Azoarcus olearius sp. nov., a nitrogen-fixing bacterium isolated from oil-contaminated soil

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A novel nitrogen-fixing strain, designated DQS-4T, was isolated from oil-contaminated soil in Taiwan and was characterized using a polyphasic taxonomic approach. Cells of strain DQS-4T stained Gram-negative, contained poly-β-hydroxybutyrate granules and were motile rods, surrounded by a thin capsule. Cells displayed a strictly aerobic type of metabolism and fixed nitrogen microaerobically. Growth occurred at 10–45 °C (optimum, 35–40 °C), at pH 7.0–8.0 (optimum, pH 7.0) and with 0–2 % NaCl (optimum, 0.5–1 %). Phylogenetic analyses based on 16S rRNA gene sequences showed that strain DQS-4T belonged to the genus Azoarcus, and its closest neighbours were Azoarcus indigens VB32T and Azoarcus communis SWub3T, with sequence similarities of 97.4 and 96.4 %, respectively. The major cellular fatty acids of strain DQS-4T were summed feature 3 (comprising C16:1ω7c and/or C16:1ω6c), C16:0 and C18:1ω7c. The major cellular hydroxy fatty acid was C10:03-OH. The DNA G+C content was 64.5 mol%. The polar lipid profile consisted of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylethanolamine and several uncharacterized aminophospholipids and phospholipids. The mean level of DNA–DNA relatedness between strain DQS-4T and A. indigens LMG 9092T was 27.4 %. On the basis of the genotypic and phenotypic data, strain DQS-4T represents a novel species in the genus Azoarcus, for which the name Azoarcus olearius sp. nov. is proposed. The type strain is DQS-4T (=BCRC 80407T=KCTC 23918T=LMG 26893T).

The genus Azoarcus (type species, Azoarcus indigens), proposed by Reinhold-Hurek et al. (1993), belongs to the family Rhodocyclaceae of the order Rhodocyclales in the class Betaproteobacteria (Reinhold-Hurek & Hurek, 2006). At the time of writing, the genus includes eight recognized species, namely A. indigens (Reinhold-Hurek et al., 1993), A. communis (Reinhold-Hurek et al., 1993), A. tolylyticus (Zhou et al., 1995), A. evansii (Anders et al., 1995), A. anaerobius (Springer et al., 1998), A. toluclasticus (Song et al., 1999), A. toluvorans (Song et al., 1999) and A. buckelii (Mechichi et al., 2002). These species were isolated from stems, roots, aquifer sediment, petroleum-contaminated soil, oxic soil and sewage sludge, indicating that members of the genus are widely distributed in various environments. Cells are straight to slightly curved rods that are highly motile using one or rarely two polar flagella (Reinhold-Hurek & Hurek, 2006). Azoarcus strains stain Gram-negative, contain poly-β-hydroxybutyrate granules and are oxidase-positive and chemo-organoheterotrophic (Reinhold-Hurek & Hurek, 2006). Members of the genus Azoarcus have a strictly respiratory metabolism with O2 as the terminal electron acceptor, except A. anaerobius. Some species fix nitrogen and then require microaerobic conditions for growth on N2 (Reinhold-Hurek & Hurek, 2006). All investigated species possess a C16:1ω6 fatty acid, and all except one have C16:1ω7 and C18:1ω9 as the predominant cellular fatty acids, and DNA G+C contents between 62 and 68 mol% (Reinhold-Hurek et al., 1993; Reinhold-Hurek &
Hurek, 2006). The present study was conducted to clarify the taxonomic position of the Azorarcus-like bacterial strain DQS-4T using a polyphasic taxonomic approach.

Strain DQS-4T was isolated and selected during the characterization of micro-organisms from oil-contaminated soil in the Chinese Petroleum Corporation Kaoshiung refinery (GPS location 22°43′18″N 120°17′47″E) located in Kaoshiung City, Taiwan. It was isolated on R2A agar (BD Difco) plates after incubation at 25 °C for 3 days, and subculturing was performed on R2A agar at 25 °C for 48–72 h. Strain DQS-4T was preserved at −80 °C in R2A broth with 20% (v/v) glycerol, or by lyophilization. A. indigenes LMG 9092T and A. communis DSM 12120T were respectively obtained from the Belgian Coordinated Collections of Microorganisms and the Deutsche Sammlung von Mikroorganismen und Zellkulturen and were grown under the same conditions and used as reference strains for phenotypic and genotypic tests.

Bacterial cells were observed by phase-contrast microscopy (DM 2000; Leica) using cells grown on R2A agar at 25 °C for 6 h (lag growth phase), 18 h (exponential phase) and 36 h (stationary phase) to ascertain their morphology. Motility was tested by the hanging drop method (Beveridge et al., 2007). A Gram-staining kit (Gram Stain Set S kit; BD Difco) and the Ryu non-staining KOH method (Powers, 1995) were used to test the Gram reaction. Poly-β-hydroxybutyrate granule accumulation was observed under light microscopy after staining of cells with Sudan black. The formation of a capsule was studied using the Hiss staining method (Murray et al., 1994). Colony morphology was observed on R2A agar using a stereoscopic microscope (SMZ 800; Nikon).

The pH range for growth was determined by measuring the OD600 of R2A broth cultures. The pH was adjusted prior to sterilization to pH 4–9 (at intervals of 1.0 pH unit) using appropriate biological buffers (Breznak & Costilow, 2007) (citrate/Na₂HPO₄ buffer, pH 4.0–5.0; phosphate buffer, pH 6.0–7.0; Tris/HCl, pH 8.0–9.0). Verification of the pH after autoclaving revealed only minor changes. The temperature range for growth was determined in R2A broth at 4–50 °C. To investigate tolerance of NaCl, R2A broth was prepared according to the formula of the BD Difco medium with the NaCl concentration adjusted to 0, 0.5 and 1.0–6.0% (w/v) (at intervals of 1.0 %). Growth under anaerobic conditions was determined after incubating the bacterial strains on R2A agar in the Oxoid AnaeroGen system. Strains were also tested for growth on nutrient agar (NA), tryptic soy agar (TSA), tryptic soy broth agar (TSBA) and Luria–Bertani (LB) agar. For nitrogenase activity tests, strain DQS-4T was inoculated in broth agar (TSBA) and Luria–Bertani (LB) agar. For AnaeroGen system. Strains were also tested for growth on R2A agar in the Oxoid for 6 h (lag growth phase), 18 h (exponential phase) and 36 h (stationary phase) to ascertain their morphology.

The effect of antibiotics on cell growth was assessed after spreading cell suspensions (0.5 McFarland) on R2A agar (BD Difco) plates. The discs (Oxoid) contained the following antibiotics (μg per disc, unless stated otherwise): ampicillin (10), chloramphenicol (30), gentamicin (10), kanamycin (30), nalidixic acid (30), novobiocin (30), rifampicin (5), penicillin G (10 U), streptomycin (10), sulfamethoxazole (23.75) plus trimethoprim (1.25), and tetracycline (30). The effect of antibiotics on cell growth was assessed after 2 days at 25 °C. The diameter of the antibiotic disc was 8 mm. The strain was considered susceptible when the diameter of the inhibition zone was >13 mm, intermediate at 10–12 mm and resistant at <10 mm, as described by Nokhal & Schlegel (1983).

Genomic DNA was isolated by using a bacterial genomic DNA purification kit (DP02-150; GeneMark Technology) and the 16S rRNA gene sequence was analysed as described previously by Chen et al. (2001). Primers FD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RD1 (5′-AAGAGGTTGATCCAGCC-3′) were used for amplification of bacterial 16S rRNA genes by PCR. These primers correspond to nucleotide positions 8–27 and 1525–1541, respectively, of the Escherichia coli 16S rRNA gene and can be used to amplify almost the full length of the 16S rRNA gene. The PCR product was purified and direct sequencing was performed by using sequencing primers FD1, RD1, 520F and 800R (Weisburg et al., 1991; Anzai et al., 1997) with a DNA sequencer (ABI Prism 3730; Applied Biosystems). An almost-complete 16S rRNA gene sequence (1456 nt) of strain DQS-4T was compared against 16S rRNA gene sequences available from EzTaxon-e (Kim et al., 2012), the Ribosomal Database Project (Maidak et al., 2001) and GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Analysis of the sequence data was performed using the software package BioEdit (Hall, 1999) and MEGA version 5.
(Tamura et al., 2011) after multiple alignments of the data by CLUSTAL_X (Thompson et al., 1997). The resulting multiple sequence alignment was corrected manually and gaps at the 5' and 3' ends of the alignment were omitted from further analyses. Distances (corrected according to Kimura's two-parameter model; Kimura, 1983) were calculated, and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were reconstructed by using the treeing algorithms contained in the PHYLIP software package (Felsenstein, 1993). In each case, bootstrap values were calculated based on 1000 replications.

The 16S rRNA gene sequence analysis indicated that strain DQS-4<sup>T</sup> belonged to the family Rhodocyclaceae of the order Rhodocyclales in the class Betaproteobacteria. Strain DQS-4<sup>T</sup> formed a distinct clade within the genus Azoarcus in the neighbour-joining tree (Fig. 1). The overall topologies of the phylogenetic trees obtained using the maximum-likelihood and maximum-parsimony methods were similar. Sequence similarity calculations (over 1400 bp) indicated that strain DQS-4<sup>T</sup> was very closely related to Azoarcus strain BH72 (99.9% 16S rRNA gene sequence similarity), but was also closely related to A. indigens VB32<sup>T</sup> (97.4%) and A. communis SWub3<sup>T</sup> (96.4%). Lower sequence similarities (<95.6%) were found to other strains listed in Fig. 1.

The nifH gene phylogeny of diazotrophic bacteria has been reported to be generally well correlated with the 16S rRNA gene phylogeny (Young, 1992; Rosado et al., 1998; Hurek et al., 1997; Bontemps et al., 2010), so the nifH gene was selected as an additional marker to elucidate phylogenetic relationships between the novel strain and related taxa. A phylogenetic tree based on partial nifH gene sequences was reconstructed as described above for the 16S rRNA gene; it revealed that strain DQS-4<sup>T</sup> and closely related Azoarcus species formed a distinct phylogenetic cluster (Fig. 2). Partial nifH gene similarity calculations indicated that the novel strain was again very closely related to Azoarcus strain BH72 (99.4% similarity), and somewhat less closely related to A. indigens VB32<sup>T</sup> (95.5%), A. communis SWub3<sup>T</sup> (87.1%), A. communis S2 (85.8%) and A. toluylticus Td-1 (79.5%). Lower sequence similarities (<79.0%) were found to representative members of other genera (Fig. 2).

![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of Azoarcus olearius sp. nov. DQS-4<sup>T</sup> and related taxa in the class Betaproteobacteria. Numbers at nodes are bootstrap percentages >70% based on the neighbour-joining (above nodes) and maximum-parsimony (below nodes) tree-making algorithms. Filled circles indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms; open circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Uliginosibacterium gangwonense 5YN10-9<sup>T</sup> was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.](http://ijs.sgmjournals.org)
Molybdatophosphoric acid was used for detection of total phospholipids by TLC according to Embley & Wait (1994).

Polar lipids were extracted and analysed by two-dimensional TLC. DNA–DNA hybridization experiments were carried out at 58 °C by the method of Ezaki et al. (1989). The separate species status of strain DQS-4T was confirmed by hybridization with A. indigens LMG 9092T, which showed 27.4 ± 0.6 % DNA–DNA relatedness. The DNA–DNA relatedness between strain DQS-4T and its closest phylogenetic neighbour was therefore below the 70 % cut-off point recommended for the assignment of strains to the same genomic species (Wayne et al., 1987), supporting the assignment of strain DQS-4T to a separate species in the genus Azoarcus.

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The fatty acid profiles of strain DQS-4T, A. indigens LMG 9092T and A. communis DSM 12120T were determined using cells grown on R2A agar at 30 °C for 2 days. The physiological age of the different bacterial cultures at the time of harvest was standardized by selection of a sector from a quadrant streak on the R2A agar plates, according to the MIDI protocol (http://www.microbialid.com/PDF/ TechNote_101.pdf). In this study, the different bacteria exhibited very similar growth rates on R2A agar. Fatty acid methyl esters were prepared and separated according to the instructions of the Microbial Identification System (MIDI) (Sasser, 1990) and identified by MIDI version 6.0 and the RTStab6.00 database. The fatty acid profile of strain DQS-4T was similar to those of A. indigens LMG 9092T and A. communis DSM 12120T, although there were differences in the proportions of some components (Table S1, available in IJSEM Online). The major fatty acids (>8 %) of strain DQS-4T were summed feature 3 (comprising C16:1ω7c and/or C16:1ω6c 39.3 %), C16:0 (31.0 %) and C18:1ω7c (9.6 %). Strain DQS-4T and the two reference strains had the same major fatty acids, and their major cellular hydroxy fatty acid was C10:0 3-OH. The DNA G+C content of strain DQS-4T, determined by HPLC according to Mesbah et al. (1989), was 64.5 ± 1.0 mol%.

Polar lipids were extracted and analysed by two-dimensional TLC according to Embley & Wait (1994). Molybdatophosphoric acid was used for detection of total lipids, ninhydrin reagent for lipids containing free amino groups, Zinzadze reagent for phosphorus-containing lipids and α-naphthol reagent for glycolipids. Strain DQS-4T exhibited a complex polar lipid profile consisting of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DMP), three uncharacterized aminophospholipids (APL1–APL3) and three uncharacterized phospholipids (PL1–PL3) (Fig. S1). Strain DQS-4T exhibited a very similar polar lipid profile to its closest relatives, A. indigens LMG 9092T and A. communis DSM 12120T; they all contained PE, PG, DPG, APL1, APL2, PL2 and PL3. However, uncharacterized phospholipid PL1 was present in strain DQS-4T and A. communis DSM 12120T, but absent from A. indigens LMG 9092T, and two further uncharacterized phospholipids (PL4 and PL5) were present in A. indigens LMG 9092T, but absent from strain DQS-4T and A. communis DSM 12120T. These results showed that, although they belong to the same genus and have generally very similar profiles, there were some differences in the polar lipid profiles of the analysed strains.

The physiological, biochemical and morphological characteristics of strain DQS-4T are given in the species description and in Tables 1 and S2. Phenotypic examination revealed many traits in common between the novel strain and its closest relatives, A. indigens LMG 9092T and A. communis DSM 12120T. Phenotypic and biochemical examination revealed many traits in common between the novel strain and its closest relatives, A. indigens LMG 9092T and A. communis DSM 12120T. Strain DQS-4T grew well in nitrogen-free SM medium, supplemented with malate as the sole carbon source, NFG medium, supplemented with D-malic acid medium, supplemented with D-malic acid as the sole carbon source. In addition, nitrogen-fixation activity of strain DQS-4T was observed using the acetylene reduction assay. Strain DQS-4T also grew on R2A agar and on TSA, as did A. indigens LMG 9092T and A. communis DSM 12120T, but, in contrast to these two close relatives, strain DQS-4T grew poorly on NA, TSBA and LB agar. Moreover, strain DQS-4T could be differentiated clearly from A. indigens LMG 9092T and A. communis DSM 12120T by its colony colour on TSBA, its ability to grow on R2A agar and its colony colour on TSBA.
well at higher NaCl concentrations (2%), the presence of cystine arylamidase, z-glucosidase and arginine dihydrolase activities, the absence of C14 lipase activity, the ability to hydrolyse aesculin and Tween 60 and the ability to assimilate mannose, mannitol, maltose and gluconate (Table 1). Strain DQS-4T could also be differentiated from A. indigens LMG 9092T by the presence of lipase activity, the ability to hydrolyse Tween 80 and the ability to assimilate citrate. Phenotypic properties such as the presence of urease activity, the absence of DNase activity, the inability to hydrolyse CM-cellulose and the ability to assimilate adipate distinguish strain DQS-4T from A. communis DSM 12120T. Interestingly, the novel strain DQS-4T could utilize more compounds as sole carbon sources, especially carbohydrates, than either of its closest relatives (Table S2).

The characteristics of strain DQS-4T are consistent with the description of the genus Azoarcus (Reinhold-Hurek et al., 1993; Reinhold-Hurek & Hurek, 2006). On the basis of the data obtained from 16S rRNA and nifH gene sequence comparisons, strain DQS-4T occupies a distinct position within the genus Azoarcus. It is clear from the phylogenetic and phenotypic data that strain DQS-4T represents a novel species of the genus Azoarcus. The name Azoarcus olearius sp. nov. is proposed for this taxon.

**Description of Azoarcus olearius sp. nov.**

Azoarcus olearius (o.le.a’ri.us. L. masc. adj. olearius of or belonging to oil, describing the environment from which the type strain was isolated).

Cells are Gram-reaction-negative, aerobic, motile, non-spore-forming rods that are surrounded by a thin capsule. Poly-β-hydroxybutyrate accumulation is observed. N₂ can be fixed under microaerobic conditions. After 48 h of incubation on R2A agar at 35 °C, cells are approximately 0.5–0.8 μm in diameter and 1.5–3.0 μm long. Colonies on R2A agar are non-pigmented, circular, smooth and convex with entire edges. Colonies on TSA and TSBA are translucent yellowish and opaque orange, respectively. Colonies are approximately 0.6–2.0 mm in diameter on R2A agar after 48 h of incubation at 35 °C. Growth occurs at 10–45 °C (optimum, 35–40 °C), at pH 7.0–8.0 (optimum, pH 7.0) and with 0–2% NaCl (optimum, 0.5–1%). Positive for oxidase, catalase, lipase (corn oil) and urease activities and hydrolysis of aesculin and Tweens 60 and 80. Negative for DNase activity and hydrolysis of casein, starch, chitin, CM-cellulose, alginate, gelatin and Tweens 20 and 40. In API 20NE tests, gives positive reactions for nitrate reduction, aesculin hydrolysis, arginine dihydrolase and urease activities and assimilation of glucose, arabinose, mannose, mannitol, N-acetylglucosamine, maltose, gluconate, adipate, malate, citrate and phenylacetate and negative reactions for indole production, glucose acidification, gelatin hydrolysis, β-galactosidase activity and assimilation of caprate. In the API ZYM kit, alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-Bl-phosphohydrolase, β-glucosidase and β-glucosidase activities are present and C14 lipase, trypsin, z-chymotrypsin, z-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, z-mannosidase and z-fucosidase activities are absent. The following compounds are utilized as sole carbon sources in the Biolog GN2 microplate: z-cyclodextrin, dextrin, glycogen, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellulobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, z-D-glucose, myo-inositol, lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, D-sorbitol, sucrose, trehalose, turanose, xyitol, pyruvic acid methyl ester, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galacturonic acid, D-gluconic acid, D-gluconaminoic acid, D-glucuronic acid.
acid, β-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, gelceronamide, L-alaninamide, D- and L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, L-histidine, L-phenylalanine, L-proline, D- and L-serine, urocanic acid, inosine, thymidine, putrescine, glycerol, DL-x-glycerol phosphate, α-D-glucose 1-phosphate and D-glucose 6-phosphate. All other substrates in the GN2 microplate are not utilized. Resistant to novobiocin and sensitive to penicillin G, ampicillin, chloramphenicol, gentamicin, rifampicin, kanamycin, tetracycline, streptomycin, sulfuramethoxazole plus trimethoprim, and nalidixic acid. The major cellular fatty acids (> 8%) are summed feature 3 (C16:1ω7c and/or C16:1ω6c), C16:0 and C18:1ω7c. The major cellular hydroxy fatty acid is C10:0 3-OH. Exhibits a complex polar lipid profile consisting of PE, PG, DPG, three characterized aminophospholipids and three uncharacterized phospholipids.

The type strain is DQS-4T (=BCRC 80407T =KCTC 23918T =LMG 26893T), which was isolated from oil-contaminated soil located in Kaoshing City, Taiwan. The DNA G+C content of the type strain is 64.5 mol%.

References


