Fodinicurvata sediminis gen. nov., sp. nov. and Fodinicurvata fenggangensis sp. nov., poly-β-hydroxybutyrate-producing bacteria in the family Rhodospirillaceae

Yong-Xia Wang,1† Ji-Hui Liu,1† Xiao-Xia Zhang,2 Yi-Guang Chen,1,3 Zhi-Gang Wang,1 Yun Chen,1 Qin-Yuan Li,1 Qian Peng1 and Xiao-Long Cui1

1Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan 650091, PR China
2Agricultural Cultural Collection of China, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100080, PR China
3College of Bio-resources and Environmental Science, Jishou University, Jishou, Hunan 416000, PR China

Two Gram-negatively stained, facultatively anaerobic, non-motile, vibrioid and rod-shaped, chemoheterotrophic bacterial strains, designated YIM D82T and YIM D812T, were isolated from a salt mine in Yunnan, south-west China. DNA–DNA hybridization, genomic DNA G+C content and phylogenetic analyses based on 16S rRNA gene sequences divided the two isolates into two distinct genospecies that were also clearly differentiated by fatty acid profiles, carbon source utilization patterns, antibiotic susceptibility and biochemical characteristics. The two isolates grew in the presence of 1.5–20 % NaCl, and optimally at 28±6°C and pH 7.5. The genomic DNA G+C contents of strains YIM D82T and YIM D812T were 61.5 and 62.3 mol%, respectively. Phylogenetic analyses based on 16S rRNA gene sequences indicated that strains YIM D82T and YIM D812T were members of the family Rhodospirillaceae and showed 90.5–90.6 % and 90.1–90.2 % similarities with their closest relatives, Rhodovibrio sodomensis and Rhodovibrio salinarum, respectively. Differential phenotypic and genotypic characteristics of the two isolates from recognized genera showed that the two strains should be classified as representing a new genus and two novel species for which the names Fodinicurvata sediminis gen. nov., sp. nov. (type strain YIM D82T =DSM 21159T=KCTC 22351T) and Fodinicurvata fenggangensis sp. nov. (type strain YIM D812T =CCTCC AA 208037T=DSM 21160T) are proposed.

The family Rhodospirillaceae, belonging to the order Rhodospirillales (Pfennig & Trüper, 1971) of the class Alphaproteobacteria, comprises 21 genera (http://www.bacterio.cict.fr). During the course of a study of the microbial diversity of the Fenggang salt mine in Yunnan, south-west China, two cream–white-pigmented bacterial strains, designated YIM D82T and YIM D812T, were isolated. The aim of the present study was to determine the exact taxonomic positions of strains YIM D82T and YIM D812T by using a polyphasic approach that included the analysis of phenotypic properties, detailed phylogenetic analysis based on 16S rRNA gene sequences and DNA–DNA relatedness.

Strains YIM D82T and YIM D812T were isolated from a sediment sample collected from the salt mine by using a standard dilution-plating technique at 28°C on Difco marine agar 2216 (MA; pH 7.2), supplemented with 3 % (w/v) NaCl. Pure cultures were maintained on nutrient agar (NA; Difco) supplemented with 5 % NaCl, and stored as 20 % (v/v) glycerol suspensions at −80°C.

Gram staining was performed using the method of Magee et al. (1975) with crystal violet (60 s), iodeine mordant (60 s), 95 % ethanol (5–10 s) and safranin counterstain (60 s), and with the 3 % KOH lysis test (Gregersen, 1978) as a supplementary test to Gram staining. Cellular morphology and motility were examined by using light microscopy (model BH 2; Olympus). Growth at various
concentrations of salt was determined on NA supplemented with NaCl at 0, 0.5, 1, 1.5, 2, 2.5, 3, 5, 7, 10, 12, 15, 20, 25 and 30 % (w/v). Growth at various pH values (4–11, in increments of 0.5 pH units) and temperatures (4, 10, 15, 20, 25, 28, 37, 42, 45, 50, 55 and 60 °C) were determined on the maintenance medium (NA supplemented with 5 % NaCl). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber (GasPak Anaerobic system; BBL) on NA supplemented with 5 % NaCl with or without nitrate. Accumulation of poly-β-hydroxybutyrate (PHB) was observed by using both negatively stained electron microscopy and Sudan black staining (Smibert & Krieg, 1994) under a light microscope. Bacteriochlorophyll a was analysed spectrophotometrically according to the procedure of Cohen-Bazire et al. (1977) and analysed by HPLC as described by Minnikin et al. (1983). Isoprenoid quinones were extracted by using the method of Collins et al. (1977) and analysed by HPLC as described by Tamaoka et al. (1983). Polar lipids were extracted according to the procedures described by Minnikin et al. (1984) and were identified by using two-dimensional TLC after spraying with the appropriate detection reagents (Collins & Jones, 1980). The presence of phosphatidylcholine was identified by spraying with Dragendorff reagent (Sigma). H₂S production was determined on Kligler iron agar (Difco). Methyl red and Voges–Proskauer tests were performed as described by Smibert & Krieg (1994). Nitrate reduction, hydrolysis of aesculin and gelatin, acid production from glucose, indole production, arginine dihydrolase, urease and β-galactosidase were tested using an API 20NE kit (bioMérieux), according to the manufacturer’s instructions. Other enzyme activities were assayed by using an API ZYM kit (bioMérieux), except that the bacterial suspensions were prepared in autoclaved 5 % NaCl solution. Carbon utilization was tested using artificial seawater medium (Cho & Giovannoni, 2006) as the basal medium with each carbon source at a final concentration of 0.5 % (w/v) or v/v. Antibiotic resistance was determined with the disc diffusion method using commercial antibiotic-impregnated discs (BBL Becton Dickinson). The results were estimated according to the formation of the inhibition zone.

Isoprenoid quinones were extracted by using the method of Collins et al. (1977) and analysed by HPLC as described by Tamaoka et al. (1983). Polar lipids were extracted according to the procedures described by Minnikin et al. (1984) and were identified by using two-dimensional TLC after spraying with the appropriate detection reagents (Collins & Jones, 1980). The presence of phosphatidylcholine was identified by spraying with Dragendorff reagent (Sigma). Biomass for quantitative fatty acid analysis of the two strains was prepared by scraping growth from NA supplemented with 5 % NaCl that had been incubated for 5 days at 28 °C. Analysis of the cellular fatty acid profiles followed the method described by Sasser (1990) using the Microbial Identification System (MIDI). The G+C content of the genomic DNA was determined by using HPLC according to Mesbah et al. (1989), after extraction of DNA using the method of Cui et al. (2001). The genomic DNA of Escherichia coli DH5α was used as a standard.

The 16S rRNA gene was amplified and sequenced as described by Cui et al. (2001). The sequence was compared to those available in GenBank using BLAST (Altschul et al., 1990). Alignments and similarities were obtained using CLUSTAL_X. Phylogenetic analyses were carried out using MEGA3 (Kumar et al., 2004). Distances (corrected according to the Kimura two-parameter model; Kimura, 1980) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). A maximum-likelihood (Felsenstein, 1981) tree (not shown) was generated using the treeing algorithm contained in the PHYLIP package (Felsenstein, 1993). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985).

Strains YIM D82T and YIM D812T were both facultatively anaerobic, Gram-negative and non-motile. Cells were vibrioid and rod-shaped. PHB granules were detected in the two strains by using both negatively stained electron microscopy and Sudan black staining. Neither flagella nor endospores were observed. Colonies were cream–white, circular, convex and opaque with irregular margins after growth on NA supplemented with 5 % at 28 °C for 5 days. Growth occurred under anaerobic conditions. The temperature range for growth was 15–42 °C (optimum, 28 °C) and the pH range for growth was 6.5–8.5 (optimum, 7.5). Growth occurred at NaCl concentrations of 1.5–20 % (w/v) (optimum, 5 %). The two strains were catalase- and oxidase-positive. They were negative for Methyl red and Voges–Proskauer reactions. The two strains did not produce H₂S or β-phenylalanine deaminase. Biochemical tests for nitrate reduction, arginine dihydrolase and urease were positive. Hydrolysis of aesculin and gelatin, indole production, glucose acidification, and phenylalanine deaminase and β-galactosidase were negative. Acetone/methanol-extractable pigments and bacteriochlorophyll a were not produced. Therefore, the energy metabolism of the two strains appeared to be exclusively non-photosynthetic chemoheterotrophy. Major characteristics that differentiate the two strains are given in Table 1. Carbon source utilization patterns and antibiotic susceptibility are given in the species descriptions.

The major cellular fatty acids of strain YIM D82T comprised C₁₈:₁ω7c (48.6 %), C₁₈:₁ 2-OH (12.2 %) and C₁₈:₀ (11.8 %), and those of strain YIM D812T comprised C₁₈:₁ω7c (46.6 %), C₁₈:₁ 2-OH (14.7 %) and C₁₆:₀ (10.6 %) (see Supplementary Table S1, available in IJSEM Online). The fatty acid C₁₈:₁ω7c was commonly found as a major component in both strains YIM D82T and YIM D812T, which is a feature shared by members of the class Alphaproteobacteria (Labrenz et al., 2000). However, moderate amounts of C₁₈:₁ 2-OH (12.2–14.7 %) and C₁₉:₀ cyclo ω8c (7.9–7.4 %) were found in strains YIM D82T and YIM D812T, but were not found at significant levels in the genus Rhodovibrio. In addition, the presence of fatty acids iso-C₁₅:₀ G, C₁₈:₁ω9c and C₂₀:₂ ω6,9c clearly differentiated the two novel isolates from the genera Rhodovibrio, Azospirillum, Tistrella, Inquilinus and
Table 1. Characteristics that differentiate strains YIM D82<sup>T</sup> and YIM D812<sup>T</sup> from other phylogenetically related genera in the family Rhodospirillaceae

Taxa: 1, strain YIM D82<sup>T</sup> (*Fodinicurvata sediminus* gen. nov., sp. nov.); 2, strain YIM D812<sup>T</sup> (*Fodinicurvata fenggangensis* sp. nov.); 3, *Rhodovibrio* (data from Nissen & Dundas, 1984; Mack et al., 1993; Imhoff et al., 1998; Garrity et al., 2005); 4, *Azospirillum* (Tarrand et al., 1978; Reinhold et al., 1987; Khammas et al., 1989; Sly & Stackebrandt, 1999; Eckert et al., 2001; Xie & Yokota, 2005; Peng et al., 2006; Mehnaz et al., 2007a, b; Young et al., 2008); 5, *Rhodocista* (Favinger et al., 1989; Kawasaki et al., 1992; Imhoff et al., 1998; Zhang et al., 2003); 6, *Defluviicoccus* (Maszenan et al., 2005); 7, *Tistrella* (Shi et al., 2002); 8, *Skermanella* (Sly & Stackebrandt, 1999); 9, *Inquilinus* (Coenye et al., 2002); 10, *Thalassobaculum* (Zhang et al., 2008).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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</thead>
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<tr>
<td>Habitat</td>
<td>Deposit of salt mine</td>
<td>Deposit of salt mine</td>
<td>Seawater, ponds of solar salt</td>
<td>Soil, root, fresh water</td>
<td>Freshwater, wastewater</td>
<td>Sludge</td>
<td>Wastewater</td>
<td>Lake water</td>
<td>Cystic fibrosis patients</td>
<td>Coastal seawater</td>
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<td>Colony colour</td>
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<td>Cream–white</td>
<td>Pink, red</td>
<td>Pink, white</td>
<td>Red, pink</td>
<td>Beige</td>
<td>NA</td>
<td>Apricot</td>
<td>Pink</td>
<td>Cream–yellow</td>
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<td>0.6–0.9 × 1.0–1.3</td>
<td>0.6–0.9 × 2–3</td>
<td>1–2 × 3.0</td>
<td>1.5–4.5</td>
<td>0.7–1.0 (width)</td>
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</tr>
<tr>
<td>Cell shape</td>
<td>Rod and vibrioid</td>
<td>Rod and vibrioid</td>
<td>Vibrio, spiral</td>
<td>Plump, vibrioid straight rod</td>
<td>Vibrio, spiral</td>
<td>Coccus</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Slightly curved and straight rod</td>
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<tr>
<td>Flagella*</td>
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<td>–</td>
<td>MP, BP</td>
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<td>6.5–8.5</td>
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<td>7–8</td>
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<td>NaCl/salt tolerance (%)</td>
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<td>3–24</td>
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<td>NA</td>
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<td>&lt;5</td>
<td>&lt;6</td>
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<tr>
<td>Poly-β-hydroxybutyrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
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<td>Bacteriochlorophyll a</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
<td>–</td>
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<td>+</td>
<td>NA</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>+</td>
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<tr>
<td>Gelatinase</td>
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<td>+</td>
<td>NA</td>
<td>V</td>
<td>NA</td>
<td>W</td>
<td>+</td>
<td>–</td>
<td>V</td>
<td>+</td>
</tr>
</tbody>
</table>

Utilization of carbon sources

| 1-Arabinose                  | + | – | NA | V | NA | + | + | + | – | + |
| d-Glucose                   | + | + | – | V | – | + | NA | + | – | – |
| Citrate                     | + | + | – | V | – | NA | NA | + | – | – |
| myo-Inositol                | + | – | NA | V | NA | NA | NA | – | – | – |
| d-Mannitol                  | + | – | NA | V | NA | NA | NA | + | – | – |
| L-Rhamnose                  | – | – | NA | V | NA | NA | NA | – | – | – |
| D-Ribose                    | – | – | NA | V | NA | NA | NA | + | NA | + |
| Sucrose                     | + | – | – | V | NA | NA | NA | – | – | + |
| Major quinone               | Q-10 | Q-10 | Q-10, MK-10 | Q-10 | Q-9 | NA | Q-10 | Q-10 | NA | Q-10 |
| DNA G + C content (mol%)    | 61.5 | 62.3 | 66.2–68.1 | 64–71 | 68.3–69.9 | 66 | 67.5 | 67.2 | 70.9 | 68.0 |

*BP, Bipolar; MP, monopolar.
Thalassobaculum. Therefore, the fatty acid profiles of strains YIM D82T and YIM D812T differed distinctly from those of related genera in the family Rhodospirillaceae. Furthermore, strains YIM D82T and YIM D812T could be further differentiated by means of the presence or absence of C13:0 2-OH, C14:1 3-OH, anteiso-C15:0, C16:0 2-OH and C17:1 iso. The isoprenoid quinone in strains YIM D82T and YIM D812T was ubiquinone 10 (Q-10), which was also found in the genera Azospirillum, Tistrella, Skermanella and Thalassobaculum. However, MK-10 and Q-9 were also found, respectively, in the genera Rhodovibrio and Rhodocista (Table 1). The polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine, except that strain YIM D82T also contained phosphatidylglycerol and three unknown phospholipids, and strain YIM D812T contained one unknown phospholipid (see Supplementary Fig. S1, available in IJSEM Online). The G+C contents of the genomic DNA of strains YIM D82T and YIM D812T were 61.5 and 62.3 mol%, respectively.

Phylogenetic analysis of almost-complete 16S rRNA gene sequences of strains YIM D82T and YIM D812T revealed that they formed a distinct lineage within the family Rhodospirillaceae (Fig. 1). The similarity between the 16S rRNA gene sequences of the two strains was 98.2%. Species of the genus Rhodovibrio were found to be the nearest phylogenetic neighbours; this relationship was supported by a high bootstrap value (92%) and also by the other tree-making algorithm used. The levels of 16S rRNA gene sequence similarities between strain YIM D82T and the type strains of Rhodovibrio sodomensis and Rhodovibrio salinarum were 90.6 and 90.5%, respectively, and 85.0–89.5% to the other type species of the family Rhodospirillaceae. The levels of 16S rRNA gene sequence similarities between strain YIM D812T and the type strains of R. sodomensis and R. salinarum were 90.2 and 90.1%, respectively, and 84.3–88.1% to the other type species of the family Rhodospirillaceae. The low sequence similarity values between strains YIM D82T and YIM D812T and the type species of genera of the family Rhodospirillaceae demonstrated that the two strains represent a distinct genus in the family Rhodospirillaceae.

DNA–DNA hybridization was performed using the photobiotin-labelling method of Ezaki et al. (1989), with a multiwell plate reader (CytoFluor; PerSeptive Biosystems). The DNA–DNA relatedness between strains YIM D82T and YIM D812T was 27.5%. Therefore, strains YIM D82T and YIM D812T should be considered as representing two separate species.

The data obtained based on the polyphasic approach used, such as fatty acid profiles, quinone and 16S rRNA gene phylogenetic analyses, demonstrated conclusively that strains YIM D82T and YIM D812T should be recognized as representing a novel genus within the family Rhodospirillaceae. In addition, the DNA–DNA hybridization values, polar lipid patterns and differences in phenotypic traits indicated that the two strains represent two novel species in the new genus, for which the names Fodinicurvata sediminis gen. nov., sp. nov. and Fodinicurvata fenggangensis sp. nov. are proposed.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strains YIM D82T and YIM D812T, and representatives of the family Rhodospirillaceae. Bootstrap per- centages (based on 1000 replications) >50% are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-likelihood method. Bar, 0.01 substi tutions per nucleotide position.
Description of Fodinicurvata gen. nov.

Fodinicurvata (Fo.di.ni.cur.va’ta. L. fem. n. fodina mine; L. adj. curvatus -a -um curved; N.L. fem. n. Fodinicurvata curved-shaped bacterium isolated from a mine).

Cells are Gram-negative, facultatively anaerobic, non-motile, vibrioid and rod-shaped. Catalase- and oxidase-positive. Bacteriochlorophyll a is not detected. Accumulate PHB granules. Nitrate is reduced. The predominant polar lipids consist of diphosphatidylglycerol, phosphatidyl-methylthanolamine and phosphatidylcholine. Phosphatidylinositol is variable among species. The DNA G+C content is 61.5–62.3 mol%. Member of the family Rhodospirillaceae. The type species is Fodinicurvata sediminis.

Description of Fodinicurvata sediminis sp. nov.

Fodinicurvata sediminis (sed.i.min’is. L. gen. n. sediminis of sediment).

Exhibits the following properties in addition to those given in the genus description. Colonies are cream–white, smooth, circular, convex and opaque, with slightly irregular margins. Cells are approximately 0.3–0.5 μm wide and 0.7–1.5 μm long. Temperature range for growth is 15–42 °C (optimum, 28 °C). pH range for growth is 6.5–8.5 (optimum, 7.5). Grows at NaCl concentrations of 1.5–20 % (w/v). Positive for urease, arginine dihydrolase and nitrate reduction, and negative for hydrolysis of gelatin and ascorbin, indole production, glucose acidification, L-phenylalanine deaminase and β-galactosidase. Utilizes L-arabinose, α-cyclodextrin, D-glucose, maltose, D-mannitol, D-sorbitol, sucrose, D-xyllose, ethanol, glycerol, acetate, citrate, L-asparagine, L-aspartic acid, L-glutamic acid and L-proline as sole carbon sources, but not adonitol, amylum, L-arabinose, D-cellulose, α-cyclodextrin, β-cyclodextrin, dextrin, L-fructose, D-galactose, myo-inositol, D-lactose, D-mannose, melibiose, methanol, raffinose, L-ramnosose, D-ribose, sucrose, trehalose, ethanol, D-mannitol, methanol, acetate, glycerol, L-histidine, L-ornithine, L-phenylalanine or D-serine. With the API ZYM system, positive for alkaline phosphatase, esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase and naphthol-AS-BI-phosphohydrolase, weakly positive for esterase (C4) and α-chymotrypsin, and negative for acid phosphatase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Sensitive to (μg per disc, unless indicated otherwise): carbenicillin (100), chloramphenicol (30) and penicillin (10 U), but resistant to amikacin (30), ampicillin (10), erythromycin (15), gentamicin (10), nalidixic acid (30), norfloxacin (10) and streptomycin (10). Polar lipids consist of diphosphatidylglycerol, phosphatidylmethylthanolamine, phosphatidylcholine, phosphatidylinositol and three unknown phospholipids. Major fatty acids are C_{18:1}ω7c, C_{18:1} 2-Oh and C_{16:0}. The isoprenoid quinone is Q-10. The DNA G+C content of the type strain is 62.3 mol%.

The type strain, YIM D812T ( = CCTCC AA 208037T = DSM 21160T), was isolated from a salt mine of Fenggang in Yunnan, south-west China.

Description of Fodinicurvata fenggangensis sp. nov.

Fodinicurvata fenggangensis (feng.gang.en’is. N.L. fem. adj. fenggangensis the locality of the salt mine from which the organism was isolated).

Exhibits the following properties in addition to those given in the genus description. Colonies are cream–white, smooth, circular, convex and opaque with slightly irregular margins. Cells are approximately 0.2–0.5 μm wide and 0.5–1.3 μm long. Temperature range for growth is 15–42 °C (optimum, 28 °C). pH range for growth is 6.5–8.5 (optimum, 7.5). Grows at NaCl concentrations of 1.5–20 % (w/v). Positive for urease, arginine dihydrolase and nitrate reduction, and negative for hydrolysis of ascesul and gelatin, indole production, glucose acidification, L-phenylalanine deaminase and β-galactosidase. Utilizes glucose, myo-inositol, maltose, citrate, L-asparagine, L-aspartic acid, L-glutamic acid, glycerol and L-proline as sole carbon sources, but not adonitol, amylum, L-arabinose, D-cellulose, α-cyclodextrin, β-cyclodextrin, dextrin, L-fructose, D-galactose, D-lactose, D-mannose, melibiose, raffinose, L-ramnosose, D-ribose, sucrose, trehalose, ethanol, D-mannitol, methanol, acetate, glycerol, L-histidine, L-ornithine, L-phenylalanine or D-serine. With the API ZYM system, positive for alkaline phosphatase, esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase and naphthol-AS-BI-phosphohydrolase, weakly positive for esterase (C4) and α-chymotrypsin, and negative for acid phosphatase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Sensitive to (μg per disc, unless indicated otherwise): carbenicillin (100), chloramphenicol (30) and penicillin (10 U), but resistant to amikacin (30), ampicillin (10), erythromycin (15), gentamicin (10), nalidixic acid (30), norfloxacin (10) and streptomycin (10). Polar lipids consist of diphosphatidylglycerol, phosphatidylmethylthanolamine, phosphatidylcholine, phosphatidylinositol and three unknown phospholipids. Major fatty acids are C_{18:1}ω7c, C_{18:1} 2-Oh and C_{16:0}. The isoprenoid quinone is Q-10. The DNA G+C content of the type strain is 62.3 mol%.

The type strain, YIM D812T ( = CCTCC AA 208037T = DSM 21160T), was isolated from a salt mine of Fenggang in Yunnan, south-west China.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (NSFC) (30860013, 30460004, 30660004, 30760006), the Ministry of Science and Technology of China (863 Program, no. 2007AA201306), the Yunnan Provincial Sciences and Technology Department (2005PY01-1, 2006C0006M, 2009DA002) and Yunnan University (2008YB005). We are grateful to Mr Wei Chen for his help during sampling, as well as Ms Xiang-Feng Cai for her excellent technical assistance.

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Fodinicurvata gen. nov., with two species


