Azospirillum rugosum sp. nov., isolated from oil-contaminated soil

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The taxonomic status of a light-orange-coloured bacterial isolate from an oil-contaminated soil sample was characterized by using a polyphasic taxonomic approach. Comparative analysis of 16S rRNA gene sequences demonstrated that the isolate belonged phylogenetically to the genus *Azospirillum*, with *Azospirillum canadense*, *Azospirillum brasilense* and *Azospirillum doebereineriae* as its closest phylogenetic relatives (97.3, 97.0 and 97.0% similarity, respectively). DNA–DNA pairing studies showed that the unidentified organism displayed 25.0, 17.0 and 19.0% relatedness to the type strains of *A. brasilense*, *A. canadense* and *A. doebereineriae*, respectively. The generic assignment was confirmed by chemotaxonomic data, which revealed a fatty acid profile that was characteristic of the genus *Azospirillum*, consisting of straight-chain saturated and unsaturated fatty acids with C_{18:1ω7c} as the major fatty acid, and ubiquinone with ten isoprene units (Q-10) as the predominant respiratory quinone. On the basis of both the phenotypic and molecular genetic evidence, it is proposed that the unknown isolate be classified as a representative of a novel species of the genus *Azospirillum*, for which the name *Azospirillum rugosum* sp. nov. is proposed. The type strain is IMMIB AFH-6^T (=CCUG 53966^T=DSM 19657^T).

The genus *Azospirillum* was proposed by Tarrand *et al.* (1978) for strains of the root-associated nitrogen fixer ‘*Spirillum lipoferum*’, which appear as vibrioid cells with a single polar flagellum and have a DNA G + C content of 69–71 mol%. Recently, 16S rRNA gene sequence analysis has revealed that members of the genus *Azospirillum* form a distinct phylogenetic line within the *Alphaproteobacteria*. Furthermore, analysis of the quinone system and cellular fatty acids revealed that *Azospirillum* species contain ubiquinone Q-10 as the major respiratory quinone and that the fatty acid profile contains straight-chain saturated and unsaturated fatty acids in addition to 3-hydroxy fatty acids. Members of the genus *Azospirillum* have been isolated from the roots of numerous wild and cultivated grasses, cereals, food crops, and soil from tropical, subtropical and temperate regions all over the world (Döbereiner *et al.*, 1976; Bally *et al.*, 1983; Ladha *et al.*,

1987; Kirchhof *et al.*, 1997; Gunarto *et al.*, 1999). Members of the genus have been reported to enhance the growth of plants by the production of phytohormones (Bashan & Holguin, 1997) and are also possible suppliers of nitrogen to their host plants (Döbereiner, 1983; Okon, 1985). In addition, it has been reported that they possess oil-oxidizing potential (Muratova *et al.*, 2005). Currently, the genus comprises 11 species, namely *Azospirillum lipoferum*, *A. brasilense*, *A. amazonense*, *A. halopraeferens*, *A. irakense*, *A. largimobile*, *A. doebereineriae*, *A. oryzae*, *A. melinis*, *A. canadense* and *A. zaeae*. In this paper, the taxonomic characterization of a strain (designated IMMIB AFH-6^T) preliminarily identified as a member of the genus *Azospirillum* is reported.

Strain IMMIB AFH-6^T was isolated on nutrient agar from oil-contaminated soil near the oil refinery located in Kaohsiung county, Taiwan. The isolate was subsequently cultivated on brain–heart infusion (BHI) agar (no. 37100; Becton Dickinson) and tryptone soy agar (CM 131; Oxoid) to determine its morphological characteristics.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMMIB AFH-6^T is AM419042.

The physiological properties of strain IMMIB AFH-6^T were determined by using the API CORYNE, API 20 STREP, API 20E and API 50CHE systems (bioMérieux). Assimilation tests were performed using the API 20NE system. Enzyme reactions and acid production from carbohydrates were determined after 3 and 7 days incubation at 37 °C. Salt tolerance was determined by cultivating the organism in tryptone soy broth supplemented with NaCl at final concentrations in the range 0.0–12.0 % (w/v).

Chemotaxonomic characteristics of strain IMMIB AFH-6^T were determined by cultivating the organism at 37 °C in shake flasks containing BHI broth for 1 week. After checking for purity at maximum growth, the organism was killed with formaldehyde (1 %, v/v), harvested by centrifugation, washed with distilled water and freeze-dried. Lipids were extracted by using acid methanolysis as described by Minnikin *et al.* (1980). Fatty acids were analysed as described previously (Yassin *et al.*, 2007). Respiratory quinones were extracted and purified according to Collins *et al.* (1977). MS analyses of the quinones were recorded in positive-ion mode on a Q-TOF 2 mass spectrometer (Micromass) equipped with a nanospray source as described previously (Yassin & Hupfer, 2006). For the compounds under study, the major ions observed with electrospray were protonated pseudo-molecular ions, [M+Na]⁺. The ubiquinone identity was verified by observing the diagnostic ion at *m/z*=197, which represents the benzylium ion.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of PCR products were carried out by using procedures described by Rainey *et al.* (1996). Purified PCR products were sequenced by using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) as described in the manufacturer's protocol. An Applied Biosystems 310 DNA Genetic Analyzer was used for electrophoresis of the sequence-reaction products.

The 16S rRNA gene sequences of species of *Azospirillum* with validly published names retrieved from GenBank were added to the ARB database (Ludwig *et al.*, 2004) and aligned by using the respective tool of the ARB package. The resulting alignment was corrected manually and evolutionary trees were inferred by using maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary-distance matrix was calculated by using the correction of Jukes & Cantor (1969). Topologies of the resultant tree were evaluated in bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings. DNA–DNA hybridization experiments were performed by using the method described by Ezaki *et al.* (1989) in triplicate (*n*=3).

To establish the phylogenetic position of strain IMMIB AFH-6^T, its 16S rRNA gene sequence was determined in this study and subjected to comparative analysis. Treeing analysis demonstrated placement of the unidentified bacterium within the genus *Azospirillum*. Highest sequence similarities were shown to the type strains of *A. canadense* (97.3 %), *A. brasilense* (97.0 %) and *A. doebereineriae* (97.0 %). Significantly lower similarity levels were shown to other members of the genus *Azospirillum* (data not shown). A tree constructed by using the maximum-likelihood method depicting the phylogenetic position of isolate IMMIB AFH-6^T within the genus *Azospirillum* is shown in Fig. 1 and demonstrates that the isolate represents a novel subline within the genus *Azospirillum*, with *A. canadense* as its closest phylogenetic relative. Branching of isolate IMMIB AFH-6^T with *A. canadense* was significant (98 % recovery in bootstrap resampling analysis). In view of the high 16S rRNA gene sequence similarities between isolate IMMIB AFH-6^T and *A. canadense*, *A. brasilense* and *A. doebereineriae*, chromosomal DNA–DNA hybridizations were performed with these organisms. Reassociation values for isolate IMMIB

Fig. 1. Maximum-likelihood tree showing the position of *Azospirillum rugosum* IMMIB AFH-6^T with respect to other members of the genus *Azospirillum*. The tree was based on a comparison of 16S rRNA gene sequences that were at least 90 % complete (with regard to the *Escherichia coli* sequence). Numbers at nodes are levels of bootstrap support (%) based on analyses of 1000 resampled datasets. Bar, 5.0 % sequence divergence.

AFH-6^T with respect to *A. canadense* LMG 23617^T, *A. brasilense* DSM 1690^T and *A. doebereinae* DSM 13131^T were 17.0, 25.0 and 19.0 %, respectively. These results demonstrate that isolate IMMIB AFH-6^T represents a separate species.

Chemotaxonomically, strain IMMIB AFH-6^T possesses chemical markers that support its assignment to the genus *Azospirillum*. Cellular fatty acid analysis revealed the presence of n-C_{14:0} (1.48 % of total fatty acids), n-C_{15:0} (1.96 %), n-C_{16:1}ω7c (18.02 %), n-C_{16:0} (12.40 %), two isomers of 9,10-methylene-hexadecanoate (0.55 % and 1.28 %), n-C_{17:0} (0.43 %), n-C_{18:2}ω6,9c (2.0 %), n-C_{18:1}ω7c (39.61 %), n-C_{18:0} (1.77 %) and n-C_{19:1}ω9c (0.54 %) as the major non-hydroxylated fatty acid methyl esters. Major hydroxylated fatty acids are n-C_{14:0} 3-OH (5.6 %), n-C_{16:0} 3-OH (3.2 %) and n-C_{18:0} 3-OH (0.89 %). The cellular fatty acid profile of strain IMMIB AFH-6^T and those of some *Azospirillum* species are shown in Table 1. According to GC-MS measurements, summed features 1, 2, 3 and 6 determined in this study (using the MIDI system)

Table 1. Comparison of the cellular fatty acid contents of strain IMMIB AFH-6^T and some *Azospirillum* species

Strains: 1, strain IMMIB AFH-6^T; 2, *A. canadense* LMG 23617^T; 3, *A. brasilense* DSM 1690^T; 4, *A. doebereinae* DSM 13131^T; 5, *A. oryzae* COC8^T (Xie & Yokota, 2005); 6, *A. lipoferum* ATCC 29707^T (Mehnaz *et al.*, 2007). Data are from this study except where indicated. —, Not detected.

Fatty acid	1	2	3	4	5	6
n-C _{13:0}	—	—	—	—	—	0.5
n-C _{14:0}	1.48	0.02	0.04	0.06	0.9	0.7
n-C _{15:0}	1.96	0.30	0.30	—	—	1.4
n-C _{16:1} ω9c	0.97	0.09	0.29	0.11	—	—
n-C _{16:1} ω7c	18.02	6.13	7.96	7.09	16.1	6.5
n-C _{16:1} ω5c	1.0	0.51	0.35	0.22	—	—
n-C _{16:0}	12.40	7.28	3.39	6.91	6.2	4.3
n-C _{17:1} ω8c	—	—	—	—	—	3.4
9,10-Methylene hexadecanoate*	0.55	0.82	0.48	0.13	—	—
9,10-Methylene hexadecanoate*	1.28	1.63	0.72	0.29	—	—
n-C _{17:0}	0.43	0.25	0.10	—	—	0.8
n-C _{18:2} ω6,9c	2.0	—	—	—	—	—
n-C _{18:1} ω7c	39.61	81.51	86.3	84.50	55.3	53.4
n-C _{18:0}	1.77	0.49	0.17	0.28	—	0.5
n-C _{19:1} ω9c	0.54	1.07	0.04	—	—	—
n-C _{19:0} cyclo ω8c	—	—	—	—	3.8	—
n-C _{14:0} 3-OH	5.6	18.43	14.18	52.67	6.7	—
n-C _{15:0} 3-OH	—	—	—	—	—	0.8
n-C _{16:0} 3-OH	3.2	55.42	38.14	45.07	4.8	4.3
n-C _{17:0} 3-OH	—	—	—	—	—	0.6
n-C _{18:0} 3-OH	0.89	1.28	1.12	2.26	—	0.5
n-C _{18:0} 2-OH	—	—	—	—	—	5.5

*Two isomers of 9,10-methylene hexadecanoate.

and in the studies of Mehnaz *et al.* (2007) and Xie & Yokota (2005) should correspond to n-C_{13:0}, n-C_{14:0} 3-OH, n-C_{16:1}ω7c and n-C_{18:2}ω6,9c, respectively. MS analysis of the main isoprenoid quinone isolated from strain IMMIB AFH-6^T showed a strong peak at *m/z* = 885.57, attributable to [M+Na]⁺ in the high-mass region. This corresponds to a ubiquinone with ten isoprene units (Q-10).

Strain IMMIB AFH-6^T consisted of Gram-negative, straight to slightly curved rods. On BHI agar, nutrient agar and tryptone soy agar, young colonies were smooth with entire margins, but older colonies become wrinkled with an irregular appearance. Colonies were light-orange-coloured. The organism grew aerobically and was catalase- and oxidase-positive. It hydrolysed gelatin, hippurate and aesculin. It reduced nitrate to nitrite. It had arginine dihydrolase, *para*-nitrophenyl-β-D-galactopyranosidase, *ortho*-nitrophenyl-β-D-galactopyranosidase and urease activities. Comparative biochemical studies (Table 2) revealed that isolate IMMIB AFH-6^T could be differentiated from its close phylogenetic relatives (*A. canadense*, *A. brasilense* and *A. doebereinae*) on the basis of carbon-source utilization, gelatin hydrolysis, nitrate reduction and arginine dihydrolase activity.

It is evident from the presented data that isolate IMMIB AFH-6^T exhibits overall cellular morphological and chemotaxonomic characteristics that are consistent with those of members of the genus *Azospirillum*, but appears not to conform to any recognized species. Phylogenetic analysis based on 16S rRNA gene sequencing confirmed this provisional assignment and demonstrated clearly that isolate IMMIB AFH-6^T represents an unknown subline within the genus *Azospirillum*. Both sequence-divergence values and branching-pattern considerations show that the unidentified organism is related more closely to *A.*

Table 2. Characteristics that differentiate strain IMMIB AFH-6^T from the most phylogenetically related *Azospirillum* species

Taxa: 1, strain IMMIB AFH-6^T; 2, *A. canadense* LMG 23617^T; 3, *A. brasilense* DSM 1690^T; 4, *A. doebereinae* DSM 13131^T. All strains are positive for assimilation of D-fructose, D-gluconate, glycerol and malic acid, but are negative for assimilation of N-acetylglucosamine, cellobiose, D-glucose, inositol, maltose, D-mannitol, D-mannose and D-sorbitol. All data are from this study and were determined by using API 20NE tests.

Characteristic	1	2	3	4
Assimilation of:				
L-Arabinose	—	+	+	+
D-Galactose	—	—	+	+
Adipic acid	+	—	—	—
Gelatin hydrolysis	+	—	+	+
Reduction of nitrate	+	+	—	—
Arginine dihydrolase	—	—	+	+

canadense than to *A. brasilense* and *A. doebereineriae*. However, chromosomal DNA–DNA pairing demonstrates that these organisms represent separate species. Support for the separateness of isolate IMMIB AFH-6^T also comes from its distinct biochemical characteristics. Therefore, based on biochemical and molecular genetic evidence, it is proposed that isolate IMMIB AFH-6^T should be classified in the genus *Azospirillum* as *Azospirillum rugosum* sp. nov.

Description of *Azospirillum rugosum* sp. nov.

Azospirillum rugosum (ru.go'sum. L. neut. adj. *rugosum* wrinkled, as the form of the colonies on the agar changes to a wrinkled appearance).

Cells are straight to slightly curved rods and motile. Gram-negative, oxidase- and catalase-positive. Ageing cells contain intracellular granules. On tryptone soy agar, nutrient agar and BHI agar, light-orange colonies are formed that are initially smooth with entire margins, becoming wrinkled and irregular with age. Aerobic. Grows at 22–37 °C and tolerates 2.0 % (w/v) NaCl. Has the salient chemotaxonomic characteristics of the genus *Azospirillum*. The fatty acid profile consists mainly of straight-chain saturated and unsaturated fatty acids, with n-C_{18:1}ω7c as the major fatty acid, as well as 3-OH hydroxylated fatty acids. The respiratory quinone consists of ubiquinone with ten isoprene units (Q-10). Gelatin, hippurate and aesculin are hydrolysed. Adipic acid, D-fructose, glycerol, potassium gluconate, 2-ketogluconate, malic acid and D-xylose are assimilated, but arbutin, D-glucose, D- and L-arabinose, D- and L-mannose, D-mannitol, N-acetylglucosamine, maltose, capric acid, phenylacetic acid and trisodium citrate are not. Acid is produced from D-fructose and D-xylose, but not from N-acetylglucosamine, D-adonitol, starch, amygdalin, D- and L-arabinose, D- and L-arabitol, arbutin, cellobiose, citrate, dulcitol, inositol, gentiobiose, glycerol, glycogen, gluconate, D-galactose, D-glucose, D- and L-fucose, inositol, inulin, D-lactose, maltose, D-mannitol, D-mannose, melibiose, melezitose, D-lactose, D-lyxose, raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, L-sorbose, sucrose, D-tagatose, trehalose, turanose, xylitol or L-xylose. Positive for nitrate reductase, alkaline phosphatase, arginine dihydrolase, α-galactosidase, 2-nitrophenyl-β-D-galactopyranosidase, 4-nitrophenyl-β-D-galactopyranosidase, lysine decarboxylase, ornithine decarboxylase, pyrazinamidase and urease activities, but negative for α-glucosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, leucine aminopeptidase, tryptophan deaminase and pyrrolidonyl arylamidase activities. Positive for acetoin production (Voges–Proskauer), but negative for indole and H₂S production.

The type strain is IMMIB AFH-6^T (=CCUG 53966^T=DSM 19657^T), isolated from oil-contaminated soil.

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