Azospirillum picis sp. nov., isolated from discarded tar

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A polyphasic taxonomic study was performed on a pink-coloured unknown bacterium isolated from discarded road tar. Comparative analysis of the 16S rRNA gene sequence demonstrated that the isolate belongs phylogenetically to the genus *Azospirillum* with *Azospirillum lipoferum*, *A. melinis* and *A. rugosum* as its closest phylogenetic relatives (96.7, 96.6 and 96.6% similarity to the respective type strains). The generic assignment was confirmed on the basis of chemotaxonomic data, which revealed a fatty acid profile characteristic for the genus *Azospirillum*, consisting of straight-chain saturated and unsaturated fatty acids, with C_{18:1ω7c} as the major unsaturated non-hydroxylated fatty acid, and C_{16:0} 3-OH as the major hydroxylated fatty acid, and a 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 should be classified within a novel species of the genus *Azospirillum*, for which the name *Azospirillum picis* sp. nov. is proposed. The type strain is IMMIB TAR-3^T (=CCUG 55431^T =DSM 19922^T).

The genus *Azospirillum* was proposed by Tarrand *et al.* (1978) for strains of the root-associated nitrogen fixer ‘*Spirillum lipoferum*’ that appear as vibrioid cells with single polar flagella and have a DNA G+C content of 69–71 mol%. Currently, the genus comprises 12 species, namely *Azospirillum lipoferum* (Tarrand *et al.*, 1978), *A. brasilense* (Tarrand *et al.*, 1978), *A. amazonense* (Magalhães *et al.*, 1983), *A. halopraeferens* (Reinhold *et al.*, 1987), *A. irakense* (Khammas *et al.*, 1989), *A. largimobile* (Ben Dekhil *et al.*, 1997), *A. doebereineriae* (Eckert *et al.*, 2001), *A. oryzae* (Xie & Yokota, 2005), *A. melinis* (Peng *et al.*, 2006), *A. canadense* (Mehnaz *et al.*, 2007a), *A. rugosum* (Young *et al.*, 2008) and *A. zeae* (Mehnaz *et al.*, 2007b). The majority of these species have been isolated from the roots of numerous wild and cultivated grasses, cereals and food crops, and from 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; Mehnaz *et al.*, 2007b) and they are possible suppliers of nitrogen to their host plants (Döbereiner, 1983; Okon, 1985). Furthermore, it has been reported that they possess oil-oxidizing potential (Muratova *et al.*, 2005). In this paper, the taxonomic characterization of strain IMMIB TAR-3^T, preliminarily identified as a member of the genus *Azospirillum*, is presented.

Strain IMMIB TAR-3^T was isolated from discarded road tar by the side of a road in Taichung City area, Taiwan. The collected tar was cut into pieces and shaken in distilled water for 30 min. Thereafter, the sample allowed to settle and the supernatant plated on nutrient agar. Isolated colonies of the strain were subsequently cultivated on tryptone soya agar (CM 0131; Oxoid) to determine its morphological characteristics. Cell morphology was observed using a LEO model 912 AB electron microscope.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMMIB TAR-3^T is AM922283.

Cellular fatty acid profiles of strain IMMIB TAR-3^T and the type strains of some *Azospirillum* species are available as supplementary material with the online version of this paper.

The biochemical properties of strain IMMIB TAR-3^T were determined using the API 20E and the API 50 CHE systems according to the manufacturer's instructions (bioMérieux) except for the time of incubation. Tests for assimilation, enzyme activities and acid production from carbohydrates were read after 3 and 7 days incubation at 37 °C. The ability of isolate IMMIB TAR-3^T to fix nitrogen was tested by using the acetylene-reduction assay, as described by Eckert *et al.* (2001). Vials (10 ml) containing 5 ml semi-solid NFB medium were inoculated with IMMIB TAR-3^T, sealed with rubber septa and incubated at 30 °C in the dark. After 36 h, 1% (v/v) of the air phase was replaced with acetylene (Burris, 1972) and the vials were reincubated. The amount of ethylene was measured every 4 h for a total of 24 h. Ethylene was measured using a gas chromatograph equipped with a flame-ionization detector and a packed column (1.0 m x 2.0 mm i.d., stainless steel, packed with Porapak-T 80–100).

Chemotaxonomic characteristics of strain IMMIB TAR-3^T were determined by cultivating the organism at 37 °C in shake flasks containing tryptone soya broth for 1 week. After checking for purity at maximum growth, the culture was killed with formaldehyde (1% v/v), harvested by centrifugation, washed with distilled water and freeze-dried. Lipids were extracted using acid methanolysis as described by Minnikin *et al.* (1980). Fatty acids were *trans*-esterified, purified and analysed as described by Yassin *et al.* (2007). Respiratory quinones were extracted and purified according to Collins *et al.* (1977). Mass spectral 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 by Yassin & Hupfer (2006). For the compounds under study, the major ions that were observed with electrospray were protonated pseudo-molecular ions, [M+Na]⁺. The identity of ubiquinone 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 the purification of PCR products were carried out as described by Rainey *et al.* (1996). Purified PCR products were sequenced 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 the electrophoresis of the sequence reaction products. The 16S rRNA gene sequences of strains of species of the genus *Azospirillum* with validly published names were retrieved from GenBank, added to the ARB database (Ludwig *et al.*, 2004) and aligned using the appropriate tool of the ARB package. The resulting alignment was corrected manually and evolutionary trees were inferred using maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix was calculated using the correction of Jukes & Cantor (1969). The topology of the resultant tree was

evaluated in bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

Strain IMMIB TAR-3^T grew aerobically on nutrient agar and tryptone soya agar, forming rough, pink-coloured colonies after 24 h incubation at 37 °C. Cells were Gram-negative, straight to slightly curved rods, which were motile by means of a single polar flagellum (Fig. 1). Ageing cells contained poly- β -hydroxybutyrate granules after 5 days of growth. The strain was able to reduce acetylene to ethylene with a mean value of 93 nmol ethylene h⁻¹ (10⁸ cells) at 30 °C. This amount is comparable with values obtained for other *Azospirillum* species (Eckert *et al.*, 2001; Reinhold *et al.*, 1987). Acetylene reduction was detected 4 h after acetylene injection. The strain was catalase- and oxidase-positive, hydrolysed aesculin but not gelatin and reduced nitrate to nitrite. The strain had *p*-nitrophenyl- β -D-galactopyranosidase and urease activities but not arginine dihydrolase activity. It assimilated D-glucose, L-arabinose, D-mannitol, *N*-acetylglucosamine, gluconate, malate and phenylacetate but not maltose, capric acid, adipate or citrate. Physiological characteristics are given in detail in the species description, and characteristics that can be used to differentiate strain IMMIB TAR-3^T from other *Azospirillum* species are given in Table 1.

Strain IMMIB TAR-3^T had chemotaxonomic characteristics that support its assignment to the genus *Azospirillum*. Cellular fatty acid analysis revealed the presence of C_{14:0} (1.32% of total fatty acids), C_{15:0} (0.33%), C_{16:1 ω 9c} (0.22%), C_{16:1 ω 7c} (6.94%), C_{16:1 ω 5c} (2.61%), C_{16:0} (19.48%), two isomers of 9,10-methylene-hexadecanoate (0.23 and 0.96%), C_{17:0} (0.26%), C_{18:1 ω 9c} (0.28%), C_{18:1 ω 7c} (57.86%), C_{18:0} (0.56%) and C_{19:1 ω 9c} (5.97%) as the major non-hydroxylated fatty acid methyl esters. Major hydroxylated fatty acids are C_{14:0} 3-OH (4.21%), C₁₆ 3-OH (47.42%) and C_{18:0} 3-OH (14.07%).

Fig. 1. Electron micrograph showing a cell of strain IMMIB TAR-3^T. The cells are straight rods bearing a single polar flagellum. Bar, 0.5 μ m.

Table 1. Characteristics that differentiate strain IMMIB TAR-3^T from the type strains of other *Azospirillum* species

Strains: 1, strain IMMIB TAR-3^T; 2, *A. lipoferum* ATCC 29707^T (unless indicated, data from Mehnaz *et al.*, 2007a); 3, *A. melinis* TMCY 0552^T (data from this study); 4, *A. rugosum* IMMIB AFH-6^T (Young *et al.*, 2008); 5, *A. canadense* LMG 23617^T; 6, *A. brasilense* DSM 1690^T; 7, *A. doebereineriae* DSM 13131^T (data in columns 5–7 from this study); 8, *A. halopraeferens* DSM 3675^T (Reinhold *et al.*, 1987); 9, *A. irakense* CIP 103311^T (Khammas *et al.*, 1989); 10, *A. amazonense* ATCC 35119^T (unless indicated, data from Reinhold *et al.*, 1987); 11, *A. largimobile* ACM 2041^T (Ben Dekhil *et al.*, 1997); 12, *A. oryzae* IAM 15130^T (Mehnaz *et al.*, 2007b; Xie & Yokota, 2005); 13, *A. zeae* LMG 23989^T (Mehnaz *et al.*, 2007b; Mehnaz & Lazarovits, 2006). +, Positive reaction; –, negative reaction; NA, no data available; v, variable. Data from this study were determined using the API 20NE and API 50 CHE kits.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Assimilation of:													
N-Acetylglucosamine	+	+	+	–	–	–	–	NA	+	v	+	–	+
Adipic acid	–	–	–	+	–	–	–	NA	NA	NA	NA	NA	–
L-Arabinose	+	+	+	–	–	+	+	+	+	+	+	+	+
D-Arabitol	+	–*	–	–	–	–	–	NA	–	–*	–	–	–
Citrate	–	+	+	–	–	–	–	+	–	–	+	+	–
L-Fucose	–	+	+	–	–	–	–	NA	+	+	–	–	+
D-Galactose	+	+	+	–	–	+	+	–	+	+	+	+	+
D-Glucose	+	+	+	–	–	–	–	–	+	+	+	+	+
D-Mannitol	+	–	+	–	–	–	–	+	–	–	+	–	+
Phenylacetic acid	+	–*	+	–	–	–	–	–	–	–	NA	NA	+
D-Ribose	+	+	+	–	–	–	–	+	–	+	+	NA	NA
D-Sorbitol	+	–	+	–	–	–	–	–	–	–	+	–	+
Sucrose	–	–	+	–	–	–	–	–	+	+	–	–	–
Gelatin hydrolysis	–	+	+	+	–	–	+	NA	NA	NA	–	+	–
Nitrate reduction	+	+	+	+	+	+	–	+	+	–	–	+	+
Arginine dihydrolase	–	–	+	–	–	+	+	NA	NA	NA	NA	NA	–

*Data from Khammas *et al.* (1989).

The cellular fatty acid profiles of strain IMMIB TAR-3^T and the type strains of some *Azospirillum* species are compared in Supplementary Table S1 (available in IJSEM Online). According to the GC-MS measurements, summed features 1, 2, 3, 6 determined in this study (using the MIDI system), and in the studies of Mehnaz *et al.* (2007a, b) and Xie & Yokota (2005), should correspond to C_{13:0}, C_{14:0} 3-OH, C_{16:1}ω7c and C_{18:2}ω6,9c, respectively. Mass-spectral analysis of the main isoprenoid quinone isolated from strain IMMIB TAR-3^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).

To investigate the phylogenetic position of isolate IMMIB TAR-3^T, the almost-complete 16S rRNA gene sequence (1445 nt) was determined in this study and subjected to comparative analysis. Sequence database searches showed that the 16S rRNA gene sequence displayed the highest similarity to those of members of the genus *Azospirillum*. Phylogenetic analysis confirmed the association of strain IMMIB TAR-3^T with the genus *Azospirillum*, with the isolate forming a distinct sublineage. A tree constructed using the neighbour-joining method and showing the phylogenetic position of strain IMMIB TAR-3^T in relation to members of the genus *Azospirillum* is presented in Fig. 2. Comparative 16S rRNA gene sequence analysis unequivocally demonstrated that the isolate represents a hitherto unknown *Azospirillum* species. Strain IMMIB TAR-3^T displayed relatively high 16S rRNA gene sequence divergence values (greater than 3%) from other recognized species within the genus *Azospirillum*. Highest sequence similarities were found with the type strains of *A. lipoferum* (96.7%), *A. rugosum* (96.6%), *A. melinis* (96.6%) and *A. oryzae* (96.3%). Significantly lower levels of similarity were shown to other members of the genus *Azospirillum* (not shown). Although strain IMMIB TAR-3^T was closely associated with *A. melinis*, bootstrap resampling analysis showed that this association was not particularly significant.

Strain IMMIB TAR-3^T has many phenotypic properties in common with *Azospirillum* species, but it can be readily distinguished biochemically from all described members of this genus. In addition, the 16S rRNA gene sequence divergence of >3.3% strongly supports the recognition of the strain as a member of novel species and it is now established that organisms displaying more than 3% sequence divergence belong to different species (Stackebrandt & Goebel, 1994). Therefore, based on both phenotypic and phylogenetic evidence, strain IMMIB TAR-3^T is considered to represent a novel species within the genus *Azospirillum*, for which the name *Azospirillum picis* sp. nov. is proposed.

Fig. 2. Neighbour-joining tree showing the position of strain IMMIB TAR-3^T in relation to members of the genus *Azospirillum*. The tree is based on a comparison of 16S rRNA gene sequences that were at least 90% complete with regard to the sequence for *Escherichia coli*. Bootstrap values based on analyses of 1000 resampled datasets are shown as percentages at each node. Bar, 5.0% sequence divergence.

Description of *Azospirillum picis* sp. nov.

Azospirillum picis (pi'cis. L. gen. fem. n. *picis* of pitch, tar).

Gram-negative, aerobic, straight to slightly curved motile rods. Grows at temperatures between 22 and 37 °C. Oxidase- and catalase-positive. Ageing cells of 5-day-old growing culture contain intracellular granules. Pink colonies on tryptone soya agar and nutrient agar. Contains the salient chemotaxonomic characteristics of the genus *Azospirillum*. The fatty acid profile consists mainly of straight-chain saturated and unsaturated fatty acids, with C_{18:1ω7c} as the major fatty acid, as well as 3-hydroxylated fatty acids. The respiratory quinone consists of ubiquinone with ten isoprene units (Q-10). Positive for nitrogen fixation. Hydrolyses aesculin but negative for gelatin liquefaction. Assimilates *N*-acetylglucosamine, L-arabinose, D-arabitol, D-fructose, D-galactose, glycerol, potassium gluconate, 2-ketogluconate, D-glucose, phenylacetic acid, malic acid, D-mannitol, D-ribose, D-sorbitol and D-xylose but not adipic acid, D-adonitol, amygdalin, L-arabitol, arbutin, D-arabinose, cellobiose, dulcitol, erythritol, D-fucose, L-fucose, gentiobiose, glycogen, inositol, inulin, D-lactose, D-lyxose, D-mannose, maltose, melezitose, melibiose, capric acid, trisodium citrate, raffinose, L-rhamnose, salicin, L-sorbose, sucrose, D-tagatose, trehalose, turanose, xylitol and L-xylose. Acid is produced from D-glucose. Positive for nitrate reductase, *p*-nitrophenyl-β-D-galactopyranosidase and urease activities. Negative for arginine dihydrolase activity and indole production.

The type strain is IMMIB TAR-3^T (=CCUG 55431^T =DSM 19922^T), isolated from discarded road tar in the Taichung City area of Taiwan.

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