mol%)	64–66	69–71	67.9	70.7	68–70	70	69–70	68.7	66.8	68.7	68.7	ND	67	67.6		

aesculin ferric citrate, D-fucose and potassium gluconate. With API ZYM, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β -glucosidase. Reduces nitrate and nitrite. Produces acid phosphatase, β -glucosidase and β -galactosidase, but not DNase. The fatty acids are mainly straight-chain saturated and unsaturated fatty acids: the major fatty acid (>50 %) is summed feature 8 (consisting of C_{18:1} ω 7c and/or C_{18:1} ω 6c) and the major hydroxyl fatty acids are C_{16:0} 3-OH and C_{18:0} 2-OH. The polar lipids are phosphatidylglycerol, phosphatidylcholine, an unknown phospholipid, phosphatidylethanolamine, phosphatidylmethylethanolamine and an unknown aminolipid. The predominant quinone system is ubiquinone (Q-10).

The type strain is CC-Nfb-7^T (=BCRC 80273^T=JCM 17639^T=DSM 24137^T), a diazotroph isolated from agricultural soil in Yunlin County, Taiwan. The DNA G+C content of the type strain is 65.5 $\pm$ 0.6 mol%.

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References

- Aziz, A., Martin-Tanguy, J. & Larher, F. (1997). Plasticity of polyamine metabolism associated with high osmotic stress in rape leaf discs and with ethylene treatment. *Plant Growth Regul* **21**, 153–163.
- Bally, R., Thomas-Bauzon, D., Heulin, T., Balandreau, J., Richard, C. & De Ley, J. (1983). Determination of the most frequent N₂-fixing bacteria in a rice rhizosphere. *Can J Microbiol* **29**, 881–887.
- Bashan, Y., Holguin, G. & de-Bashan, L. E. (2004). *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). *Can J Microbiol* **50**, 521–577.
- Ben Dekhil, S., Cahill, M., Stackebrandt, E. & Sly, L. I. (1997). Transfer of *Conglomeromonas largomobilis* subsp. *largomobilis* to the genus *Azospirillum* as *Azospirillum largomobile* comb. nov., and elevation of *Conglomeromonas largomobilis* subsp. *parooensis* to the new type species of *Conglomeromonas*, *Conglomeromonas parooensis* sp. nov. *Syst Appl Microbiol* **20**, 72–77.
- Collins, M. D. (1985). Isoprenoid quinone analysis in classification and identification. In *Chemical Methods in Bacterial Systematics*, pp. 267–287. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Döbereiner, J. & Day, J. M. (1976). Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. In *Proceedings of the First International Symposium on N₂ Fixation*, pp. 518–538. Edited by W. E. Newton & C. J. Nyman. Pullman: Washington State University Press.
- Eckert, B., Weber, O. B., Kirchhof, G., Halbritter, A., Stoffels, M. & Hartmann, A. (2001). *Azospirillum dobereineriae* sp. nov., a nitrogen-fixing bacterium associated with the C₄-grass *Miscanthus*. *Int J Syst Evol Microbiol* **51**, 17–26.
- Falk, E. C., Döbereiner, J., Johnson, J. L. & Krieg, N. R. (1985). Deoxyribonucleic acid homology of *Azospirillum amazonense* Magalhães *et al.* 1984 and emendation of the description of the genus *Azospirillum*. *Int J Syst Bacteriol* **35**, 117–118.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Hardy, R., Burns, R. C. & Holsten, R. D. (1973). Application of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biol Biochem* **5**, 47–81.
- Heiner, C. R., Hunkapiller, K. L., Chen, S. M., Glass, J. I. & Chen, E. Y. (1998). Sequencing multimegabase-template DNA with BigDye terminator chemistry. *Genome Res* **8**, 557–561.
- Helsel, L. O., Hollis, D. G., Steigerwalt, A. G. & Levett, P. N. (2006). Reclassification of *Roseomonas fauriae* Rihs *et al.* 1998 as a later heterotypic synonym of *Azospirillum brasilense* Tarrand *et al.* 1979. *Int J Syst Evol Microbiol* **56**, 2753–2755.
- Hung, M.-H., Bhagwath, A. A., Shen, F.-T., Devasya, R. P. & Young, C.-C. (2005). Indigenous rhizobia associated with native shrubby legumes in Taiwan. *Pedobiologia (Jena)* **49**, 577–584.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Khammas, K. M., Ageron, E., Grimon, P. A. D. & Kaiser, P. (1989). *Azospirillum irakense* sp. nov., a nitrogen-fixing bacterium associated with rice roots and rhizosphere soil. *Res Microbiol* **140**, 679–693.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kirchhof, G., Reis, V. M., Baldan, J. I., Eckert, B., Döbereiner, J. & Hartmann, A. (1997). Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. *Plant Soil* **194**, 45–55.
- Koch, B. & Evans, H. J. (1966). Reduction of acetylene to ethylene by soybean root nodules. *Plant Physiol* **41**, 1748–1750.
- Ladha, J. K., So, R. B. & Watanabe, I. (1987). Composition of *Azospirillum* species associated with wetland rice plants grown in different soils. *Plant Soil* **102**, 127–129.
- Lavrinenko, K., Chernousova, E., Gridneva, E., Dubinina, G., Akimov, V., Kuever, J., Lysenko, A. & Grabovich, M. (2010). *Azospirillum thiophilum* sp. nov., a diazotrophic bacterium isolated from a sulfide spring. *Int J Syst Evol Microbiol* **60**, 2832–2837.
- Lin, S.-Y., Young, C.-C., Hupfer, H., Siering, C., Arun, A. B., Chen, W.-M., Lai, W.-A., Shen, F.-T., Rekha, P. D. & Yassin, A. F. (2009). *Azospirillum picis* sp. nov., isolated from discarded tar. *Int J Syst Evol Microbiol* **59**, 761–765.
- Mehnaz, S. & Lazarovits, G. (2006). Inoculation effects of *Pseudomonas putida*, *Gluconacetobacter azotocaptans* and *Azospirillum lipoferum* on corn plant growth under green house conditions. *Microb Ecol* **51**, 326–335.
- Mehnaz, S., Weselowski, B. & Lazarovits, G. (2007a). *Azospirillum canadense* sp. nov., a nitrogen-fixing bacterium isolated from corn rhizosphere. *Int J Syst Evol Microbiol* **57**, 620–624.
- Mehnaz, S., Weselowski, B. & Lazarovits, G. (2007b). *Azospirillum zae* sp. nov., a diazotrophic bacterium isolated from rhizosphere soil of *Zea mays*. *Int J Syst Evol Microbiol* **57**, 2805–2809.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

- Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, K. & Parlett, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* 2, 233–241.
- Murray, R. G. E., Doetsch, R. N. & Robinow, F. (1994). Determinative and cytological light microscopy. In *Methods for General and Molecular Bacteriology*, pp. 21–41. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Okon, Y. & Itzigsohn, R. (1992). Poly- β -hydroxybutyrate metabolism in *Azospirillum brasilense* and the ecological role of PHB in the rhizosphere. *FEMS Microbiol Lett* 103, 131–139.
- Okon, Y. & Vanderleyden, J. (1997). Root-associated *Azospirillum* species can stimulate plants. *ASM News* 63, 366–370.
- Patriquin, D. G., Döbereiner, J. & Jain, D. K. (1983). Sites and processes of association between diazotrophs and grasses. *Can J Microbiol* 29, 900–915.
- Peng, G., Wang, H., Zhang, G., Hou, W., Liu, Y., Wang, E. T. & Tan, Z. (2006). *Azospirillum melinis* sp. nov., a group of diazotrophs isolated from tropical molasses grass. *Int J Syst Evol Microbiol* 56, 1263–1271.
- Poly, F., Monrozier, L. J. & Bally, R. (2001). Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol* 152, 95–103.
- Reinhold, B., Hurek, T., Fendrik, I., Pot, B., Gillis, M., Kersters, K., Thielemans, S. & De Ley, J. (1987). *Azospirillum halopraeferens* sp. nov., a nitrogen-fixing organism associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth). *Int J Syst Bacteriol* 37, 43–51.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4, 406–425.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Saxena, B., Modi, M. & Modi, V. V. (1986). Isolation and characterization of siderophores from *Azospirillum lipoferum* D-2. *J Gen Microbiol* 132, 2219–2224.
- Seldin, L. & Dubnau, D. (1985). Deoxyribonucleic acid homology among *B. polymyxa*, *B. macerans*, *B. azotofixans* and other nitrogen-fixing *Bacillus* strains. *Int J Syst Bacteriol* 35, 151–154.
- Seshadri, S., Muthukumarasamy, R., Lakshinarasimhan, C. & Ignacimuthu, S. (2000). Solubilization of inorganic phosphates by *Azospirillum halopraeferans*. *Curr Sci* 79, 565–567.
- 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.
- Steenhoudt, O. & Vanderleyden, J. (2000). *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiol Rev* 24, 487–506.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24, 1596–1599.
- Tarrand, J. J., Krieg, N. R. & Döbereiner, J. (1978). A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can J Microbiol* 24, 967–980.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25, 4876–4882.
- Thuler, D. S., Floh, E. I., Handro, W. & Barbosa, H. R. (2003). Plant growth regulators and amino acids released by *Azospirillum* sp. in chemically defined media. *Lett Appl Microbiol* 37, 174–178.
- Tien, T. M., Gaskins, M. H. & Hubbell, D. H. (1979). Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Appl Environ Microbiol* 37, 1016–1024.
- Watts, D. & MacBeath, J. R. (2001). Automated fluorescent DNA sequencing on the ABI PRISM 310 Genetic Analyzer. *Methods Mol Biol* 167, 153–170.
- Xie, C. H. & Yokota, A. (2005). *Azospirillum oryzae* sp. nov., a nitrogen-fixing bacterium isolated from the roots of the rice plant *Oryza sativa*. *Int J Syst Evol Microbiol* 55, 1435–1438.
- Young, C.-C., Hupfer, H., Siering, C., Ho, M.-J., Arun, A. B., Lai, W.-A., Rekha, P. D., Shen, F.-T., Hung, M.-H. & other authors (2008). *Azospirillum rugosum* sp. nov., isolated from oil-contaminated soil. *Int J Syst Evol Microbiol* 58, 959–963.