Azospirillum zeae sp. nov., a diazotrophic bacterium isolated from rhizosphere soil of *Zea mays*

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Two free-living nitrogen-fixing bacterial strains, N6 and N7^T, were isolated from corn rhizosphere. A polyphasic taxonomic approach, including morphological characterization, Biolog analysis, DNA–DNA hybridization, and 16S rRNA, *cpn60* and *nifH* gene sequence analysis, was taken to analyse the two strains. 16S rRNA gene sequence analysis indicated that strains N6 and N7^T both belonged to the genus *Azospirillum* and were closely related to *Azospirillum oryzae* (98.7 and 98.8 % similarity, respectively) and *Azospirillum lipoferum* (97.5 and 97.6 % similarity, respectively). DNA–DNA hybridization of strains N6 and N7^T showed reassociation values of 48 and 37 %, respectively, with *A. oryzae* and 43 % with *A. lipoferum*. Sequences of the *nifH* and *cpn60* genes of both strains showed 99 and ~95 % similarity, respectively, with those of *A. oryzae*. Chemotaxonomic characteristics (Q-10 as quinone system, 18 : 1ω7*c* as major fatty acid) and G+C content of the DNA (67.6 mol%) were also similar to those of members of the genus *Azospirillum*. Gene sequences and Biolog and fatty acid analysis showed that strains N6 and N7^T differed from the closely related species *A. lipoferum* and *A. oryzae*. On the basis of these results, it is proposed that these nitrogen-fixing strains represent a novel species. The name *Azospirillum zeae* sp. nov. is suggested, with N7^T (=NCCB 100147^T=LMG 23989^T) as the type strain.

Diverse nitrogen-fixing bacteria have been isolated from the roots of numerous wild and cultivated grasses grown in tropical, subtropical and temperate regions all over the world. Among these bacteria, *Azospirillum* spp. have been isolated from various geographical regions of the world. Members of this genus are known to be associated with roots of numerous wild and cultivated grasses, cereals, food crops and soils in various regions (Peng *et al.*, 2006). The genus *Azospirillum* was first described by Tarrand *et al.* (1978) and comprised two species: *Azospirillum lipoferum* and *Azospirillum brasilense*. The genus currently comprises 10 species, including *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), *Azospirillum melinis* (Peng *et al.*, 2006) and *Azospirillum canadense* (Mehnaz *et al.*, 2007). In studies aimed at identifying the bacterial rhizosphere

Abbreviation: IAA, indole acetic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *cpn60* and *nifH* gene sequences of strain N6 are DQ682469, DQ682474 and DQ682471, respectively; the corresponding accession numbers for strain N7^T are DQ682470, DQ682473 and DQ682472.

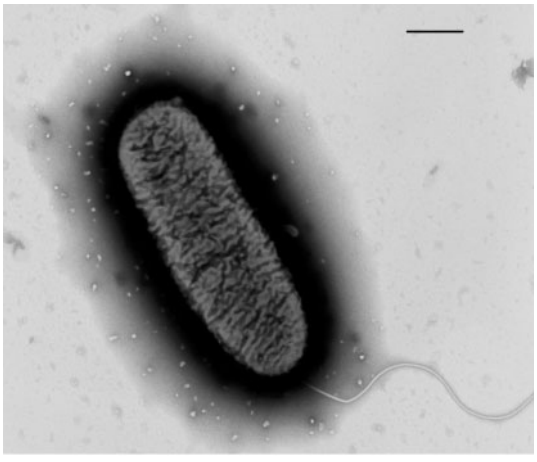

Fig. 1. Electron micrograph showing cell morphology of corn isolate N6. Bar, 0.6 μm.

Table 1. Physiological differences between *Azospirillum zeae* sp. nov. isolates N6 and N7^T and other *Azospirillum* species

Taxa: 1, *A. zeae* N7^T and N6; 2, *A. oryzae* IAM 15130^T; 3, *A. lipoferum* ATCC 29707^T; 4, *A. melinis* TMCY 0552^T; 5, *A. doebereinerae*. +, Positive; –, negative; V, variable; ND, not determined. Data for carbon source utilization by *A. oryzae*, *A. lipoferum* and *A. melinis* are from Mehnaz *et al.* (2007); data for N6, N7^T and *A. doebereinerae* are from this study. Other data for *A. oryzae*, *A. lipoferum*, *A. melinis* and *A. doebereinerae* are taken from Eckert *et al.* (2001), Peng *et al.* (2006) and Xie & Yokota (2005).

Characteristic	1	2	3	4	5*
Motility	+	+	+	−	+
Biotin requirement	−	+	+	−	−
Growth with 2 % NaCl	−	−	−	+	−
Growth at 41 °C	+	−	−	−	−
Optimum growth temperature (°C)	30	30	37	20–33	30
pH range for growth	5.0–7.0	6.0–7.0	6.0–7.0	4.0–8.0	6.0–7.0
Gelatin hydrolysis	−	+	+	ND	−
Carbon source utilization:					
N-Acetyl-D-glucosamine	V	−	+	+	−
L-Arabinose	+	+	+	+	+
D-Cellobiose	−	−	−	−	−
D-Fructose	+	+	+	+	+
L-Fucose	+	−	+	+	−
D-Galactose	+	+	+	+	+
Gentiobiose	−	−	−	−	−
D-Glucose	V	+	+	+	+
Glycerol	+	+	+	+	+
myo-Inositol	−	−	+	−	−
Lactose	−	−	−	−	−
Maltose	−	−	−	+	−
D-Mannitol	+	−	+	+	+
D-Mannose	−	−	+	−	−
L-Rhamnose	−	−	−	−	−
D-Sorbitol	+	−	−	+	+
Sucrose	−	−	−	−	−
Trehalose	−	−	−	−	−
DNA G+C content (mol%)	67.6	66.8	69–70	68.7	70.7

*DNA G+C content and carbon source utilization data are for *A. doebereinerae* GSF71^T; all other data refer to more than one strain.

residents of corn plants grown in Southern Ontario, Canada, several bacterial strains were isolated from soil adhering to corn roots. Some of them were found to fix nitrogen and form pink dry colonies on malate medium. These bacteria were compared with *Azospirillum* spp. by using microbial and molecular biology techniques. Previously, on the basis of partial 16S rRNA gene sequencing, isolate N7^T was identified and reported as *A. lipoferum* (Mehnaz & Lazarovits, 2006). In this study, this isolate has been re-identified and isolation and identification of another *Azospirillum* strain, N6, from corn rhizosphere using a polyphasic approach is reported.

Isolates N6 and N7^T were isolated on M medium (Xie &Yokota, 2005) except that biotin was not added and the medium was pH 7.2–7.4. Subcultivation was done on the same medium at 30 °C for 48–72 h. The isolates formed flat pink colonies on M medium. With time, these colonies became dried and wrinkled. Cell morphology was observed by transmission electron microscopy (Zeiss EM902). Cell morphology of N6 and N7^T was similar. Both strains showed a single polar flagellum in liquid medium, although N7^T cells were bigger than those of N6; cells of N6 were 0.9–1.1 × 1.9–3.3 μm (Fig. 1) and those of N7^T were 1.5 × 3.4–6.8 μm. Bacterial growth at different temperatures (20–41 °C), pH values (4–10) and various NaCl concentrations (0.5–3.0 %) was determined on 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, including carbon source utilization tests, suitable for the differentiation of isolates N6 and N7^T from known *Azospirillum* species is presented in Table 1. In Table 1, N6, N7^{T}and *A.*

Table 2. Cellular fatty acid composition of corn rhizosphere isolates N6 and N7^T and closely related *Azospirillum* species

Strains: 1, N6; 2, N7^T; 3, *A. oryzae* IAM 15130^T (data from Mehnaz *et al.*, 2007); 4, *A. lipoferum* ATCC 29707^T (data from Mehnaz *et al.*, 2007). Fatty acid values are given as percentages of the total peak area. ND, Not detected.

Fatty acid	1	2	3	4
13 : 1 at 12–13	ND	0.34	0.6	0.7
14 : 0	0.7	0.6	0.5	0.7
15 : 0	0.7	0.8	0.5	1.4
16 : 0	5.6	5.6	6.9	4.3
17 : 0	0.4	0.5	0.3	0.8
18 : 0	0.8	0.4	0.7	0.5
15 : 0 3-OH	0.3	0.4	ND	0.8
16 : 0 3-OH	4.3	4.2	4.1	4.3
17 : 0 3-OH	ND	0.3	ND	0.6
18 : 0 3-OH	0.6	0.4	0.5	0.5
18 : 1 2-OH	6.3	5.7	5.0	5.5
15 : 1ω8*c*	0.3	0.4	ND	0.6
17 : 1ω8*c*	1.9	2.1	0.8	3.4
17 : 1ω6*c*	3.2	3.7	1.4	7.1
18 : 1ω7*c*	54.4	54.9	57.2	53.4
19 : 0 cyclo ω8*c*	ND	ND	0.8	1.6
Summed feature 2*	5.6	5.5	5.3	5.9
Summed feature 3*	14.4	13.9	14.6	6.5

*Summed features: summed feature 2, 12 : 0 aldehyde (unknown), 16 : 1 iso I/14 : 0 3-OH; summed feature 3, 16 : 1ω7*c*/16 : 1ω6*c*.

melinis showed the same pattern for carbon source utilization, but there are 28 carbon sources that can be used by *A. melinis*, but not by N6 and N7^T (data not shown). Phosphate solubilization on NBRIP medium (Nautiyal, 1999) was not observed. Indole acetic acid (IAA) production in the presence of 100 mg tryptophan l^{-1} in CCM (Rennie, 1981) was quantified by HPLC as described by Mehnaz *et al.* (2001). The amount of IAA produced by both strains was 6.0–6.5 μg ml^{-1}.

Cellular fatty acid profiles of isolates N6 and N7^T were determined by GC using the Sherlock Microbial Identification system (MIDI) according to a standard protocol (Paisley, 1996); data are shown in Table 2.

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 N7^T was 67.6 mol%, which is in accordance with the values given for members of the genus *Azospirillum* (64–71 mol%; Ben Dekhil *et al.*, 1997). The 16S rRNA genes of N6 and N7^T were amplified using the primers (FGPS4–281 bis, AGAGTTTGATCCTGGCTCAG; FGPS1509–153, AAGGAGGTGATCCAGCCGCA; Normand, 1995) and PCR conditions described by Mehnaz *et al.* (2001). PCR products of 1.5 kb were obtained. Phylogenetic analysis was performed using the software package BIONUMERICS (Applied Maths, Belgium) after including the consensus sequence in an alignment of small ribosomal subunit sequences collected from EMBL. The alignment was

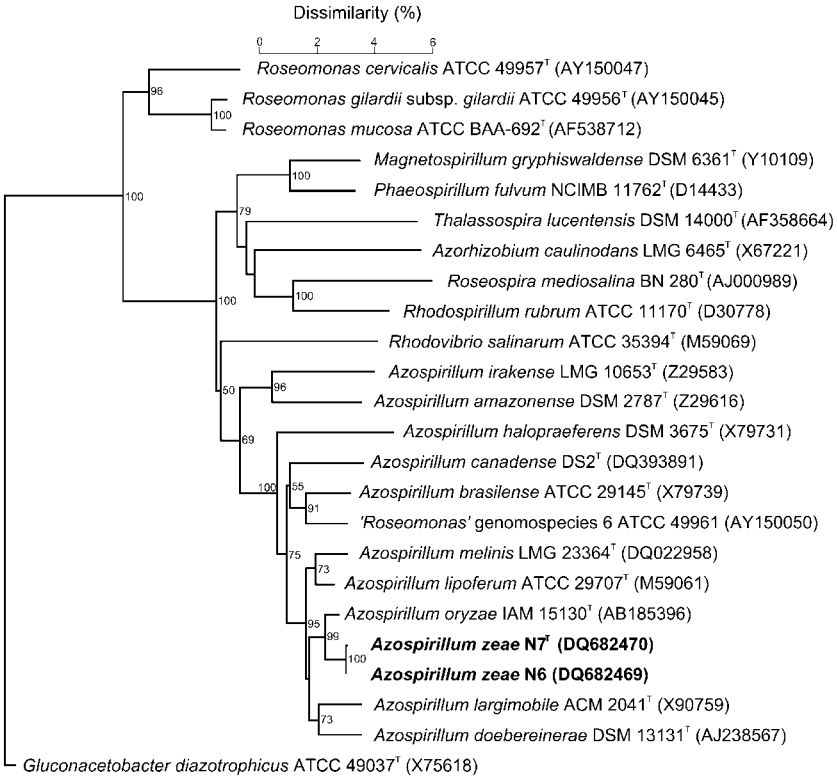

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences constructed by the neighbour-joining method showing the close relationship between strains N7^T and N6 and the nearest relatives of the genus *Azospirillum*. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 50 % are shown.

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. The resulting tree, based on a comparison of 1482 and 1488 bases of N6 and N7^T, respectively, was constructed using the 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 the distance matrix, 16S rRNA gene sequence similarity values indicated that the closest relatives to strains N6 and N7^T are *A. oryzae* IAM 15130^T (98.8 and 98.7 % similarity, respectively), *A. lipoferum* ATCC 29707^T (97.6 and 97.5 % similarity, respectively), *A. melinis* TMCY 0552^T (97.3 and 97.2 % similarity, respectively), *A. largimobile* ACM 2041^T (96.8 and 96.9 % similarity, respectively) and *A. doebereinerae* DSM 13131^T (96.7 and 96.7 % similarity, respectively). The phylogenetic tree based on 16S rRNA gene sequences constructed by using neighbour-joining method is shown in Fig. 2.

DNA–DNA hybridization was performed at 38.5 °C according to a modification of the method described by Ezaki *et al.* (1989). For isolate N7^T, DNA–DNA hybridization results showed reassociation values of 37 % with *A. oryzae* IAM 15130^T and 43 % with *A. lipoferum* ATCC 29707^T; isolate N6 showed 48 % reassociation with *A. oryzae* IAM 15130^T and 43 % reassociation with *A. lipoferum* ATCC 29707^T. Isolates N6 and N7^T showed 98 % reassociation with each other.

The chaperonin gene (*cpn60*) was amplified from bacterial genomic DNA of isolates N6 and N7^T using universal *cpn60* degenerate primers and PCR conditions described by Mehnaz *et al.* (2007). The DNA sequences of N6 and N7^T showed 95.1 and 93.9 % similarity, respectively, with the *cpn60* sequence of *A. oryzae* IAM 15130^T (GenBank accession no. DQ813649), 82.8 and 82.1 % similarity, respectively, with that of *A. lipoferum* ATCC 29707^T (GenBank accession no. DQ813650), and 81 and 80.7 % similarity, respectively, with that of *A. melinis* TMCY 0552^T (GenBank accession no. EF428027). Strains N6 and N7^T showed 98.7 % sequence similarity with each other.

Semi-solid M medium was used for the acetylene reduction assay, which was carried out as described by Mehnaz & Lazarovits (2006). Veil-like subsurface pellicle formation and very high nitrogenase activity, i.e. 5.9–6.6 μmol ethylene h^{-1} (mg protein)$^{-1}$, was observed. The *nifH* gene was 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. Comparison of results using BLAST (NCBI) revealed highest sequence similarities with the *nifH* gene of *A. oryzae* IAM 15130^T (98.5 and 97.6 %, respectively, for strains N6 and N7^T), *A. lipoferum* ATCC 29707^T (96.6 and 96.3 %, respectively) and *A. brasilense* sp. 7^T (93.5 and 93.3 %, respectively).

Description of *Azospirillum zeae* sp. nov.

Azospirillum zeae [L. gen. n. *zeae* of spelt, of *Zea mays*, referring to its isolation from rhizosphere soil of corn (*Zea mays*)].

Cells are rods, 0.9–1.5 × 1.9–6.8 μm in size, Gram-negative, motile via a single polar flagellum. Pink colonies form after 48–72 h, which become wrinkled and dried with time. Growth occurs on M medium at 20–41 °C, pH 5–7 and in 0.5–1.0 % NaCl. Optimum growth occurs at 30 °C and pH 5–7. Positive for nitrogen fixation and IAA production; negative for phosphate solubilization. L-Arabinose, D-fructose, L-fucose, D-galactose, D-glucose, D-mannitol, D-sorbitol, D-psicose, D- and L-alanine, L-asparagine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, acetic acid, α-aminobutyric acid, L-hydroxybutyric acid, L-aspartic acid, bromosuccinic acid, formic acid, α- and β-hydroxybutyric acid, D-galacturonic acid, L-glutamic acid, α-ketoglutaric acid, DL-lactic acid, malic acid, phenylacetic acid, propionic acid, D-saccharic acid, succinic acid, urocanic acid, *N*-acetyl-D-glucosamine, potassium gluconate, Tweens 80 and 40, pyruvic acid methyl ester, succinic acid monomethyl ester, *p*-hydroxyphenylacetic acid, 2-aminoethanol, 2,3-butanediol, glucuronamide, L-alaninamide and glycerol can be used as unique carbon sources. D-Arabitol, D-cellobiose, *myo*-inositol, D-lactose, D-mannose, D-melibiose, D-raffinose, L-rhamnose, trehalose, gentiobiose, sucrose, maltose, xylitol, succinamic acid, quinic acid, malonic acid, D-glucuronic acid, *cis*-aconitic acid, citric acid, L-erythritol, D-gluconic acid, α-ketobutyric acid, L-histidine, D-serine, trisodium citrate, capric acid and adipic acid are not utilized. Positive for catalase, oxidase, urease, nitrate reduction, aesculin hydrolysis and β-galactosidase. Negative for indole production, arginine dihydrolase and gelatin hydrolysis. Variable response for acetoin production. Biotin is not required for growth. Major cellular fatty acids are 18 : 1ω7*c*, 18 : 1 2-OH and 16 : 0. The predominant quinone system is ubiquinone (Q-10). The DNA G+C content of the type strain is 67.6 mol%.

The type strain, N7^T (=NCCB 100147^T=LMG 23989^T), was isolated from rhizosphere soil of corn (*Zea mays*) from London, Ontario, Canada.

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