Flavobacterium banpakuense sp. nov., isolated from leaf-and-branch compost

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A strictly aerobic, Gram-stain-negative, yellow-pigmented, non-spore-forming, motile (by gliding), rod-shaped bacterium, designated strain 15F3^T, was isolated from leaf-and-branch compost. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain 15F3^T was most closely related to *Flavobacterium reichenbachii* WB 3.2-61^T and formed a distinct phyletic lineage within the genus *Flavobacterium*, the type genus of the family *Flavobacteriaceae*. Growth was observed at 10–34 °C (optimum, 30 °C) and pH 6.0–8.0 (optimum, pH 7.0). No growth occurred in the presence of ≥2% (w/v) NaCl. Strain 15F3^T reduced nitrate to nitrogen and showed catalase activity but no oxidase activity. The predominant cellular fatty acids were iso-C_{15:0} and summed feature 3 (comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH). The major isoprenoid quinone was menaquinone-6. The G+C content of the genomic DNA was 31.1 mol%. On the basis of data from this polyphasic study, strain 15F3^T may be classified as a representative of a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium banpakuense* sp. nov. is proposed; the type strain is 15F3^T (=KACC 14225^T =JCM 16466^T).

The genus *Flavobacterium* is the type genus of the family *Flavobacteriaceae* in the phylum ‘Bacteroidetes’. Species of the genus *Flavobacterium* are defined as Gram-stain-negative, aerobic, yellow-pigmented, rod-shaped bacteria that are non-motile or motile by gliding. DNA G+C contents range from 30 to 52 mol% and menaquinone-6 (MK-6) is the major respiratory quinone (Bernardet & Bowman, 2006; Liu *et al.*, 2010). Strains representing the genus *Flavobacterium* have been found in a variety of environments such as soil, freshwater, seawater, sediments and diseased fish (Cousin *et al.*, 2007; Yoon *et al.*, 2009). At the time of writing, the genus *Flavobacterium* contained over 60 recognized species, including the recently described species *Flavobacterium reichenbachii* (Ali *et al.*, 2009), *Flavobacterium chungangense* (Kim *et al.*, 2009), *Flavobacterium sasangense* (Yoon *et al.*, 2009) and *Flavobacterium resistens* (Ryu *et al.*, 2008). They show very diverse physiological characteristics being either psychrophilic or mesophilic and either halophilic or sensitive to salts. The variety of enzymes they produce, some of them having potential applications in industry and bioremediation

(Humphry *et al.*, 2001; Tamaki *et al.*, 2003; Riffel & Brandelli, 2002; Singh, 2009), suggests that they have important roles in the environment.

Most strains of the genus *Flavobacterium* are able to degrade polysaccharides such as carboxymethyl-cellulose (McCammon & Bowman, 2000; Bernardet *et al.*, 1996; Park *et al.*, 2007), xylan (Lee *et al.*, 2006), chitin (Bernardet *et al.*, 1996) and β-1,3-glucan (Rasmussen *et al.*, 2008). For instance, *Flavobacterium johnsoniae* has been shown to degrade chitin and CM-cellulose (Reichenbach, 1989; Bernardet *et al.*, 1996; McBride *et al.*, 2003). Here, the isolation and identification of a novel polysaccharide-degrading (e.g. starch and CM-cellulose) strain that represents a novel species in the genus *Flavobacterium* are reported.

Strain 15F3^T was isolated from aerobic leaf-and-branch compost at EXPO Park in Osaka, Japan. The compost was made from leaves and branches cut from trees. These leaves and branches were piled up in an open space to 2 m high. Urea was added to the stack as a nitrogen source at the beginning of composting and the stack was agitated every month. The compost matured by spontaneous fermentation. At the time of sampling, the compost was 4 months old and pH 7.5 and 67 °C. The compost sample was

Abbreviations: MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 15F3^T is GQ281770.

enriched with M9 minimal medium (Sambrook & Russell, 2001) containing 1.0 % insoluble crystalline cellulose (Avicel; Merck) at 15 °C for 5 days. After three successive steps of enrichment cultivation, the enriched samples were diluted with 0.8 % (w/v) saline, spread on M9-Avicel plates containing 1.5 % gellan gum as a gelling agent and incubated at 15 °C for 5 days under aerobic conditions. Gellan gum was used instead of agar because improved culturability of certain bacteria has been reported using this gelling agent (Janssen *et al.*, 2002; Stott *et al.*, 2008). The isolate was cultured routinely on Luria–Bertani (LB) plates containing 1.5 % gellan gum at 30 °C for 3 days under aerobic conditions.

The 16S rRNA gene was amplified by PCR using two universal primers, 9F and 1542R (Yoon *et al.*, 1998). The PCR product was purified using the Zymoclean Gel DNA Recovery kit (Zymo Research) and the sequence of the amplified 16S rRNA gene was determined by using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit and an ABI PRISM 310 genetic analyser (both from Applied Biosystems). Phylogenetic analysis was initially carried out with the programs BLAST (Altschul *et al.*, 1997) and MEGABLAST (Zhang *et al.*, 2000) against the database of prokaryotic type strains with validly published names to determine an approximate phylogenetic affiliation using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). The nearly complete 16S rRNA gene sequence of strain 15F3^T (1472 bp) was aligned with sequences of representatives of the genus *Flavobacterium* using the software CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic trees for the datasets were inferred from the neighbour-joining (NJ) and maximum-parsimony (MP) methods using MEGA version 4.0 (Tamura *et al.*, 2007). Distances were calculated based on the NJ method according to the Kimura two-parameter model. The stability of relationships was assessed by a bootstrap analysis of 1000 datasets. Sequence similarity values were calculated with the sequences of closely related members of the genus *Flavobacterium* using the EzTaxon server (Chun *et al.*, 2007).

16S rRNA gene sequence analysis indicated that strain 15F3^T formed a distinct phyletic clade within the genus *Flavobacterium* (Fig. 1). The topology of the MP tree was essentially the same as that of the NJ tree (data not shown). 16S rRNA gene sequence comparisons using the Ribosomal Database Project and EzTaxon servers indicated that strain 15F3^T showed the highest sequence similarity (96.9 %) to *F. reichenbachii* WB 3.2-61^T. Sequence similarities with other members of the genus *Flavobacterium* were 91.3–96.7 %. 16S rRNA gene sequence similarity values less than 97.0 % have been proposed by Stackebrandt & Goebel (1994) as a criterion for differentiating bacteria at the species level.

Cell morphology of strain 15F3^T was examined by using light microscopy (SE; Nikon) after growth on LB gellan gum at 30 °C for 3 days. Growth was examined on

LB gellan gum at 4–38 °C (at 2 °C intervals). The pH range for growth was determined on plates of LB gellan gum adjusted to pH 4–10 (at unit intervals) with NaOH and HCl solutions. The requirement of and tolerance to NaCl were tested on modified LB gellan gum plates containing 0–4 % (w/v) NaCl (at 0.5 % intervals). Growth was tested on R2A agar (Reasoner & Geldreich, 1985), LB agar, nutrient agar (NA) and trypticase-soy agar (TSA; bioMérieux). Laboratory-prepared R2A agar, LB agar and NA were used in this experiment. Anaerobic growth was assessed on LB gellan gum in an anaerobic jar (Mitsubishi Gas Chemical) using the AnaeroPack anaerobic system [20 % (v/v) CO₂, <0.1 % (v/v) O₂; Mitsubishi Gas Chemical; Kim *et al.*, 2008]. Anaerobic conditions were monitored using anaerobic indicator tablets (Mitsubishi Gas Chemical). All above-mentioned growth tests were performed at 30 °C for 7 days. The Gram-reaction was determined by: (i) using a Gram stain kit (Difco) according to the manufacturer's instructions; and (ii) observation of a visible amount of cells from a colony mixed in a loopful of 3 % (w/v) aqueous KOH solution on a glass slide (Powers, 1995). Oxidase activity was tested by oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux) and catalase activity was evaluated by production of oxygen bubbles in 3 % (v/v) hydrogen peroxide solution. Production of flexirubin-type pigments and extracellular glycans were determined by using the KOH and Congo red tests, respectively, and gliding motility was investigated using the hanging drop technique with R2A broth culture, according to the minimal standards for describing novel taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002).

Hydrolysis of skim milk, chitin, starch, gelatin and tributyrin was tested on LB agar containing 1 % (w/v) substrate after 7 days of incubation according to previously described methods (Lányi, 1987; Smibert & Krieg, 1994). CM-cellulose degradation was tested by two different methods: the CM-cellulose-trypan blue plate method; and the Congo red flooding method. In the first method, LB agar plates containing 0.5 % (w/v) CM-cellulose and 0.01 % (w/v) trypan blue were inoculated and CM-cellulose degradation was revealed by clear haloes around the colonies (Han *et al.*, 1995). In the Congo red flooding method, colonies grown on LB agar plates containing 0.5 % (w/v) CM-cellulose were flooded with 0.2 % (w/v) aqueous Congo red solution for 30 min and washed several times with a 1 M NaCl solution to reveal clear zones around the colonies (Li *et al.*, 2009). The ability to degrade crystalline cellulose was determined by placing strips (1.0 × 6.0 cm) of Whatman no. 1 filter paper in tubes of carbohydrate-free broth and observing the disintegration of the paper strip during growth of the culture (Smibert & Krieg, 1981).

Additional biochemical characteristics and enzyme activities were tested by using API 20NE and API ZYM kits (bioMérieux) according to the manufacturer's instructions except that the kits were incubated at 30 °C and read after 4 days and 16 h of incubation, respectively. *F. reichenbachii*

Fig. 1. NJ phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the phylogenetic relationships of strain 15F3^T and representative members of the genus *Flavobacterium*. 16S rRNA gene sequence accession numbers of strains are shown in parentheses. Bootstrap values are shown as percentages at nodes based on NJ analysis of 1000 resampled datasets (only values >70 % are shown). The sequence from *Dokdonia donghaensis* DSW-1^T (DQ003276) was used as an outgroup. Bar, 0.01 changes per nucleotide position.

WB 3.2-61^T, *Flavobacterium anhuiense* D3^T and *F. johnsoniae* ATCC 17061^T were grown under the same culture conditions and used as reference strains in most phenotypic tests. Biochemical and physiological properties of strain 15F3^T are given in the species description and compared to those of reference strains in Table 1.

Fatty acid methyl ester analysis of strain 15F3^T, *F. reichenbachii* WB 3.2-61^T, *F. anhuiense* D3^T and *F. johnsoniae* ATCC 17061^T was performed according to the instructions of the Microbial Identification System (MIDI; Microbial ID; Lee *et al.*, 2009) using cells grown on TSA at 30 °C for 3 days except that *F. reichenbachii* WB 3.2-61^T was cultured at 25 °C for 3 days because it does not grow at 30 °C. The DNA G+C content of strain 15F3^T was determined by HPLC analysis of deoxyribonucleosides using a reverse-phase column (COSMOSIL 5C18-AR-II, 4.6 × 150 mm; Nacalai Tesque) after hydrolysis with nuclease P1 (EC 3.1.30.1; Yamasa) and bacterial alkaline phosphatase (EC 3.1.3.1; Takara). The isoprenoid quinones of strain 15F3^T were determined by HPLC analysis using a model LC-20A (Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reverse-phase column (250 × 4.6 mm, Kromasil; Akzo Nobel) as described previously (Komagata & Suzuki, 1987).

The cellular fatty acid profiles of strain 15F3^T and the type strains of the most closely related species of the genus *Flavobacterium* are shown in Table 2. The major cellular fatty acids of strain 15F3^T were iso-C_{15:0} (31.4 %) and summed feature 3 (comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 14.0 %). The overall fatty acid compositions of the

novel isolate and three reference strains grown under the same conditions were very similar and differed only in the proportions of some fatty acids. The major isoprenoid quinone of strain 15F3^T was MK-6 and the genomic DNA G+C content was 31.1 mol%. The fatty acid composition, major isoprenoid quinone and DNA G+C content of strain 15F3^T were consistent with those of recognized members of the genus *Flavobacterium* (Bernardet *et al.*, 1996; Cousin *et al.*, 2007; Bernardet & Bowman, 2006; Zhang *et al.*, 2006).

Data from this polyphasic taxonomic study warrant the description of strain 15F3^T as a representative of a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium banpakuense* sp. nov. is proposed.

Description of *Flavobacterium banpakuense* sp. nov.

Flavobacterium banpakuense [ban.pa.ku.en'se. N.L. neut. adj. *banpakuense* of or belonging to banpaku, the short name of 'Bankoku Hakurankai Koen' (the Japanese name of EXPO Park), where the type strain was isolated].

Cells are Gram-stain-negative rods, 0.4–0.8 µm in diameter and 1.6–3.2 µm in length, motile by gliding, strictly aerobic, chemoheterotrophic, catalase-positive and oxidase-negative. Spores are not formed. Colonies on LB gellan gum are circular with entire margins, convex, smooth, slightly raised and yellow-pigmented. Congo red is not absorbed by colonies. Flexirubin-type pigments are produced. Growth occurs on LB gellan gum at 10–34 °C

Table 1. Differential characteristics of strain 15F3^T and related members of the genus *Flavobacterium*

Strains: 1, 15F3^T; 2, *F. reichenbachii* WB 3.2-61^T; 3, *F. anhuiense* D3^T; 4, *F. johnsoniae* ATCC 17061^T. All data are from this study except for the DNA G+C contents of the reference strains (taken from: Ali *et al.*, 2009; Liu *et al.*, 2008; Bernardet *et al.*, 1996). All strains are positive for the following characteristics: catalase activity, gliding motility, production of flexirubin-type pigments, growth on NA, LB agar, R2A agar and TSA, growth at 25 °C, glucose utilization, degradation of starch, gelatin and casein, and β -galactosidase activity (ONPG hydrolysis). All strains are negative for absorption of Congo red by colonies and degradation of agar. +, Positive; –, negative; (+), weakly positive.

Characteristic	1	2	3	4
Growth at:				
30 °C	+	(+)	+	+
37 °C	–	–	+	–
Acid production from glucose	–	(+)	–	(+)
Degradation of:				
Gelatin	+	+	+	+
CM-cellulose	+	+	–	+
Oxidase activity	–	+	–	+
Nitrate reduction	+	–	–	–
DNA G+C content (mol%)	31.1	34.3	31.4	35.2

**F. anhuiense* D3^T has been reported previously as negative for gelatin degradation (Liu *et al.*, 2008).

†Degradation of nitrate to nitrogen is an important feature and differentiates strain 15F3^T from other species of the genus *Flavobacterium*. *F. denitrificans* is currently the only other *Flavobacterium* species that has been reported to degrade nitrate to nitrogen (Horn *et al.*, 2005).

(optimum, 30 °C), at pH 6–8 (optimum, pH 7) and in the presence of 0–1.5 % (w/v) NaCl (optimum, 1 %). Weak growth occurs at 4 °C, but no growth occurs at 37 °C. No growth occurs in 2 % (w/v) NaCl. Grows on LB agar, NA, TSA and R2A agar at 30 °C. Colonies on R2A agar show irregular spreading shapes. Skim milk, CM-cellulose and starch are degraded, but not agar, chitin, tributyrin or filter paper. In the API 20NE strip: nitrate is reduced to nitrogen; indole is produced; glucose is not fermented; β -galactosidase activity is present; arginine dihydrolase and urease activities are absent; aesculin and gelatin are hydrolysed; and glucose, arabinose, mannose, *N*-acetylglucosamine and maltose are assimilated, but mannitol, gluconate, caprate, adipate, malate, citrate and phenylacetate are not. In the API ZYM strip, alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase, β -glucosidase and *N*-acetyl- β -glucosaminidase activities are present, but esterase (C4), lipase (C14), α -galactosidase, α -mannosidase and α -fucosidase activities are absent. Weak activities are observed for esterase lipase (C8), cystine arylamidase and β -glucuronidase.

Table 2. Cellular fatty acid profiles of strain 15F3^T and the type strains of related species of the genus *Flavobacterium*

Strains: 1, 15F3^T; 2, *F. reichenbachii* WB 3.2-61^T; 3, *F. anhuiense* D3^T; 4, *F. johnsoniae* ATCC 17061^T. All data are from this study. Data are expressed as percentages of total fatty acids. Fatty acids with percentages of less than 1.0 % in all species are not shown. tr, Trace (less than 1.0 % of total); –, not detected.

Fatty acid	1	2	3	4
Saturated				
C _{13:0}	–	–	tr	1.1
C _{14:0}	1.2	tr	1.8	2.3
C _{15:0}	6.1	7.0	6.8	7.3
C _{16:0}	5.3	3.5	7.6	9.9
Hydroxy				
C _{15:0} 3-OH	1.3	–	1.3	1.1
C _{16:0} 3-OH	3.8	1.0	6.8	6.5
Branched				
iso-C _{14:0}	–	–	tr	1.6
iso-C _{15:0}	31.4	30.2	29.7	30.5
iso-C _{15:0} 3-OH	7.3	8.6	7.9	5.9
anteiso-C _{15:0}	1.6	2.9	1.1	1.7
iso-C _{15:1} G	3.5	5.8	2.3	1.1
iso-C _{17:0}	tr	1.0	tr	tr
iso-C _{17:0} 3-OH	6.2	6.1	4.4	4.2
iso-C _{17:1} ω 9c	2.3	5.9	1.7	1.5
Unsaturated				
C _{13:1} AT 12-13	1.8	tr	–	–
C _{15:1} ω 6c	2.8	4.4	2.4	2.0
C _{17:1} ω 8c	tr	1.1	tr	tr
C _{17:1} ω 6c	1.2	2.3	tr	tr
Unknown				
Unknown 13.565	3.8	3.7	–	–
Summed features*				
Summed feature 2	1.0	tr	1.7	2.3
Summed feature 3	14.0	10.2	17.4	14.6
Summed feature 4	–	2.0	–	–

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 contains C_{14:0} 3-OH and/or iso-C_{16:1} I; summed feature 3 contains C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH; summed feature 4 contains iso-C_{17:1} I and/or anteiso-C_{17:1} B.

The predominant cellular fatty acids (>10 % of the total fatty acids) are iso-C_{15:0} and summed feature 3 (comprising C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH). The major isoprenoid quinone is MK-6.

The type strain is 15F3^T (=KACC 14225^T =JCM 16466^T), isolated from leaf-and-branch compost at EXPO Park, Osaka, Japan. The DNA G+C content of the type strain is 31.1 mol% (HPLC).

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