Flavobacterium beibuense sp. nov., isolated from marine sediment

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A taxonomic study was carried out on strain F44-8^T, which was isolated from a crude-oil-degrading consortium, enriched from marine sediment of the Beibu Gulf, PR China. The 16S rRNA gene sequence of strain F44-8^T showed highest similarities to those of *Flavobacterium frigoris* LMG 21922^T (93.3%), *Flavobacterium terrae* R2A1-13^T (93.3%) and *Flavobacterium gelidilacus* LMG 21477^T (93.1%). Sequence similarities to other members of the genus *Flavobacterium* were <93.0%. The dominant fatty acids of strain F44-8^T were iso-C_{15:0}, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), iso-C_{15:1} G and iso-C_{17:0} 3-OH. The DNA G+C content of strain F44-8^T was 38.6 mol%. These results are consistent with characteristics of members of the genus *Flavobacterium*. Strain F44-8^T could, however, be readily distinguished from all known *Flavobacterium* species by a number of phenotypic features. Therefore, according to the phenotypic and 16S rRNA gene sequence data, strain F44-8^T represents a novel species in the genus *Flavobacterium*, for which the name *Flavobacterium beibuense* sp. nov. is proposed (type strain F44-8^T = CCTCC AB 209067^T = LMG 25233^T = MCCC 1A02877^T).

In an attempt to investigate crude-oil-degrading bacteria in the marine sediment of the Beibu Gulf, PR China, many bacterial strains were isolated and characterized taxonomically. One of these isolates, designated strain F44-8^T, is described in this study. 16S rRNA gene sequence analysis indicated that strain F44-8^T formed a distinct lineage in the genus *Flavobacterium*. The genus *Flavobacterium* (Bergey *et al.*, 1923; Bernardet *et al.*, 1996; Bernardet & Bowman, 2006) is the type genus of the family *Flavobacteriaceae*, which currently contains 88 genera with validly published names (<http://www.bacterio.cict.fr/>). *Flavobacterium* strains have been isolated from a variety of environments (Bernardet & Bowman, 2006) and they have previously been reported from soil samples contaminated with engine

oil (Mandri & Lin, 2007). The aim of the present study was to determine the exact taxonomic position of strain F44-8^T by using a polyphasic characterization including the determination of phenotypic properties and a detailed phylogenetic analysis based on 16S rRNA gene sequences.

Marine sediment was sampled at site no. 44 (21° 26' 12.50" N 109° 40' 21.90" E) in the Beibu Gulf in October 2008. The sample was enriched with crude oil (1%, w/v) and the strains were isolated on 216L marine agar (sodium acetate, 1.0 g; tryptone, 10.0 g; yeast extract, 2.0 g; sodium citrate, 0.5 g; NH₄NO₃, 0.2 g; seawater, 1 l; pH 7.5) according to Lai *et al.* (2009). For morphological and biochemical characterization, strain F44-8^T was cultivated on marine agar 2216 (MA; BD Difco).

Genomic DNA was prepared according to the method of Ausubel *et al.* (1995); the 16S rRNA gene was amplified by PCR using previously described primers (Liu & Shao, 2005). Sequences of related taxa were obtained from GenBank. Phylogenetic analysis was performed using MEGA4 (Tamura *et al.*, 2007) and DNAMAN (version 5.1;

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Abbreviation: NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain F44-8^T is GQ245972.

A supplementary figure is available with the online version of this paper.

Lynnon Biosoft) according to Lai *et al.* (2009). Evolutionary distances were calculated using the algorithm of Kimura's two-parameter model (Kimura, 1980). Distances (distance options according to Kimura's two-parameter model) and clustering with the neighbour-joining (NJ; Saitou & Nei, 1987) and minimum-evolution (Rzhetsky & Nei, 1992, 1993) methods were determined by using bootstrap values based on 1000 replications.

A nearly full-length 16S rRNA gene sequence (1480 bp) of strain F44-8^T was determined and about 1360 positions were used for sequence alignment and phylogenetic analysis. The 16S rRNA gene sequence of strain F44-8^T showed highest similarities to those of *Flavobacterium frigidis* LMG 21922^T (93.3%), *Flavobacterium terrae* R2A1-13^T (93.3%) and *Flavobacterium gelidilacus* LMG 21477^T (93.1%). Lower similarities were shown to other members of the genus *Flavobacterium* (<93.0%). A tree constructed with the NJ method is shown in Fig. 1. Topology of the minimum evolution tree was essentially the same as that of the NJ tree (not shown). Phylogenetic analyses with all members of the genus *Flavobacterium* and representative members of the family *Flavobacteriaceae* revealed that strain F44-8^T formed a distinct lineage in the genus *Flavobacterium*. The 16S rRNA gene sequence divergences between strain F44-8^T and type strains of recognized species were all $\geq 6.7\%$ and, thus, the data support the view that strain F44-8^T represents a novel species (Stackebrandt & Goebel, 1994).

The Gram reaction, catalase, oxidase and lipase (Tween 80) activities, hydrolysis of aesculin, starch, CM-cellulose, agar and DNA, optimal growth temperature (in 216L broth incubated at 4, 10, 16, 20, 25, 28, 30, 37, 41 and 42 °C) and pH (in 216L broth adjusted with HCl and NaOH to pH 4–12 at 1 pH unit intervals), and tolerance of NaCl (in 216L broth adjusted to 0, 0.5, 1, 3, 5, 7, 10, 12 and 15 % NaCl) were studied as described previously (Dong & Cai, 2001). The presence of flexirubin-type pigments and gliding

motility were tested as recommended by Bernardet *et al.* (2002). Other biochemical tests were carried out at 28 °C using API 20NE, API 20E, API ZYM and API 50CH strips (bioMérieux) according to the manufacturer's instructions, except that the NaCl concentration was adjusted to 3.0 % in all tests. Antibiotic susceptibility tests were performed on MA (at 28 °C for 48 h) by using the disc diffusion method as described by Shieh *et al.* (2003). Oxid discs were used in this study. General cell morphology was studied under an inverted microscope (Olympus IX70) using a day-old culture on MA. For transmission electron microscopy, exponential phase cells were harvested, suspended in double-distilled water, absorbed on a Formvar-carbon-coated grid and stained with phosphotungstic acid (Supplementary Fig. S1, available in IJSEM Online). *F. gelidilacus* LMG 21477^T was grown under the same conditions and included as a reference in the API 20NE and API ZYM tests, as well as in a number of other phenotypic tests. The phenotypic features of strain F44-8^T are given in the species description and in Table 1.

Analysis of the respiratory quinones of strain F44-8^T was carried out by the Identification Service of the DSMZ (Braunschweig, Germany). The major quinone was MK-6 (99.5%), which is in accordance with all members of the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006). Minor amounts of MK-5 (0.5%) were also present. Fatty acids of whole cells grown on MA at 28 °C for 48 h were extracted, saponified and esterified; this was followed by GC analysis of the fatty acid methyl esters according to the instructions of the MIDI system (Sasser, 1990). *F. gelidilacus* LMG 21477^T was tested under the same conditions. As shown in Table 2, the major fatty acids of strain F44-8^T were iso-C_{15:0} (23.7%), summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 16.7%), iso-C_{15:1} G (11.0%) and iso-C_{17:0} 3-OH (10.7%). The overall fatty acid composition of strain F44-8^T was similar to that of *F. gelidilacus* LMG 21477^T grown under the same conditions.

Fig. 1. Neighbour-joining tree showing the estimated phylogenetic positions of strain F44-8^T and representatives of related taxa within the family *Flavobacteriaceae*, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. The sequences of *Weeksellia virosa* ATCC 43766^T and *Chryseobacterium koreense* Chj707^T were used as an outgroup. Bar, 0.01 substitution rate (K_{nuc}) units.

Table 1. Differential characteristics of strain F44-8^T and related *Flavobacterium* type strains

Strains: 1, F44-8^T (data from this study); 2, *F. gelidilacus* LMG 21477^T (unless indicated, data from this study); 3, *F. frigoris* LMG 21922^T (Van Trappen *et al.*, 2004); 4, *F. terrae* R2A1-13^T (Weon *et al.*, 2007); 5, *F. ceti* 454-2^T (Vela *et al.*, 2007). +, Positive; –, negative; ND, no data available. Some of the published data listed in this table were obtained using growth conditions and methods different from those used in this study.

Characteristic	1	2	3	4	5
Catalase activity	–	+	+	–	+
Growth at 37 °C	+	–	–	+	+
Tolerance of 3 % NaCl	+	+	+	–	+
Flexirubin-type pigments	–	–	–	+	+
Acid production from carbohydrates	+	–*	–	ND	–
Hydrolysis of:					
Gelatin	+	+	–	+	+
Starch	–	+	+	+	+
Agar	–	–	–	ND	–
Aesculin	+	–	+	–	–
β-Galactosidase activity	+	–	–	–	–
DNA G + C content (mol%)	38.6	30*	34.5	34	36.7

*Data from Van Trappen *et al.* (2003).

The two strains differed, however, in their respective proportions of a number of fatty acids, particularly iso-C_{15:0}, anteiso-C_{15:0} and summed feature 3.

The G + C content of the chromosomal DNA was determined according to the method described by Mesbah & Whitman (1989) using reversed-phase HPLC. The DNA G + C content of strain F44-8^T was 38.6 mol%, which is consistent with the range reported for the genus *Flavobacterium* (30–41 mol%; Liu *et al.*, 2008).

Strain F44-8^T was allocated to the genus *Flavobacterium* on the basis of 16S rRNA gene sequence analysis, supported by morphological and physiological characteristics, respiratory quinones, fatty acid composition and G + C content. Table 1 shows the characteristics used to distinguish strain F44-8^T from related species. On the basis of these data, strain F44-8^T represents a novel species in the genus *Flavobacterium*, for which the name *Flavobacterium beibuense* sp. nov. is proposed.

Description of *Flavobacterium beibuense* sp. nov.

Flavobacterium beibuense (bei.bu.en'se. N.L. neut. adj. *beibuense* referring to Beibu Gulf in the China Sea, the site where the type strain was isolated).

Cells are Gram-reaction-negative rods, 3.0–3.2 µm long and 1.3–1.4 µm in diameter, devoid of flagellar and gliding motility. On 216L agar, colonies are smooth, yellow, circular with regular edges and a slightly raised centre,

Table 2. Cellular fatty acid contents of strain F44-8^T and related *Flavobacterium* type strains

Strains: 1, F44-8^T (data from this study); 2a, *F. gelidilacus* LMG 21477^T (data from this study); 2b, *F. gelidilacus* (mean values for 22 strains; Van Trappen *et al.*, 2003); 3, *F. frigoris* (n=19; Van Trappen *et al.*, 2004); 4, *F. terrae* R2A1-13^T (Weon *et al.*, 2007); 5, *F. ceti* 454-2^T (Vela *et al.*, 2007). Values are percentages of total fatty acids. tr, Trace (<1 %); ND, not detected/not reported. Fatty acids amounting to <1 % of the total fatty acids in all strains are not shown.

Fatty acid	1	2a	2b	3	4	5
Straight chain						
C _{16:0}	tr	3.0	ND	1.8 ± 0.5	ND	ND
C _{15:0}	ND	ND	10	6.9 ± 1.2	ND	1.8
C _{18:0}	tr	3.2	ND	ND	ND	ND
Unsaturated						
C _{15:1ω6c}	tr	4.0	6	11.3 ± 2.2	1.1	ND
C _{17:1ω6c}	3.5	2.3	3	3.7 ± 1.2	ND	ND
C _{17:1ω8c}	tr	tr	ND	1.1 ± 0.3	ND	ND
C _{18:1ω5c}	tr	tr	1	ND	ND	ND
Branched						
iso-C _{14:0}	1.8	4.3	4	3.3 ± 1.0	4.7	ND
anteiso-C _{15:1}	ND	ND	1	tr	ND	ND
iso-C _{15:1}	ND	ND	10	2.7 ± 0.7	ND	ND
iso-C _{15:1} G	11.0	6.1	ND	ND	10.7	ND
iso-C _{15:0}	23.7	10.5	12	7.4 ± 1.4	18.4	45.9
anteiso-C _{15:0}	tr	12.7	8	10.2 ± 2.7	2.8	ND
iso-C _{16:1}	ND	ND	4	4.4 ± 1.2	ND	ND
iso-C _{16:1} H	2.1	4.5	ND	ND	ND	ND
iso-C _{16:1} G	ND	ND	ND	ND	3.0	ND
iso-C _{16:0}	5.7	10.1	8	6.9 ± 1.6	16.6	ND
iso-C _{17:1ω9c}	5.5	1.1	2	2.0 ± 0.5	6.2	18.9
Hydroxy						
C _{15:0} 3-OH	1.4	1.6	1	1.2 ± 0.5	2.2	ND
C _{16:0} 3-OH	tr	1.9	ND	1.8 ± 0.5	1.3	ND
C _{17:0} 3-OH	tr	tr	ND	ND	1.5	ND
iso-C _{14:0} 3-OH	tr	2.0	ND	ND	1.1	ND
iso-C _{15:0} 2-OH	1.3	1.5	ND	ND	ND	ND
iso-C _{15:0} 3-OH	5.9	7.5	6	3.9 ± 0.9	6.7	3.7
iso-C _{16:0} 3-OH	3.4	8.8	10	7.8 ± 2.0	5.0	ND
iso-C _{17:0} 3-OH	10.7	4.9	6	3.4 ± 1.3	10.3	11.6
Summed feature 3*	16.7	4.1	2	14.5 ± 3.0	1.3	11.5

*Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

1–2 mm in diameter after 72 h incubation at 28 °C. Positive for oxidase and gelatinase activities and for hydrolysis of aesculin; weakly positive for glucose fermentation. Negative for catalase, amylase, urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities, indole and H₂S production, citrate utilization, acetoin production (Voges–Proskauer), lipase activity (Tween 80), flexirubin-type pigments, nitrate reduction and hydrolysis of agar, DNA and CM-cellulose. Growth occurs with 0–9 % NaCl

(optimum, 3 %) and at 4–41 °C (optimum, 28 °C). No growth is observed at 42 °C after 7 days. Growth occurs at pH 6–8 (optimum, pH 6–7). Sensitive (μg per disc, unless otherwise indicated) to ampicillin (10), carbenicillin (100), cefalexin (30), cefoperazone (30), cephadrin (30), chloramphenicol (30), ciprofloxacin (5), clindamycin (2), cotrimoxazole (25), erythromycin (15), furazolidone (15), norfloxacin (10), ofloxacin (5), penicillin G (10), piperacillin (100), rifampicin (5), ceftriaxone (30), tetracycline (30), vancomycin (30) and doxycycline (30). Resistant to cefazolin (30), gentamicin (10), kanamycin (30), lincomycin (2), metronidazole (5), minocycline (30), neomycin (10), oxacillin (1), polymyxin B (30 IU) and streptomycin (10). In the API ZYM strip, alkaline phosphatase, esterase lipase (C8), esterase (C4), leucine arylamidase, trypsin, valine arylamidase, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, β -galactosidase, β -glucosidase, acid phosphatase and α -glucosidase activities are present; weak cystine arylamidase, lipase (C14) and α -galactosidase activities are present and *N*-acetyl- β -glucosaminidase, α -fucosidase, α -mannosidase and β -glucuronidase activities are absent. Maltose is the only carbon source utilized in the API 20NE and API 20E strips. Starch, amygdalin, arbutin, cellobiose, D-fructose, D-mannose, sucrose, D-sorbitol, trehalose, aesculin ferric citrate, gentiobiose, glycogen, L-rhamnose, potassium 5-ketogluconate, potassium gluconate, salicin and xylitol are utilized in the API 50CH kit. The major fatty acids are iso-C_{15:0}, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), iso-C_{15:1} G and iso-C_{17:0} 3-OH. MK-6 is the major respiratory quinone.

The type strain is F44-8^T (=CCTCC AB 209067^T =LMG 25233^T =MCCC 1A02877^T), isolated from marine sediment of the Beibu Gulf, PR China. The DNA G + C content of the type strain is 38.6 mol%.

Acknowledgements

This work was supported financially by the Sci & Tech Project of Fujian Province of China (no. 2009H0029) and the National Infrastructure of Natural Resources for Science and Technology Program of China (2005DKA21209).

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