Sphingobium rhizovicinum sp. nov., isolated from rhizosphere soil of *Fortunella hindsii* (Champ. ex Benth.) Swingle

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The taxonomic status of a pale-yellow-coloured bacterial isolate from rhizosphere soil of *Fortunella hindsii* (Champ. ex Benth.) Swingle was characterized using a polyphasic taxonomic approach. Comparative analysis of the 16S rRNA gene sequence showed that the isolate constituted a distinct branch within the genus *Sphingobium*. The generic assignment was confirmed by chemotaxonomic data, which revealed the presence of a fatty acid profile that was characteristic for the genus *Sphingobium*, consisting of straight-chain saturated and unsaturated as well as 2-OH fatty acids and the lack of 3-OH fatty acids, ubiquinone with ten isoprene units (Q-10) as the predominant respiratory quinone, and a polar lipid pattern that consisted of the predominant compounds phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmonomethylethanolamine, phosphatidylmethylethanolamine, diphosphatidylglycerol, sphingoglycolipid and an unknown glycolipid. Spermidine was the major polyamine component. The genotypic and phenotypic data (physiology and fatty acid and polar lipid profiles) showed that the isolate merits classification as representing a novel species of the genus *Sphingobium*, for which the name *Sphingobium rhizovicinum* sp. nov. is proposed. The type strain is CC-FH12-1^T (=CCM 7941^T=BCRC 17770^T).

The genus *Sphingobium* proposed by Takeuchi *et al.* (2001) contains strictly aerobic, chemo-organotrophic, yellow- to whitish-brown-pigmented, Gram-negative, rod-shaped bacteria. Members of the genus are characterized chemotaxonomically by having fatty acid profiles that contain *n*-C_{18:1ω7c} as the major fatty acid as well as 2-OH *n*-C_{14:0} as the main hydroxylated fatty acid, but never 3-OH fatty acids. They contain ubiquinone Q-10 as the main respiratory quinone and spermidine as the major polyamine. The polar lipid pattern consists of diphosphatidylglycerol, phosphatidylglycerol, sphingoglycolipid, phosphatidylethanolamine, phosphatidylmethylethanolamine

and phosphatidylcholine (Busse *et al.*, 1999; Stolz *et al.*, 2000; Pal *et al.*, 2005; Prakash & Lal, 2006; Wittich *et al.*, 2007). Members of the genus *Sphingobium* represent environmental isolates that play an important role in bioremediation and the biodegradation of pollutants. At the time of writing, the genus *Sphingobium* comprised 13 recognized species (Young *et al.*, 2007). In this paper we report on the taxonomic characterization of a yellow-pigmented isolate (designated strain CC-FH12-1^T), identified preliminarily as a member of the genus *Sphingobium*, from rhizosphere soil samples of *Fortunella hindsii* (Champ. ex Benth.) Swingle after appropriate dilutions. The isolate was subsequently cultivated on R2A (Oxoid) and tryptone soya agar (Oxoid) to determine its morphological characteristics. Fermentation and enzymic tests were performed by using API 20E and API ZYM (bioMérieux) systems and assimilation tests were performed using the API 20NE (bioMérieux) and Biolog systems, according to the manufacturers' instructions. All the strips were read

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CC-FH12-1^T is EF465534.

A figure showing the polar lipid profile of strain CC-FH12-1^T and a table showing the cellular fatty acid contents of strain CC-FH12-1^T and recognized *Sphingobium* species are available as supplementary material with the online version of this paper.

after 72 h incubation at 37 °C. Antibiotic susceptibility testing was carried out using ATB Staph 5 strips (bioMérieux), according to the manufacturer's recommendations. Salt tolerance was determined by cultivating the organism in tryptone soya broth supplemented with NaCl at final concentrations in the range 0.0–12.0 %. Flagellum staining was performed using Spot Test Flagella stain (BD Difco). Poly- β -hydroxybutyrate granule accumulation was observed under light microscopy after staining cells with Sudan black (Smibert & Krieg, 1994).

DNA for the determination of the G+C content was isolated by using an UltraClean microbial DNA isolation kit (MOBIO), following the instructions given by the manufacturer. The G+C content of the DNA was determined using HPLC as described by Mesbah *et al.* (1989) using a Waters Symmetry Shield C8 column. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

Fatty acid analyses were performed according to Kämpfer & Kroppenstedt (1996) after subcultivation of the strain on tryptic soy agar (BD Difco) for 48 h at 28 °C. Respiratory quinones and polar lipids were extracted as described by Tindall (1990a, b). Quinones were analysed using HPLC as reported by Altenburger *et al.* (1996) and Stolz *et al.* (2007). Polar lipids were separated by using two-dimensional thin-layer chromatography according to Tindall (1990a, b). The total lipid profile was visualized by spraying with molybdatophosphoric acid and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates) and α -naphthol (specific for sugars). For polyamine analysis, two cultures of strain CC-FH12-1^T were grown on PYE medium (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2), harvested at approximately 20 and 50 %, respectively, of the maximum optical density and, after lyophilization, polyamines were extracted and analysed as described by Busse & Auling (1988), Busse *et al.* (1997) and Stolz *et al.* (2007).

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR products were carried out according to the methods of 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 electrophoresis of the sequence reaction products. The 16S rRNA gene sequences of recognized species of the genus *Sphingobium* retrieved from GenBank were added to the ARB database (Ludwig *et al.*, 2004) and aligned using the respective tool of the ARB package. The resulting alignment was corrected manually by removing ambiguous bases and evolutionary trees were inferred using maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood algorithms (Felsenstein, 1981). The 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.

An almost-complete 16S rRNA gene sequence (1450 nt) of strain CC-FH12-1^T was determined in this study. A tree depicting the phylogenetic affinity of strain CC-FH12-1^T within the genus *Sphingobium* is shown in Fig. 1. It is evident from the tree that strain CC-FH12-1^T formed a distinct subline within the genus *Sphingobium*, branching proximal to the base of a subcluster of species, which included *Sphingobium cloacae*, *S. yanoikuyae* and *S. amiense*. However, bootstrap resampling showed that the association of strain CC-FH12-1^T with this subcluster of species was not supported by a high bootstrap value and, from the tree construction analysis, it was evident that strain CC-FH12-1^T does not exhibit a significant affinity with any recognized species. The highest sequence similarities were shown with the type strains of *Sphingobium japonicum* (97.4 %), *S. amiense* (97.3 %), *S. francense* (97.2 %), *S. indicum*, *S. chungbukense* (96.9 %) and *S. fuliginis* (96.8 %), with other species showing lower levels (<96.5 %) of similarity. DNA–DNA hybridization experiments were performed with strain CC-FH12-1^T and *S. japonicum* DSM 16413^T, *S. amiense* DSM 16289^T and *S. francense* CCUG 53833^T using the method described by Ziemke *et al.* (1998), except that, for nick translation, 2 μ g DNA was labelled during a 3 h incubation at 15 °C. Strain CC-FH12-1^T showed relatively low DNA–DNA relatedness to all these strains (<50 %). Further support for the distinctiveness of strain CC-FH12-1^T was also evident from phenotypic analyses (Table 1).

Chemotaxonomically, strain CC-FH12-1^T possesses chemical markers that support its assignment to the genus *Sphingobium*. Cellular fatty acids analyses revealed the presence of C_{14:0} 2-OH (5.8 %) and C_{18:1} ω 7c (67.5 %) as major hydroxylated and non-hydroxylated fatty acids, respectively. The detailed fatty acid profile of strain CC-FH12-1^T in comparison with those of other recognized *Sphingobium* species is shown in Supplementary Table S1 (available in IJSEM Online). The quinone system of strain CC-FH12-1^T consisted of the major compound ubiquinone Q-10 (93 %) and minor amounts of Q-9 (7 %). The polar lipid profile exhibited the major compounds phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, sphingoglycolipid (SGL) and an unknown glycolipid (GL2). In addition, minor amounts of an unknown aminolipid, two unknown phospholipids, two unknown phosphoglycolipids, an unknown glycolipid (GL1) and five unknown polar lipids were detected (see Supplementary Fig. S1, available in IJSEM Online). It is worth mentioning here that the spot identified as sphingoglycolipid and the unknown glycolipid GL1 developed a flimsy blue colour after spraying with molybdenum blue spray but, due to a deviation from the behaviour of typical phospholipids, it is not clear whether the colour development indicates the presence of phos-

Fig. 1. Maximum-likelihood tree showing the position of strain CC-FH12-1^T (*Sphingobium rhizovicinum* sp. nov.) among recognized *Sphingobium* species. The tree was based on a comparison of 16S rRNA gene sequences that were at least 90% complete (with regard to *Escherichia coli* sequence). Asterisks indicate branches that were also recovered using maximum-parsimony. Bar, 2.0% sequence divergence.

phate groups in these lipids or only a non-specific reaction. As sugar-containing lipids usually develop a reddish to violet colour after spraying with α -naphthol it should be mentioned here that, similar to sphingoglycolipid and the unknown glycolipid GL1, the two unknown phosphoglycolipids and unknown glycolipid GL2 exhibited a grey colour and hence they were identified as sugar-containing lipids as well. Based on their chromatographic behaviour, lipid SGL corresponds to the alkali stable lipid GL and lipid GL1 to the alkali stable GL' (Yabuuchi *et al.*, 1990) and also to lipids with identical designations found in several sphingomonads (Busse *et al.*, 1999). This polar lipid profile shares the majority of characteristics that have also been reported for other *Sphingobium* species. However, the lack of phosphatidylcholine has not been shown for any recognized *Sphingobium* species and the detection of an unknown aminolipid, two unknown phosphoglycolipids and unknown phospholipid PL2 are unique features of strain CC-FH12-1^T that are useful for differentiation from other *Sphingobium* species for which polar lipid profiles have been obtained (Busse *et al.*, 1999; Stolz *et al.*, 2000; Pal

et al., 2005; Prakash & Lal, 2006; Wittich *et al.*, 2007). During two-dimensional thin-layer chromatography only a single yellow spot was detected, which exhibited a highly hydrophobic behaviour (Supplementary Fig. S1, in IJSEM Online). Strain CC-FH12-1^T exhibited a polyamine pattern that contained the predominant compound spermidine [49–61 μmol (g dry weight)⁻¹] and moderate amounts of spermine [8–10 μmol (g dry weight)⁻¹]. This polyamine pattern is in agreement with the characteristics of members of the genus *Sphingobium* (Busse *et al.*, 1999; Takeuchi *et al.*, 2001), but the relatively high amounts of spermine distinguishes strain CC-FH12-1^T from other species of the genus.

Strain CC-FH12-1^T was examined for a range of phenotypic characteristics. The organism consisted of Gram-negative, rod-shaped cells. On nutrient agar, Brain Heart Infusion (BHI) agar (BD Difco) and tryptone soya agar colonies were pale yellow, slightly elevated and smooth with entire margins. The organism grew aerobically and was catalase- and oxidase-negative. It hydrolysed gelatin,

Table 1. Differential biochemical characteristics of strain CC-FH12-1^T and recognized *Sphingobium* species

Strains: 1, CC-FH12-1^T (*Sphingobium rhizovicinum* sp. nov.); 2, *S. olei* IMMIB HF-1^T (data from Young *et al.*, 2007); 3, *S. amiense* YT^T (Ushiba *et al.*, 2003); 4, *S. cloacae* JCM 10874^T (Ushiba *et al.*, 2003); 5, *S. yanoikuyae* JCM 7371^T (Ushiba *et al.*, 2003); 6, *S. chlorophenolicum* DSM 8671^T (Ushiba *et al.*, 2003); 7, *S. herbicidovorans* DSM 11019^T (Ushiba *et al.*, 2003); 8, *S. chungbukense* KCTC 2955^T (Kim *et al.*, 2000); 9, *S. indicum* B90A^T (Pal *et al.*, 2005); 10, *S. japonicum* UT26^T (Pal *et al.*, 2005); 11, *S. francense* SP4^T (Pal *et al.*, 2005); 12, *S. xenophagum* BN6^T (Stolz *et al.*, 2000); 13, *S. aromaticiconvertens* DSM 12677^T (Wittich *et al.*, 2007); 14, *S. fuliginis* DSM 18781^T (Prakash & Lal, 2006). Strain CC-FH12-1^T is positive for the assimilation of L-rhamnose and negative for sucrose, whereas *S. japonicum* is positive for the assimilation of sucrose and negative for L-rhamnose (Pal *et al.*, 2005). +, Positive reaction; –, negative reaction; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Assimilation of:														
D-Glucose	+	+	+	–	+	+	+	+	+	+	+	+	–	+
L-Arabinose	+	+	–	–	+	–	+	+	+	+	+	+	–	+
Fructose	+	–	ND	–	–	–	–	+	+	+	–	–	+	ND
D-Mannose	+	–	–	–	–	–	–	+	–	–	–	–	–	ND
N-Acetylglucosamine	–	–	–	–	+	–	–	–	ND	ND	ND	–	–	ND
Malate	+	+	–	–	+	–	+	–	ND	ND	ND	+	–	ND
Gluconate	+	+	–	–	+	–	+	+	ND	ND	ND	ND	–	ND
Maltose	+	+	+	–	+	–	+	+	ND	ND	ND	+	–	+
Aesculin hydrolysis	+	+	–	–	+	+	+	–	–	+	+	+	–	–
Reduction of nitrate to nitrite	+	–	–	–	–	–	–	–	ND	ND	ND	ND	–	+
Production of catalase	–	–	+	+	+	+	+	+	+	+	+	+	+	+
β-Galactosidase	+	–	–	–	+	–	–	–	ND	ND	ND	–	+	–

hippurate and aesculin. It had alkaline phosphatase, α-glucosidase, pyrrolidonyl arylamidase, leucine aminopeptidase and pyrazinamidase activities. It showed positive urease activity and was capable of reducing nitrate to nitrite. The detailed biochemical properties of strain CC-FH12-1^T are given in the species description below. Differential biochemical characteristics of strain CC-FH12-1^T and recognized species of the genus *Sphingobium* are given in Table 1.

Based on both phenotypic and genetic criteria, these results suggest that strain CC-FH12-1^T represents a novel species within the genus *Sphingobium*, for which the name *Sphingobium rhizovicinum* sp. nov. is proposed.

Description of *Sphingobium rhizovicinum* sp. nov.

Sphingobium rhizovicinum (rhi.zo.vi.ci'num. Gr. n. *rhiza* root; L. masc. adj. *vicinus* -a -um neighbouring; N.L. neut. adj. *rhizovicinum* neighbouring a root, referring to the rhizosphere, soil closely related to plant roots, from where the type strain was isolated).

Cells are Gram-negative and rod-shaped (0.4–0.6 × 1.0–1.5 µm). Motile by means of a single polar flagellum. On nutrient agar, tryptone soya agar and BHI agar, pale-yellow, slightly elevated colonies with entire margins are formed. Aerobic, catalase- and oxidase-negative, grows at temperatures ranging between 22 and 37 °C, and tolerates pH in the range 5.5–8 and salt concentration of 3.0% NaCl. Cells accumulate poly-β-hydroxybutyrate granules. The DNA G+C content is 59.4 ± 0.2 mol%. Exhibits the salient chemotaxonomic characteristic of the genus *Sphingobium*. The detailed fatty acid profile is presented

in Supplementary Table S1 (available in IJSEM Online). Respiratory quinones consist of the major compound ubiquinone Q-10 and minor amounts of Q-9. Polar lipid profile consists of the major compounds phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, sphingoglycolipid and an unknown glycolipid. In addition, minor amounts of an unknown aminolipid, two unknown phospholipids, two unknown phosphoglycolipids, an unknown glycolipid and five unknown polar lipids are detected. Polyamine pattern contains the major compound spermidine and moderate amounts of spermine. Gelatin, hippurate and aesculin are hydrolysed. Positive (Biolog GN2 system) for dextrin, α-cyclodextrin, Tween 80, Tween 40, L-arabinose, cellobiose, D-fructose, L-fucose, D-galactose, α-D-glucose, maltose, D-mannose, D-melibiose, L-rhamnose, trehalose, pyruvic acid methyl ester, succinic acid monomethyl ester, citric acid, D-gluconic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, propionic acid, quinic acid, succinic acid, bromosuccinic acid, L-alaninamide, D-alanine, L-alanyl glycine, L-aspartic acid, L-glutamic acid, hydroxy-L-proline and L-proline.

Employing Biolog GN2 the following substrates are not oxidized: adonitol, D-arabitol, glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, i-erythritol, gentiobiose, α-D-lactose, lactulose, m-inositol, D-mannitol, methyl β-D-glucoside, raffinose, sucrose, turanose, cis-aconitic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, D-psicose, D-sorbitol, xylitol,

itaconic acid, α -ketovaleric acid, malonic acid, acetic acid, formic acid, D-saccharic acid, sebacic acid, succinamic acid, glucuronamide, glycyl L-aspartic acid, glycyl L-glutamic acid, L-alanine, L-asparagine, L-histidine, L-leucine, L-phenylalanine, L-pyrogutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ -aminobutyric acid, L-ornithine, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL- α -glycerol phosphate, α -D-glucose 1-phosphate and D-glucose 6-phosphate.

Using ATB Staph (bioMérieux), sensitive to cotrimoxazole, tetracycline, minocycline and gentamicin (intermediately sensitive) and resistant to rifampicin, norfloxacin, levofloxacin, nitrofurantoin, penicillin, clindamycin, erythromycin, fusidic acid, plindamycin, vancomycin, teicoplanin, quinupristin-dalfopristin, coag-oxacillin and oxacillin.

In API 20NE, positive for nitrate reduction, aesculin, gelatin hydrolysis, β -galactosidase, and assimilation of glucose, arabinose, mannose, maltose, potassium-glucuronate, capric acid, malic acid and trisodium citrate, but negative for L-tryptophan, arginine, urease, glucose fermentation, mannitol, N-acetylglucosamine, phenylacetic acid and adipic acid. In API 20E, positive for β -galactosidase, arginine dihydrolase, citrate utilization, acetoin production (Voges-Proskauer), gelatinase and fermentation of glucose, mannitol, inositol, sorbitol, melibiose and amygdalin, but not indole, H₂S production, urease or oxidase.

In API ZYM, positive for alkaline acid phosphatase, butyrate esterase (C4), caprylate esterase (C8), myristate lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase and β -glucosidase, and negative for trypsin, α -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase.

The type strain (CC-FH12-1^T=CCM 7941^T=BCRC 17770^T) was isolated from rhizosphere soil of *Fortunella hindsii* (Champ. ex Benth.) Swingle.

Acknowledgements

We are grateful to Professor Dr Hans-Georg Trüper for nomenclatural advice and W. S. Huang, for technical assistance. This research work was kindly supported by a grant from the National Science Council and Council of Agriculture, Executive Yuan, Taiwan, Republic of China.

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