Azospirillum canadense sp. nov., a nitrogen-fixing bacterium isolated from corn rhizosphere

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A free-living diazotrophic strain, DS2^T, was isolated from corn rhizosphere. Polyphasic taxonomy was performed including morphological characterization, Biolog analysis, and 16S rRNA, *cpn60* and *nifH* gene sequence analyses. 16S rRNA gene sequence analysis indicated that strain DS2^T was closely related to the genus *Azospirillum* (96 % similarity). Chemotaxonomic characteristics (DNA G+C content 67.9 mol%; Q-10 quinone system; major fatty acid 18 : 1ω7*c*) were also similar to those of the genus *Azospirillum*. In all the analyses, including phenotypic characterization using Biolog analysis and comparison of cellular fatty acids, this isolate was found to be different from the closely related species *Azospirillum lipoferum*, *Azospirillum oryzae* and *Azospirillum brasilense*. On the basis of these results, a novel species is proposed for this nitrogen-fixing strain. The name *Azospirillum canadense* sp. nov. is suggested with the type strain DS2^T (=NCCB 100108^T=LMG 23617^T).

The genus *Azospirillum* was first described by Tarrand *et al.* (1978) with two species, *Azospirillum lipoferum* and *Azospirillum brasilense*. At present the genus comprises nine species, including, in addition to *A. lipoferum* and *A. brasilense*, *Azospirillum amazonense* (Magalhães *et al.*, 1983), *Azospirillum halopraeferens* (Reinhold *et al.*, 1987), *Azospirillum irakense* (Khammas *et al.*, 1989), *Azospirillum largimobile* (Ben Dekhil *et al.*, 1997), *Azospirillum doebereinerae* (Eckert *et al.*, 2001), *Azospirillum oryzae* (Xie & Yokota, 2005) and *Azospirillum melinis* (Peng *et al.*, 2006). A few *Azospirillum* strains including *A. brasilense* and *A. oryzae* were isolated from rhizosphere of corn, growing in Western Ontario, Canada. Strain DS2^T was isolated from rhizosphere soil. It was identified as *Azospirillum* due to its cell shape, colony morphology, nitrogen fixation and 96 % similarity for the 16S rRNA gene sequence but the species was not identified as it did not show close similarity to any known species of this genus. Strain DS2^T was screened for its growth-promoting activity and it showed significant growth promotion in two corn varieties and vegetables, under greenhouse conditions. This strain is on field trial for growth promotion in corn at two research stations in Ontario, Canada. In the present study, we characterized this strain by using a polyphasic approach, including phylogenetic analyses of the 16S rRNA, *cpn60* and *nifH* gene sequences.

Isolate DS2^T was isolated on M medium (Xie & Yokota, 2005) except that biotin was not added and pH 7.2–7.4 was used. Subcultivation was done on the same medium at 30 °C for 48–72 h. The bacterium formed wet, white colonies which later turned light-pink. Cell morphology was observed using a scanning electron microscope. Cells of the bacterium were short rods 0.9 μm in width and 1.8–2.5 μm in length, with a single polar flagellum (Fig. 1). Bacterial growth at different temperatures (20–41 °C) and pH values (4–10) and with various NaCl concentration (0.5–3 %) ranges was determined in M medium. The Biolog analysis system and API 20NE bacterial identification kit were used for physiological characterization. Results of the analyses are given in the species description. A summary of the results of carbon-source utilization suitable for the differentiation of isolate DS2^T from known *Azospirillum* species is presented in Table 1. Phosphate solubilization on NBRIP medium (Nautiyal, 1999) was not observed. Indole acetic acid production in the presence of 100 mg l^{-1} tryptophan in CCM (Rennie, 1981) was ~6.5 μg ml^{-1}.

Cellular fatty acid profiles of isolate DS2^T, *A. oryzae* IAM 15130^T, *A. lipoferum* ATCC 29707^T and *A. brasilense* ATCC 29145^T were determined with a gas chromatograph, using the Sherlock Microbial Identification System (MIDI), according to a standard protocol (Paisley, 1996), and data are provided in Table 2. The fatty acid profile of strain DS2^T was composed of 18 : 1ω7*c* (54.9 %), 16 : 1ω7*c* (12.0 %), 16 : 0 (12.3 %) and 19 : 0 cyclo ω8*c* (0.9 %); and hydroxy fatty acids 16 : 0 3-OH (2.2 %) and 18 : 1 2-OH (2.2 %). Fatty acids 12 : 0 (1.3 %) and summed feature 6 (1.5 %) were detected in DS2^T but not in *A. lipoferum*, *A. oryzae* or *A. brasilense*. Its ratio for 18 : 1ω7*c* is closest to that of *A. lipoferum* (53.4 %) but values for 19 : 0 cyclo ω8*c*, 16 : 0 3-OH, 18 : 1 2-OH, 17 : 1ω8*c* and 17 : 1ω6*c* are almost two times lower than those of *A. lipoferum* (1.6, 4.3, 5.5, 3.4 and

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *cpn60* and *nifH* gene sequences of strain DS2^T are DQ393891, DQ914833 and DQ393890, respectively.

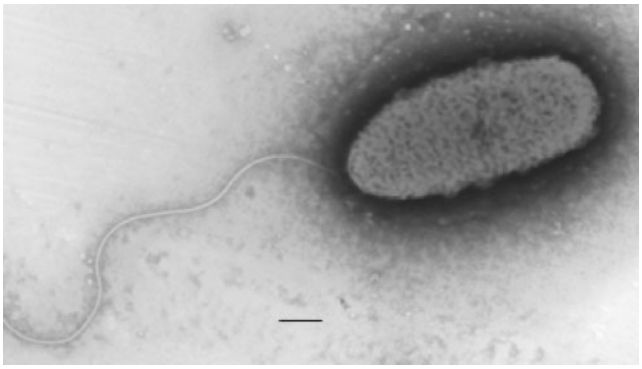

Fig. 1. Cell morphology of corn isolate DS2^T observed under an electron microscope. Bar, 0.6 μm.

7.1 %). For fatty acids 14 : 0, 16 : 0 and 18 : 0, isolate DS2^T has threefold higher values than those for *A. lipoferum*. *A. oryzae* has similar a ratio to DS2^T for 19 : 0 cyclo ω8*c* (0.8 %) and 18 : 1ω7*c* (57.2 %) but values for 16 : 0 3-OH (4.1 %) and 18 : 1 2-OH (5.5 %) are higher and values for 14 : 0 (0.7 %), 16 : 0 (4.3 %), 18 : 0 (0.5 %), 17 : 1ω8*c* (0.8 %) and 17 : 1ω6*c* (1.4 %) are two- to threefold lower than those of DS2^T. *A. brasilense* has a very different profile from that of *A. lipoferum*, *A. oryzae* and isolate DS2^T and values for all the fatty acids used for comparison are either very high or very low compared to those for DS2^T.

Determination of DNA base composition was carried out using the HPLC technique described by Mesbah *et al.* (1989). The DNA G+C content of strain DS2^T was 67.9 mol%, which is in accordance with values for the genus *Azospirillum* (64–71 mol%; Ben Dekhil *et al.*, 1997). The 16S rRNA gene was amplified by using the primers and PCR conditions previously described by Mehnaz *et al.* (2001). The sequence was deposited in GenBank (accession no. DQ393891). Phylogenetic analysis was performed using the software package Bionumerics (Applied Maths) after including the consensus sequence in an alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library EMBL. The alignment was pairwise, calculated by using an open gap penalty of 100 % and a unit gap penalty of 0 %. A similarity matrix was created by homology calculation with a gap penalty of 0 % and after discarding unknown bases. A resulting tree based on comparison of 1475 bases was constructed using the

Table 1. Physiological differences between *Azospirillum canadense* sp. nov. isolate DS2^T and other *Azospirillum* species

Strains: 1, DS2^T; 2, *A. oryzae* IAM 15130^T; 3, *A. lipoferum* ATCC 29707^T; 4, *A. brasilense* ATCC 29145^T; 5, *A. dobereinerae*; 6, *A. largimobile*; 7, *A. halopraeferens*; 8, *A. irakense*; 9, *A. amazonense* LMG 22237^T; 10, *A. melinis* TMCY 0552^T. +, Positive; −, negative; V, variable; ND, not determined. Data for DS2^T, *A. oryzae*, *A. lipoferum*, *A. brasilense* and *A. melinis* are from this study. The remaining data were taken from Peng *et al.* (2006).

Characteristics	1	2	3	4	5	6	7	8	9	10
Biotin requirement	−	+	+	−	−	−	+	−	−	−
Growth in 3 % NaCl	−	−	−	−	−	−	+	+	−	−
Carbon source										
N-Acetylglucosamine	−	−	+	−	−	+	ND	+	V	+
L-Arabinose	−	+	+	+	ND	+	V	+	+	+
D-Cellobiose	−	−	−	−	−	−	ND	+	+	−
D-Fructose	−	+	+	+	+	+	+	V	+	+
L-Fucose	−	−	+	−	ND	−	ND	+	+	+
D-Galactose	−	+	+	−	V	+	−	+	+	+
Gentiobiose	−	−	−	−	−	+	ND	+	+	−
D-Glucose	−	+	+	−	V	+	−	+	+	+
Glycerol	−	+	+	+	+	+	+	−	−	+
myo-Inositol	−	−	+	−	−	−	ND	−	+	−
Lactose	−	−	−	−	−	−	ND	+	V	−
Maltose	−	−	−	−	−	−	ND	+	+	+
D-Mannitol	−	−	+	−	+	+	+	−	−	+
D-Mannose	−	−	+	−	−	−	+	+	+	−
L-Rhamnose	−	−	−	−	−	−	ND	+	V	−
D-Sorbitol	−	−	−	−	+	+	−	−	−	+
Sucrose	−	−	−	−	−	ND	−	+	+	−
D-Trehalose	−	−	−	−	−	−	ND	+	+	−
Optimal temperature for growth (°C)	25–30	30	37	37	30	28	41	33	30	20–33
DNA G+C content (mol%)	67.9	66.8	69–70	69–71	70.7	70	68–70	64–67	66–68	68.7

Table 2. Comparison of the cellular fatty acid content of corn rhizosphere isolate DS2^T and related *Azospirillum* species

Data for all organisms are from this study. Fatty acid values are given as a percentage of the total peak area. Summed feature 1 = 13 : 0 3-OH/15 : 1 isoH; summed feature 2 = 12 : 0 aldehyde?16 : 1 iso I/14 : 0 3-OH; summed feature 3 = 16 : 1ω7*c*/16 : 1ω6*c*; summed feature 6 = 18 : 0 ante/18 : 2ω6, 9*c*. ND, Not detected.

Fatty acid	DS2^T	*A. oryzae* IAM 15130^T	*A. lipoferum* ATCC 29707^T	*A. brasilense* ATCC 29145^T
13 : 1 at 12-13	ND	0.6	0.7	0.3
12 : 0	1.3	ND	ND	ND
14 : 0	2.3	0.5	0.7	0.3
15 : 0	1.3	0.5	1.4	0.2
16 : 0	12.3	6.9	4.3	5.0
17 : 0	ND	0.3	0.8	0.1
18 : 0	2.2	0.7	0.5	0.2
15 : 0 3-OH	ND	ND	0.8	0.2
16 : 0 3-OH	2.2	4.1	4.3	3.2
17 : 0 3-OH	ND	ND	0.6	ND
18 : 0 3-OH	ND	0.5	0.5	0.1
18 : 1 2-OH	2.2	5.0	5.5	5.2
15 : 1ω8*c*	ND	ND	0.6	0.1
15 : 1ω6*c*	ND	ND	0.2	ND
17 : 1ω8*c*	1.7	0.8	3.4	0.8
17 : 1ω6*c*	2.8	1.4	7.1	1.5
18 : 1ω7*c*	54.9	57.2	53.4	62.0
19 : 0 cyclo ω8*c*	0.9	0.8	1.6	ND
Summed feature 1	ND	ND	0.5	ND
Summed feature 2	0.7	5.3	5.9	4.7
Summed feature 3	12.0	14.6	6.5	14.9
Summed feature 6	1.5	ND	ND	ND

neighbour-joining method. Bootstrap analysis was performed using the same software package to test the statistical reliability of the topology of the neighbour-joining tree with 1000 bootstrap resamples of the data. On the basis of distance matrix, the percentage 16S rRNA gene sequence similarity indicated that the closest relatives to strain DS2^T are '*Roseomonas*' genomospecies 6 ATCC 49961^T (96.1 %), *A. oryzae* IAM 15130^T (95.9 %), *A. lipoferum* ATCC 29707^T (95.9 %) and *A. brasilense* Sp7^T (95.5 %). The phylogenetic tree based on 16S rRNA gene sequence, constructed by using the neighbour-joining method, is shown in Fig. 2.

A chaperonin gene (*cpn60*) was amplified from bacterial genomic DNA of isolate DS2^T, *A. lipoferum* ATCC 29707^T, *A. oryzae* IAM 15130^T and *A. brasilense* ATCC 29145^T by using universal *cpn60* degenerate primers. The primers were H729, H730 (Hill *et al.*, 2006), H1610 (5′-CGCCAGGGTTTTCCCAGTCACGACGAIIIIGCIGGYGACGGYACSACSAC-3′) and H1611 (5′-AGCGGATAACAATTTCACACAGGACGRCGRTCRCCGAAGCCSGGIGCCTT-3′). For PCR, a primer mixture (forward mix 1 part H729 and 3 parts H1610; reverse mix 1 part H730 and 3 parts H1611) was used. The PCR conditions were 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 50 °C, 45 s at 72 °C and 5 min at 72 °C. The DNA sequence of DS2^T showed 97.3 % (539/554 identities), 93.6 % (510/545 identities) and 83.2 % (445/535 identities) sequence similarity with *cpn60* sequences of *A. brasilense* (accession no. DQ854727), *A. lipoferum* (accession no. DQ813650) and *A. oryzae* (accession no. DQ813649), respectively.

Semi-solid M medium was used for the acetylene reduction assay and it was carried out as described by Mehnaz & Lazarovits (2006). Veil-like subsurface pellicle formation was observed and an ethylene peak was detected. The *nifH* gene was also amplified by PCR using the primer set PolF/PolR and the conditions described by Poly *et al.* (2001). The expected 360 bp amplification product was observed. This PCR product was purified and sequenced. The sequence was deposited in GenBank (accession no. DQ393890). Comparison of the results through an NCBI BLAST search revealed highest sequence similarities with the *nifH* gene of *A. lipoferum* ATCC 29707^T (95.4 %), *A. brasilense* Sp7^T (94.8 %) and *A. oryzae* IAM 15130^T (93.9 %). However, the similarities with the *nifH* gene of other diazotrophic bacteria were 89–91 %.

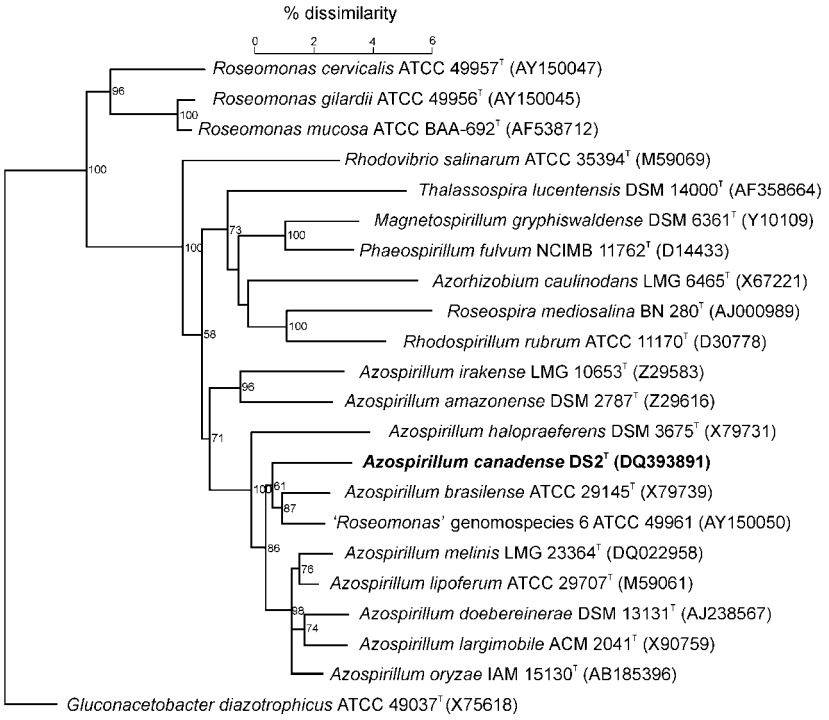

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences, constructed by using the neighbour-joining method, showing close relationship between strain DS2^T and the nearest relatives of the genus *Azospirillum*. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 60 % are shown.

Description of *Azospirillum canadense* sp. nov.

Azospirillum canadense (can.ad.en′se. N.L. neut. adj. *canadense* pertaining to Canada, the region of isolation, referring to its isolation from Canadian soil).

Cells are short rods, 0.9 × 1.8–2.5 μm in size, Gram-negative, motile via a single polar flagellum. White to light-pink, rounded, wet colonies form after 48–72 h. Growth occurs on M medium at 20–37 °C, pH 5–7 and 0.5–1 % NaCl concentration. Optimum temperature is 25–30 °C and optimum pH is 5–7. Positive for nitrogen fixation and indole acetic acid production; negative for phosphate solubilization. Malic acid, potassium gluconate, acetic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid, D-glucuronic acid, α and β-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, D-alanine, L-asparagine and L-aspartic acid can be use as single carbon source. Sucrose, D-glucose, L-arabinose, D-arabitol, D-cellobiose, L-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, *myo*-inositol, D-lactose, D-mannose, D-mannitol, maltose, D-melibiose, D-raffinose, L-rhamnose, D-sorbitol, D-trehalose, xylitol, D-gluconic acid, α-ketobutyric acid, L-alanine, L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, D-serine, L-serine, L-threonine, *N*-acetyl D-glucosamine, trisodium acetate, capric acid, adipic acid and phenylacetic acid are not utilized. Positive for catalase, oxidase, nitrate reduction, β-glucosidase, β-galactosidase and acetoin production and negative for indole production, arginine dihydrolase, urease and gelatin hydrolysis. Biotin is not required for growth. Major cellular fatty acids are 18 : 1ω7*c*, 16 : 1ω7*c*, 16 : 0. The DNA G+C content is 67.9 mol%. The predominant quinone system is ubiquinone Q-10.

The type strain, DS2^T (=NCCB 100108^T=LMG 23617^T), was isolated from rhizosphere of corn (*Zea mays*) from Delhi, Ontario, Canada.

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