Azospirillum oryzae sp. nov., a nitrogen-fixing bacterium isolated from the roots of the rice plant *Oryza sativa*

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The taxonomic position of the free-living diazotrophic strain COC8^T isolated from rice was investigated based on phylogenetic analyses. 16S rRNA gene sequence analyses indicated that strain COC8^T was closely related to the genus *Azospirillum* (96 % similarity). Chemotaxonomic characteristics (G + C content of the DNA 66.8 mol%, Q-10 quinone system, 18 : 1ω7c as the major fatty acid and 14 : 0 3-OH and 16 : 0 3-OH as the major hydroxy fatty acids) were also similar to those of the genus *Azospirillum*. Based on its physiological characteristics, strain COC8^T can clearly be differentiated from recognized species of *Azospirillum*. The name *Azospirillum oryzae* sp. nov. is proposed to accommodate this bacterial strain; the type strain is COC8^T (=IAM 15130^T = CCTCC AB204051^T).

Members of the genus *Azospirillum* Tarrand *et al.* 1978 are associated with grasses, cereals and crops. The genus currently comprises seven species; the type species is *Azospirillum lipoferum*. Cells are nitrogen-fixing, curved rods that are motile with a single polar flagellum; they have been found previously in association with the rice plant (Khammas *et al.*, 1989; Ladha *et al.*, 1987). Strain COC8^T was isolated from the paddy soil of a rice plant in 1982, and was reported to be similar to *A. lipoferum* based on its phenotypic characteristics (Oyaizu-Masuchi & Komagata, 1988). However, the strain has remained unidentified at the species level. The purpose of this study was to clarify the taxonomic position of this strain based on phylogenetic analysis of its 16S rRNA gene sequence together with those of other representatives of the genus *Azospirillum*.

All strains investigated were incubated on M medium (5.0 g sodium malate, 0.02 g CaCl₂·2H₂O, 0.2 g MgSO₄·7H₂O, 0.1 g K₂HPO₄, 0.4 g KH₂PO₄, 0.1 g NaCl, 10 mg FeCl₃, 2 mg Na₂MoO₄·2H₂O, 0.1 g yeast extract, 2 µg biotin, 1.0 l distilled water, pH 6.8) and NFG medium (10.0 g glucose, 20 mg CaCl₂·2H₂O, 0.2 g MgSO₄·7H₂O, 1.0 g K₂HPO₄, 5 g CaCO₃, 50 mg FeSO₄·7H₂O, 1 mg Na₂MoO₄·2H₂O, 1.0 l distilled water, pH 7.3). Methods used for phenotypic characterization (determination of the DNA base composition and quinone system and acetylene reduction assay)

were as described by Oyaizu-Masuchi & Komagata (1988). Cellular fatty acid methyl esters were prepared, separated and identified using the Microbial Identification system as described by Xie & Yokota (2003). The fatty acid composition could not be clearly identified by the MIDI system (Microbial ID, Inc.). For example, summed features 2 and 3 were further analysed as follows: fatty acid samples and standard non-polar fatty acids and hydroxy fatty acids used for comparison were developed on a TLC plate (silica-gel F254; Merck) with hexane/ethyl ether (1 : 1), sprayed with a 0.02 % dichlorofluorescein ethanol solution and dried and detected under UV light. The separated spots of non-polar fatty acids and hydroxy fatty acids were scraped from the plates, transferred to tubes and extracted with ethyl ether. These extracts were then concentrated under a N₂ gas stream and dissolved in hexane/methyl tert-butyl ether (1 : 1). The separated and purified non-polar fatty acids and hydroxy fatty acids were then again identified by using the MIDI system. Summed features 2 and 3 were identified to be 14 : 0 3-OH and 16 : 1ω7c, respectively. PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Xie & Yokota (2003). A 420-base fragment of the *nifH* gene (encoding dinitrogenase reductase) was amplified from the extracted DNA using the forward primer IKG (5'-TACGGYAA-RGGBGGYATCGG) and the backward primer AQE (5'-GACGATGATYTCCTG) (Y = C/T; S = G/C; R = A/G; B = C/G/T) (Xie & Yokota, 2004). The DNA sequences were compared with sequences obtained from DDBJ/GenBank and aligned with the CLUSTAL W software package (Thompson *et al.*, 1994); evolutionary distances and the *K*_{nuc} value (Kimura, 1980) were then calculated. Alignment gaps and ambiguous bases were excluded from the

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Abbreviation: PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nifH* gene sequences of strain COC8^T are AB185396 and AB185395, respectively.

Fig. 1. Neighbour-joining analysis of the phylogenetic tree based on the 16S rRNA gene showing close relationships between strain COC8^T and the nearest relatives of the genus *Azospirillum*. *Rhodospirillum rubrum* was used as the outgroup. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 60 % are shown.

calculations. A phylogenetic tree based on comparison of 1108 bases was constructed using the neighbour-joining method (Saitou & Nei, 1987). The topology of this phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates, and similarity values were calculated using PAUP 4.0b1 (Swofford, 1998). Using similar methodology, 408-base partial *nifH* sequences were also aligned and a phylogenetic tree was constructed.

16S rRNA gene sequence analyses indicated that the closest relatives to strain COC8^T were *A. lipoferum* and *Azospirillum largimobile* (96.0 % similarity); values of not more than 95.0 % were obtained against other recognized species of *Azospirillum*. When alignment gaps and ambiguous bases in the 16S rRNA gene sequences were excluded from the calculations, similarity values for strain COC8^T against *A. lipoferum* and *A. largimobile* were 96.3 and 96.8 %, respectively. *A. lipoferum* was the first species assigned to *Azospirillum* (Tarrand *et al.*, 1978), and *A. largimobile* was subsequently reclassified from the genus *Conglomeromonas* (Skerman *et al.*, 1983). Unfortunately, the type strain ACM 2041^T of this species is no longer extant, either with the authors who originally described it (Ben Dekhil *et al.* 1997) or with the ACM Bacteria Collection, where the strain was deposited. However, based on the fact that the 16S rRNA gene sequences differed by more than 3 %, which is a level sufficient to allow the proposal of a new species (Stackebrandt & Goebel, 1994), strain COC8^T was considered to represent a novel species of the genus *Azospirillum*. The results of the neighbour-joining analysis of the 16S rRNA gene sequence are shown in Fig. 1.

Activity in the acetylene-reduction assay for strain COC8^T was found to be greater when the air concentration was

increased from 10 to 90 % (Oyaizu-Masuchi & Komagata, 1988). Moreover, a partial fragment of the *nifH* gene was amplified in this study, suggesting that strain COC8^T is a nitrogen-fixing bacterium. The expected 420-bp *nifH* sequence of strain COC8^T was used as a database search, and highest sequence similarities (91 %) were shown by *A. lipoferum* and *Azospirillum brasilense*; however, similarities with other diazotrophic bacteria were not more than 89 %. The results of the neighbour-joining analysis of the *nifH* sequence (Fig. 2) revealed that strain COC8^T was closely related to *Azospirillum* species (69 % bootstrap support).

Cells of the bacterium are spiral or vibrioid, 1.0 × 1.5–5.0 µm in size and have a single polar flagellum. Poly-β-hydroxybutyrate (PHB) granules were observed in the cells. These properties matched the general characteristics of the genus *Azospirillum*. The physiological properties of strain COC8^T were similar to those of its nearest relatives *A. largimobile* and *A. lipoferum*: acid production from glucose aerobically and anaerobically; growth with glucose, D-galactose, D-fructose, D-ribose and L-arabinose as the sole carbon source; positive for catalase, oxidase, urease, DNase and nitrate reduction; aesculin hydrolysis; and no growth with lactose or D-cellobiose, or on 3 % NaCl. However, they could be differentiated from each other based on several physiological properties (Table 1). Cells of strain COC8^T have only one polar flagellum whereas those of *A. largimobile* have 1–10 distinctive lateral flagella (Ben Dekhil *et al.*, 1997). On media containing sodium ions, multicellular conglomerates of *A. largimobile* develop from a single cell and become immobile (Ben Dekhil *et al.*, 1997); strain COC8^T shows no such characteristic. Strain COC8^T can utilize hydrogen for growth (Oyaizu-Masuchi & Komagata, 1988).

Fig. 2. Neighbour-joining analysis of the phylogenetic tree based on the *nifH* gene showing that strain COC8^T is most closely related to *Azospirillum lipoferum* and *Azospirillum brasilense*. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 60 % are shown.

Table 1. Physiological differences between strain COC8^T and its closest relatives within the genus *Azospirillum*

Strain: 1, COC8^T; 2, *A. largimobile* ACM 2041^T; 3, *A. lipoferum* DSM 1691^T. Data were compiled from Ben Dekhil *et al.* (1997), Eckert *et al.* (2001) and this study. +, Positive; –, negative; w, weak.

Characteristic	1	2	3
Optimal temperature for growth (°C)	30	28	37
Carbohydrate utilization:			
D-Mannitol	–	+	+
D-Sorbitol	–	+	+
D-Xylose	+	–	+
L-Rhamnose	+	–	–
Malonate	–	+	–
Maltose	–	–	+
Sucrose	–	+	–
Inositol	–	–	+
Gelatin hydrolysis	–	–	+
Denitrification	–	–	+
Phosphatase	+	w	+
Biotin requirement	+	–	+

The G+C content of the DNA of strain COC8^T was 66.8 mol%, which is within the published range for the genus *Azospirillum* (approximately 64–71 mol%; Ben Dekhil *et al.*, 1997). As with *A. lipoferum* and *A. brasilense*, strain COC8^T had a ubiquinone (Q-10) quinone system. The fatty acid profile of strain COC8^T included 18:1 ω 7c (55.3%), 16:1 ω 7c (16.1%), 16:0 (6.2%), 19:0 cyclo ω 8c (3.8%), 13:1 at 12–13 (3.3%), unknown ECL 10:922 (3.0%) and 14:0 (0.9%); hydroxy fatty acids were 14:0 3-OH (6.7%) and 16:0 3-OH (4.8%). This Sherlock MIS result differs slightly from that identified by using GLC (Oyaizu-Masuchi & Komagata, 1988). However, the two methods are in broad agreement that the genus *Azospirillum* has a large amount of 18:1 ω 7c (55.3%), contains 16:1 ω 7c, 16:0 as a major component, and that the major hydroxy fatty acids are 14:0 3-OH and 16:0 3-OH.

The results obtained from the chemotaxonomic analyses were consistent with phylogenetic analysis, indicating that strain COC8^T belongs to the genus *Azospirillum* and can be differentiated from all recognized species. The name *Azospirillum oryzae* sp. nov. is therefore proposed for strain COC8^T.

Description of *Azospirillum oryzae* sp. nov.

Azospirillum oryzae (o.ry'zae. L. gen. n. *oryzae* of rice, from where the type strain was isolated).

Cells are spiral or vibrioid, 1.0 × 1.5–5.0 µm in size and motile via a single polar flagellum. PHB granules are present in the cells. Nitrogen can be fixed, and the cells can grow on nitrogen-free medium or nutrient medium. Temperature range for growth is 4–37 °C (optimum

30 °C). Optimum pH for growth is between 6.0 and 7.0. Cells do not tolerate 3% NaCl. Acids are produced from L-arabinose, D-xylose, D-ribose, D-glucose, D-fructose, D-galactose and L-rhamnose but not from sorbitol, lactose, maltose, mannitol, inositol, cellobiose or sucrose. Positive for nitrate reduction but negative for nitrite reduction. Positive reactions for catalase, oxidase, urease, phosphatase, DNase, gelatin liquefaction, aesculin hydrolysis and growth on citrate as a carbon source. Negative reactions for the Voges–Proskauer test and indole production. Malonate and phenylalanine are not assimilated. Biotin is required for growth. Hydrogen is utilized. Major cellular fatty acids are 18:1 ω 7c, 16:1 ω 7c and 16:0 and major hydroxy fatty acids are 14:0 3-OH and 16:0 3-OH. The G+C content of the DNA is 66.8 mol%. The predominant quinone system is ubiquinone (Q-10).

The type strain, COC8^T (=IAM 15130^T=CCTCC AB204051^T), was isolated in 1982 from the roots of *Oryza sativa*.

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