Flavobacterium saliperosum sp. nov., isolated from freshwater lake sediment

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An aerobic, yellow-pigmented, Gram-negative bacterium, designated strain S13^T, was isolated from freshwater sediment of Taihu Lake in central China. The taxonomy of strain S13^T was studied by using phenotypic and phylogenetic methods. Cells of strain S13^T were rod-shaped, non-motile and with a size range of 0.35–0.55 × 1.5–2.5 µm. The nearly complete 16S rRNA gene of strain S13^T was amplified and sequenced. A BLAST search and phylogenetic analysis based on 16S rRNA gene sequence similarity showed that strain S13^T was related to members of the genus *Flavobacterium*, with the highest sequence similarity of 93.8% to *Flavobacterium columnare* (ATCC 23463^T). Cells contained menaquinone-6 (MK-6) as the major respiratory quinone and the genomic DNA G + C content was 41 mol%. The major fatty acids were iso-C_{15:0} (28.2%) and iso-C_{17:1ω9c} (19.0%). It is proposed that S13^T (=CGMCC 1.3801^T=JCM 13331^T) represents the type strain of a novel species, *Flavobacterium saliperosum* sp. nov.

The genus *Flavobacterium* was established by Frankland in 1889 (see Bergey *et al.*, 1923), since when many amendments to its description have been made. Bernardet *et al.* (1996) defined that *Flavobacterium* species are Gram-negative rods, are motile by gliding, contain menaquinone-6 (MK-6) as the sole respiratory quinone, and have DNA G + C contents in the range 32–37 mol% (among other properties). The 26 species within the genus *Flavobacterium* that are recognized at the time of writing appear in Fig. 1 (see later). *Flavobacterium* species are physiologically diverse: they can be psychrophilic, psychrotolerant or mesophilic, and can be halophilic, halotolerant or sensitive to salts. As a reflection of their physiological diversity, *Flavobacterium* species have been isolated from various habitats such as freshwater sediments and soil (Bernardet *et al.*, 1996; Tamaki *et al.*, 2003; McCammon & Bowman, 2000), a glacier (Zhu *et al.*, 2003) and Antarctic lakes (Van Trappen *et al.*, 2003, 2004; McCammon *et al.*, 1998; McCammon & Bowman, 2000; Humphry *et al.*, 2001; Yi *et al.*, 2005).

Taihu Lake (also known as Lake Tai; 120° 02' 16.8" E, 31° 27' 10.7" N), in Jiangsu Province, China, is the third largest freshwater lake in China and plays an important role in the region's agriculture, aquaculture and industry. Owing to

accelerating economic development and lack of environmental protection, the water quality of the lake is declining, with algal blooms occurring regularly in recent years. In an attempt to understand the microbial contribution to the cycling of nutrient elements (carbon, nitrogen, sulfur), analysis of the microbial community was conducted, and a genetic clone library of 16S rRNA genes derived from DNA samples of Taihu Lake sediment indicated that sequences related to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group accounted for 12% of the total clones (Dai *et al.*, 2005). Thus, efforts to isolate these CFB-related bacteria were made in our laboratory. We report here the isolation and identification of one of these organisms, designated strain S13^T.

To isolate *Flavobacterium* species, a method that combines plate culture with PCR-guided screening was established. First, colonies on agar plates were obtained by plating 10-fold dilutions (10⁻¹–10⁻⁵) of lake sediment samples on dilute nutrient medium (DNM; 0.5 g beef extract l⁻¹, 1 g fish peptone l⁻¹, 0.5 g NaCl l⁻¹, 15 g agar l⁻¹) and incubating at 25 °C for 10 days. Second, yellow-pigmented colonies were checked by PCR using a forward primer (designed here according to alignment of 16S rRNA gene sequences of some CFB group members: 5'-ACGGGTGG-CGGAACACGTACAG-3') and a reverse primer (1468R, a general primer for bacteria: 5'-CTGGCCACAGACGTGG-AG-3'). Eight of 40 colonies gave positive signals during PCR detection. DNA sequences of these eight strains showed that they have almost identical 16S rRNA genes (500 bp at the 5'-end). One representative strain, S13^T, was selected for

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Abbreviation: CFB, *Cytophaga-Flavobacterium-Bacteroides*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Flavobacterium saliperosum* S13^T is DQ021903.

Fig. 1. Phylogenetic tree constructed with the neighbour-joining method according to 16S rRNA gene sequences of strain S13^T and species of the genus *Flavobacterium*. Numbers at nodes indicate percentages of bootstrap support based on 1000 resampled datasets. *Cytophaga marinoiflava* (ATCC 19326^T) was used as outgroup. GenBank accession numbers are given in parentheses. Bar, evolutionary distance (K_{nuc}) of 0.05.

phylogenetic analysis and for biochemical and physiological characterization.

Routine cultivation was conducted at 25 °C with modified M1 medium (ATCC) or Shieh medium (Song *et al.*, 1988). Gram reactions were determined according to the method described by Gerhardt *et al.* (1994). Cell flagellation and morphology were examined by transmission and scanning electron microscopy. Chitin hydrolysis was tested as described by Hsu & Lockwood (1975). Hydrolysis of CM-cellulose (0.5 %, w/v), gelatin (0.5 %, w/v), casein (50 % skimmed milk, v/v), agar (1.5 %, w/v), alginate (0.5 %, w/v), pectin (0.5 %, w/v), aesculin (0.5 %, w/v), starch (0.5 %, w/v), L-tyrosine (0.5 %, w/v), Tween 80 (1 %, v/v) and egg yolk (5 %, w/v) was tested using the standard mineral base of Stanier *et al.* (1966). After the mineral base was autoclaved, each compound was added. Growth was examined after incubation at 25 °C for 1, 3, 7 and 14 days. Catalase and oxidase activities, Voges–Proskauer reaction, brown diffusible pigment on L-tyrosine agar and production of H₂S were also investigated according to the methods of Dong & Cai (2001). Aerobic and anaerobic production of acids (OF reaction) from carbohydrates was determined in OF basal medium (Hugh & Leifson, 1953). Carbohydrate solution sterilized by filtration was added at final concentration of 1 % (w/v), and acid production was recorded after 7 and 14 days incubation.

Flexirubin-type pigments were detected by using 20 % KOH (Fautz & Reichenbach, 1980) and extracellular glycans were

identified with the Congo red absorption test (McCammon & Bowman, 2000). Growth temperature was determined with a TN3F temperature-gradient incubator (Advantec). Growth on seawater agar, nutrient agar and trypticase soy agar was tested at 25 °C after 14 days growth. The presence of gliding motility was determined as described by Zhu *et al.* (2003). Salt tolerance was tested in M1 medium supplemented with 0–2 % NaCl (spun at 100 r.p.m., 25 °C) incubated for 3 days. Duplicate antibiotic-sensitivity tests were performed using filter-paper discs (ø3.25 × 0.75 mm) containing each of the following: ceftazidime (30 µg), ceftriaxone (30 µg), tobramycin (10 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg); penicillin (10 IU), erythromycin (15 µg), tetracycline (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), azithromycin (15 µg), kanamycin (30 µg), streptomycin (10 µg) and rifampicin (5 µg). Discs were placed on M1 medium plates spread with S13^T culture and were then incubated at 25 °C for 18–24 h.

Cells of strain S13^T were Gram-negative and aerobic, with a size range of 0.35–0.55 × 1.5–2.5 µm. Flagella were not observed. Colonies were yellow, smooth, circular and 2–4 mm in diameter. Growth was observed over a temperature range of 20–34 °C and a pH range of 6.5–8.5. Gelatin and casein were hydrolysed but starch, CM-cellulose, agar, alginate, aesculin, chitin and pectin were not. A detailed description of assimilation of sugars, sensitivity to antibiotics, etc., is provided in the species description. Growth occurred without adding NaCl to the medium and was severely inhibited in the presence of more

than 1% NaCl; the optimal concentration of NaCl for growth was 0.1% in medium. Differential phenotypic characteristics of strain S13^T and related *Flavobacterium* species are detailed in Table 1.

Biomass for chemotaxonomic analysis was harvested from modified M1 medium cultures at 25 °C for 24 h. Strain S13^T was further characterized for its cellular fatty acid profile. Cellular fatty acids were extracted and analysed by the Sherlock system (Midi) following the manufacturer's instructions. The most abundant cellular fatty acids of strain S13^T were iso-C_{15:0} (28.2%), iso-C_{17:1}9c (19.0%), iso-C_{17:0} 3-OH (8.6%) and iso-C_{15:1}G (6.7%). The cellular fatty acid profile of strain S13^T is given in the species description. Menaquinones were extracted and purified according to the method of Collins (1985) and were analysed by HPLC (Wu *et al.*, 1989), with the MK-6 from *Salgentibacter holothuriorum* (NBRC 100249^T) used as a reference. *S. holothuriorum* NBRC 100249^T was cultivated in liquid Zobell 2216E medium (150 r.p.m., 25 °C) for 36 h. Strain S13^T was shown to have MK-6 as the major respiratory quinone. DNA base composition was determined by thermal denaturation (Marmur & Doty, 1962) using DNA from *Escherichia coli* K-12 as a control. The DNA

G + C content of strain S13^T was 41 mol%, which is higher than that of other *Flavobacterium* species (32–37 mol%).

The nearly complete 16S rRNA gene of strain S13^T (1400 bp) was amplified and sequenced as described by Zhang *et al.* (2003). Alignments of 16S rRNA gene sequences were performed with the CLUSTAL_X program, version 1.64b (Thompson *et al.*, 1997). A phylogenetic tree (Fig. 1) was constructed by the neighbour-joining method (Saitou & Nei, 1987) with Kimura's two-parameter calculation model (Kimura, 1980). 16S rRNA gene sequence analysis indicated that strain S13^T was phylogenetically related to members of the genus *Flavobacterium*, with similarity ranging from 89.1 to 93.8%. Strain S13^T had highest 16S rRNA gene sequence similarity to *Flavobacterium columnare* (93.8%) and to *Flavobacterium aquatile* (93.3%). The phylogenetic tree indicated that strain S13^T clustered with *F. columnare*, and that this cluster was further grouped with the remaining recognized species of the genus *Flavobacterium* with strong support (100%). Based on this phylogenetic analysis and the phenotypic properties, we concluded that strain S13^T represents a novel, aquatic species within the genus *Flavobacterium*. Because it is sensitive to NaCl (1% NaCl completely halted growth), we propose the name *Flavobacterium saliperosum* sp. nov.

Table 1. Phenotypic characteristics that differentiate *Flavobacterium saliperosum* sp. nov. from related *Flavobacterium* species

Taxa: 1, *Flavobacterium saliperosum* sp. nov. (data from this study); 2, *Flavobacterium columnare*; 3, *Flavobacterium aquatile*; 4, *Flavobacterium branchiophilum* (data for 2–4 from Bernardet *et al.*, 1996); 5, *Flavobacterium hibernum* (McCammon *et al.*, 1998); 6, *Flavobacterium gelidilacus* (Van Trappen *et al.*, 2003). +, Positive; –, negative; (+), weakly positive; v, variable among strains.

Characteristic	1	2	3	4	5	6
Growth on:						
Seawater agar	–	–	–	–	–	+
Nutrient agar	+	–	–	–	+	+
Trypticase soy agar	(+)	–	(+)	–	+	+
Gliding motility	–	+	+	–	+	–
Flexirubin pigments	+	+	–	–	+	–
Congo red absorption	–	+	–	–	–	–
Acid from carbohydrates	–	–	+	+	+	–
Degradation of:						
Gelatin	+	+	v	+	+	+
Starch	–	–	v	+	+	+
Aesculin	–	–	v	–	+	–
Tyrosine	+	–	v	+	+	–
Brown pigment on tyrosine agar	+	v	–	–	–	–
Precipitation on egg-yolk medium	+	+	+	+	–	–
H ₂ S production	–	+	–	–	–	–
Nitrate reduction	–	v	v	–	+	–
Production of cytochrome oxidase	–	+	+	+	–	+
DNA G + C content (mol%)	41	32	33	33	36	30

Description of *Flavobacterium saliperosum* sp. nov.

Flavobacterium saliperosum (sal.i.per.o'sum. L. n. *sal*, *salis* salt; L. adj. *perosum* detesting, hating greatly; N.L. neut. adj. *saliperosum* salt-hating).

Gram-negative, aerobic and heterotrophic, with cell size of 0.35–0.55 × 1.5–2.5 µm. Non-flagellated and non-gliding. Colonies are yellow, smooth and circular with entire margins. Grows at temperatures of 20–34 °C (optimum growth at 28 °C). Grows over pH range 6.5–8.5 (optimum growth at pH 7.5). A concentration of NaCl above 1% severely inhibits growth (optimum growth is at 0.1% NaCl in medium). Grows on nutrient agar and trypticase soy agar but not on seawater agar. Voges–Proskauer reaction and oxidase are negative but catalase and lipase are positive. Cells contain flexirubin pigments. Does not reduce nitrate or sulfate. Forms a precipitate on egg-yolk agar and produces brown pigment on tyrosine agar. Hydrolyses L-tyrosine, gelatin and casein, but not agar, alginate, aesculin, starch, CM-cellulose, chitin or pectin. Does not produce acid from the following sugars: lactose, arabinose, rhamnose, raffinose, ribose, galactose, melibiose, D-melezitose, sucrose, xylose, mannose, fucose, fructose, glucose, cellobiose, maltose, salicin, laetrite, mannitol or sorbitol. Filter-paper disc tests indicate resistance to ceftazidime, ceftriaxone, tobramycin and trimethoprim/sulfamethoxazole, but susceptibility to penicillin, erythromycin, tetracycline, gentamicin, chloramphenicol, azithromycin, kanamycin, streptomycin and rifampicin. Cells contain menaquinone-6 (MK-6). Cellular fatty acids are iso-C_{15:0} (28.2%), iso-C_{17:1}9c (19.0%), iso-C_{17:0} 3-OH (8.6%), iso-C_{16:0} (6.9%), iso-C_{15:1}G

(6.7%), iso-C_{15:0} 3-OH (5.0%), C_{15:0} (4.6%), anteiso-C_{15:0} (3.9%), C_{16:1}ω7c (2.6%), iso-C_{16:1}H (2.3%), iso-C_{16:0} 3-OH (2.0%), iso-C_{14:0} (1.3%), C_{17:0} 2-OH (1.0%), iso-C_{15:0} 2-OH (0.9%), iso-C_{13:0} (0.5%), C_{15:1}ω6c (0.42%) and C_{17:1}ω6c (0.3%). DNA G+C content is 41 mol%.

The type strain, S13^T (=CGMCC 1.3801^T=JCM 13331^T), was isolated from freshwater lake sediment.

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