Flavobacterium compostarboris sp. nov., isolated from leaf-and-branch compost, and emended descriptions of Flavobacterium hercynium, Flavobacterium resistens and Flavobacterium johnsoniae

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A strictly aerobic, Gram-negative, yellow-pigmented, non-spore-forming rod, designated 15C3T, was isolated from aerobic leaf-and-branch compost at EXPO Park in Osaka, Japan. Growth was observed at 9–33 °C (optimum 25 °C) and pH 5.6–7.9 (optimum pH 6.1–7.0). No growth occurred with >2 % (w/v) NaCl. Strain 15C3T reduced nitrate to nitrogen and showed catalase activity but not oxidase activity. The predominant fatty acids were iso-C15 : 0, iso-C17 : 03-OH and summed feature 3 (comprising C16 : 1v7c and/or iso-C15 : 02-OH). The isolate contained phosphatidylethanolamine as the major polar lipid and menaquinone-6 as the major respiratory quinone. The G+C content of the genomic DNA of strain 15C3T was 33.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain 15C3T belonged to the genus Flavobacterium and was most closely related to Flavobacterium hercynium WB 4.2-33T (96.9 % sequence similarity). On the basis of phenotypic and phylogenetic distinctiveness, strain 15C3T is considered to represent a novel species in the genus Flavobacterium, for which the name Flavobacterium compostarboris sp. nov. is proposed. The type strain is 15C3T (=KACC 14224T =JCM 16527T). Emended descriptions of F. hercynium, Flavobacterium resistens and Flavobacterium johnsoniae are also given.

The genus Flavobacterium, which belongs to the phylum ‘Bacteroidetes’, is the type genus of the family Flavobacteriaceae. Members of the genus are Gram-negative, aerobic, yellow-pigmented rods that are predominantly motile by gliding, contain menaquinone-6 (MK-6) as the major respiratory quinone and have a DNA G+C content in the range 30–52 mol% (Bernardet et al., 1996; Bernardet & Bowman, 2006, 2011; Liu et al., 2010).

Along with the genera Chitinophaga, Pedobacter and Chryseobacterium, the genus Flavobacterium has shown one of the highest increases in the number of species for the family Flavobacteriaceae. Recently, many novel species of the genus Flavobacterium have been isolated from a variety of habitats, such as soil (Lim et al., 2010; Yang et al., 2011), freshwater (Sheu et al., 2011), seawater (Yoon et al., 2011), sediments (Fu et al., 2011; Lee et al., 2010), glacier-frozen soil (Xu et al., 2011), wastewater treatment systems (Zhang et al., 2010; Liu et al., 2010), marine algae (Miyashita et al., 2010), rhizospheres (Xiao et al., 2011; Madhaiyan et al., 2010), leaf-and-branch compost (Kim et al., 2011) and reed roots (Liu et al., 2011). Some strains produce enzymes that are able to degrade a variety of biopolymers and/or are active at low temperatures (Kim et al., 2011). These enzymes have a potential use in biotechnological applications.

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain 15C3T is GQ281769.

A supplementary figure is available with the online version of this paper.
In a study of cellulose-degrading bacteria, a Gram-negative CM-cellulose-degrading rod was isolated from aerobic leaf-and-branch compost at EXPO Park in Osaka, Japan. Strain 15C3T was isolated from 4-month-old, spontaneously fermented compost (pH 7.5; 67 °C) made from tree leaves and branches. Details of the compost profile and isolation procedure have been described by Kim et al. (2011). Following isolation on M9 minimal medium (Sambrook & Russell, 2001) containing 1.0% insoluble crystalline cellulose (Avicel; Merck), strain 15C3T was routinely maintained on laboratory-prepared Luria–Bertani (LB) plates containing 1.5% gellan gum (LBG plates) at 25 °C under aerobic conditions, with subcultivation at 3-day intervals. *Flavobacterium hercynium* KACC 14934T (Cousin et al., 2007), *Flavobacterium resistens* KACC 14246T (Ryu et al., 2008) and *Flavobacterium johnsoniae* KACC 11414T (Bernardet et al., 1996) were obtained from the Korean Agricultural Culture Collection and used as reference strains in most phenotypic and biochemical tests.

DNA extraction and amplification of the 16S rRNA gene was carried out as described by Kim et al. (2011). The PCR products were purified using a Zymoclean Gel DNA Recovery kit (Zymo Research), according to the manufacturer’s instructions. Sequencing and phylogenetic analysis were carried out as described by Kim et al. (2011). The resulting nearly complete 16S rRNA gene sequence (1461 nt) was compared with available sequences in the EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007) to determine an approximate phylogenetic affiliation. The 16S rRNA gene sequence of strain 15C3T was aligned with those of representatives of the genus *Flavobacterium* and phylogenetic trees for the datasets were inferred using the neighbour-joining and maximum-parsimony methods with MEGA version 4.0 (Tamura et al., 2007). Distances were calculated based on the neighbour-joining method according to the Kimura two-parameter model. The stability of relationships was assessed by a bootstrap analysis of 1000 datasets. Sequence similarity was calculated using the EzTaxon server. Strain 15C3T was most closely related to *F. hercynium* WB 4.2-33T (96.9 %), *F. resistens* BD-b365T (96.7 %) and *F. johnsoniae* UW101T (96.7 %). Sequence similarities with other members of the genus *Flavobacterium* were <96.7 %. The neighbour-joining tree based on 16S rRNA gene sequences indicated that strain 15C3T formed a distinct lineage in the genus *Flavobacterium* (Fig. 1). Because <97.0% 16S rRNA gene sequence similarity has been proposed as a criterion for differentiating bacteria at the species level (Stackebrandt & Goebel, 1994), we abstained from performing DNA–DNA hybridization experiments.

Cell morphology and cell dimensions of strain 15C3T were studied by light microscopy (SE; Nikon) equipped with Image-Pro Express version 6.0 (Media Cybernetics) after growth on LBG at 25 °C for 3 days. The Gram-reaction was determined by the conventional Gram-staining method using a Gram stain kit (Difco), according to the manufacturer’s instructions, and by the KOH method (Powers, 1995). For the KOH method, a visible amount of cells was taken from a colony with an inoculation loop and mixed with 3% (w/v) aqueous KOH solution on a glass slide. 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 a 3% (v/v) aqueous hydrogen peroxide solution. The production of flexirubin-type pigments and extracellular glycan was tested by the KOH and Congo red tests, respectively, and gliding motility was determined by the hanging-drop technique, according to the minimal standards for describing novel taxa in the family *Flavobacteriaceae* (Bernardet et al., 2002). Growth on the following bacteriological media was tested at 25 °C, with 1.5% gellan gum used as the gelling agent (Kim et al., 2011): R2A (R2AG; Reasoner & Geldreich, 1983), nutrient (NG) (all laboratory prepared) and trypticase soy (TSG; bioMérieux). The temperature range for growth was examined at 5–35 °C (at intervals of 2 °C) on LBG. The pH range for growth was determined on LBG with the pH adjusted to pH 4.5–9.0 (at intervals of 0.5 pH unit) using NaOH and H2SO4 solutions; the pH values after autoclaving were pH 4.5, 5.1, 5.6, 6.1, 6.6, 7.0, 7.5, 7.9, 8.4 and 8.8. NaCl concentration for growth was tested on LBG supplemented with 0–4% (w/v) NaCl (at intervals of 0.5%). Results were recorded after 3 days of incubation. Colony morphology was observed on R2AG, LBG, NG and TSG after 3 days. Anaerobic growth was assessed in LB broth supplemented with 0.1% KNO3 in an anaerobic jar (Mitsubishi Gas Chemical) with the Anaeropack anaerobic system (20%, v/v, CO2; <0.1%, v/v, O2; Mitsubishi Gas Chemical; Kim et al., 2011) at 25 °C for 7 days. Anaerobic conditions were monitored using an anaerobic indicator tablet (Mitsubishi Gas Chemical). Hydrolysis of skimmed milk (Smibert & Krieg, 1994), chitin, starch (Smibert & Krieg, 1994), tributyrin, gelatin (Lányi, 1988; Smibert & Krieg, 1994) and CM-cellulose (Han et al., 1995; Li et al., 2009; Kim et al., 2011) were tested on LBG containing 0.5% (w/v) substrate after 7 days. Hydrolysis of agar was tested on LB agar. The ability to degrade crystalline cellulose was determined by placing 1.0 × 6.0 cm strips of Whatman No. 1 filter paper in inoculated carbohydrate-free R2A broth and observing the disintegration of the paper strip during growth (Smibert & Krieg, 1981). Additional physiological and biochemical properties were determined using the API 20 NE and API ZYM kits (bioMérieux), according to the manufacturer’s instructions except that the galleries were incubated at 25 °C and results were recorded after 3 days and 16 h, respectively. Nitrate reduction was confirmed by the colorimetric method (Smibert & Krieg, 1981).

The physiological and biochemical features of strain 15C3T are given in the species description and compared to those of the reference strains in Table 1. Cells of strain 15C3T were Gram-negative, straight or slightly curved rods (0.3–0.7 μm wide and 1.1–7.2 μm long). The isolate showed catalase activity but not oxidase activity, although the reference strains exhibited both activities (Table 1). The
isolate was able to reduce nitrate to nitrogen and we found that *F. hercynium* KACC 14934<sup>T</sup> and *F. resistens* KACC 14246<sup>T</sup> were also able to do so, in contrast with the original descriptions (Cousin et al., 2007; Ryu et al., 2008). In line with previously published data (Lim et al., 2010), *F. johnsoniae* KACC 11414<sup>T</sup> did not reduce nitrate to nitrite. Other members of the genus *Flavobacterium* reportedly reduce nitrate to nitrogen, including *Flavobacterium banpakuense* 15F3<sup>T</sup> and *Flavobacterium denitrificans* ED5<sup>T</sup> (Kim et al., 2011; Horn et al., 2005; Bernardet & Bowman, 2006).

For fatty acid methyl ester analysis, cells of strain 15C3<sup>T</sup>, *F. hercynium* KACC 14934<sup>T</sup>, *F. resistens* KACC 14246<sup>T</sup> and *F. johnsoniae* KACC 11414<sup>T</sup> were grown on trypticase soy agar (TSA) for 3 days at 25°C. Fatty acids were extracted, methylated and separated by GC (HP 6890; Hewlett Packard) and the methyl esters were identified and quantified using the TSBA50 library (version 5.0) of the Sherlock Microbial Identification System (Microbial ID; Lee et al., 2009). The cellular fatty acid composition of strain 15C3<sup>T</sup> is compared with those of its closest phylogenetic relatives in Table 2. Strain 15C3<sup>T</sup> contained iso-C<sub>15:0</sub>, iso-C<sub>16:1</sub>ω7c and/or iso-C<sub>15:0</sub> 2-OH as the major cellular fatty acids. These fatty acids are generally present in recognized members of the genus *Flavobacterium* (Bernardet et al., 1996; Cousin et al., 2007; Bernardet & Bowman, 2006, 2011; Zhang et al., 2006). The fatty acid composition of the isolate was similar to those of the reference strains, although there were differences in the proportions of some fatty acids.

The polar lipids of strain 15C3<sup>T</sup> and the reference strains were extracted and analysed by two-dimensional TLC as described by Komagata & Suzuki (1988) on cells harvested from LBG plates after 3 days at 25°C. The predominant polar lipid was phosphatidylethanolamine, but small amounts of uncharacterized polar lipids were also present. The polar lipid profiles of the isolate and the reference strains were very similar; they only differed by the presence of...
and number of unidentified aminolipids (Fig. S1, available in IJSEM Online).

The DNA G+C content of strain 15C3\textsuperscript{T}, \textit{F. hercynium} KACC 14934\textsuperscript{T} and \textit{F. resistens} KACC 14246\textsuperscript{T} were estimated from \( T_m \) values obtained by a modified thermal denaturation fluorimetric method (Xu et al., 2000; Gonzalez & Saiz-Jimenez, 2002, 2004) employing SYBR Green I and a real-time PCR system (Rotor-Gene Q; Qiagen). Genomic DNA from \textit{F. johnsoniae} KACC 11414\textsuperscript{T} (34.1 mol\%); \textit{Bacillus subtilis} DSM 402 (43.5 mol\%; Kunst et al., 1997) and \textit{Beijerinckia indica} subsp. \textit{indica} ATCC 9039\textsuperscript{T} (57.0 mol\%; Tamas et al., 2010), for which the genomes have been completely sequenced, were used as the calibration references. Thermal denaturation was performed with 50 \( \mu \)l reaction mixture containing approximately 2.5 \( \mu \)g DNA, 0.1 \( \times \) standard saline citrate and SYBR Green I (Invitrogen) at a dilution of 1:100,000. The thermal condition consisted of a ramp from 55 to 99 \(^\circ\text{C} \) at 1 \( \text{C} \) min\(^{-1} \). Reactions were prepared in triplicate. The DNA G+C content for strain 15C3\textsuperscript{T} was calculated using a linear regression analysis of melting temperature (\( T_m \) value) against the DNA G+C content of the calibration references. The DNA G+C content of strain 15C3\textsuperscript{T} was estimated to be 33.57±0.92 mol\%, which is within the range reported for the genus \textit{Flavobacterium} (Bernardet & Bowman, 2011).

The major respiratory quinones of strain 15C3\textsuperscript{T} were determined by HPLC coupled with HPTLC. Quinone extracts in ethanol were separated by TLC on HPTLC silica gel 60 F\textsubscript{254} aluminium sheets (Merck) using \( n \)-hexane/diethyl ether (90:10, \( v/v \)) as the developing solvent. Quinone spots were scraped from the TLC plate and extracted twice with
*n*-hexane/water (1:1, v/v) and resolved in ethanol. HPLC analysis was performed using a model LC-20A (Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250 × 4.6 mm, Kromasil; Akzo Nobel) as described previously (Kim et al., 2011; Komagata & Suzuki, 1988). The major respiratory quinone of strain 15C3T was MK-6, which is in line with the family Flavobacteriaceae.

The data obtained in this study support the assignment of strain 15C3T to a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium compostarboris* sp. nov. is proposed. On the basis of the data obtained in this study, emended descriptions of *F. hercynium*, *F. resistens* and *F. johnsoniae* are also given.

**Description of Flavobacterium compostarboris sp. nov.**

*Flavobacterium compostarboris* (com.post.ar.bo’ris. N.L. n. compostum -i compost; L. n. arbor -oris tree; N.L. gen. n. compostarboris compost made of trees, from which the type strain was isolated).

Gram-negative rods (approx. 0.3–0.7 × 1.1–7.2 μm), motile by gliding, strictly aerobic. Catalase-positive and oxidase-negative. Spores are not formed. Flexirubin-type pigments are present. On LBG, grows at 9–33 °C (optimum 25 °C), at pH 5.6–7.9 (optimum pH 6.1–7.0) and with 0–2% NaCl (optimum 0%). Grows on R2AG, NG and TSG at 25 °C. Colonies on LBG are translucent, smooth, orange–yellow, raised, glistening, non-spreading and circular with slightly lobate margins. Colonies on NG are translucent, irregular, yellow, spreading with thinly lobate margins and raised centres. Colonies on TSG are irregular, smooth, orange–yellow, raised, glistening, non-spreading and circular. Colonies on R2AG are transparent, yellow, flat, glistening and spreading. Congo red is not absorbed by colonies. Skimmed milk, CM-cellulose and starch are degraded, but agar, chitin, tributyrin, gelatin and filter paper are not. Nitrate is reduced to nitrogen. With API 20 NE, positive for esterase lipase (C8), but negative for esterase (C4), lipase (C14), trypsin, α-galactosidase, β-mannosidase and z-fucosidase. The predominant polar lipid is phosphatidylethanolamine. The major fatty acids (>10% of the total) are iso-C_{15:0}, iso-C_{17:0} 3-0H and summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-0H). The major respiratory quinone is MK-6.

The type strain, 15C3T (=KACC 14224T=JCM 16527T), was isolated from leaf-and-branch compost at EXPO Park, Osaka, Japan. The DNA G+C content of the type strain is 33.6 mol%.

**Emended description of Flavobacterium hercynium Cousin et al. 2007**

The description is as given by Cousin et al. (2007) with the following amendments. The major polar lipid of the type strain is phosphatidylethanolamine. CM-cellulose is weakly degraded. Reduces nitrate to nitrogen. The DNA G+C content of the type strain is 33.9 mol%.

**Emended description of Flavobacterium resistens Ryu et al. 2008**

The description is as given by Ryu et al. (2008) with the following amendments. Facultatively anaerobic. The major polar lipid of the type strain is phosphatidylethanolamine. CM-cellulose is weakly degraded. Reduces nitrate to nitrogen. The DNA G+C content of the type strain is 33.0 mol%.

**Emended description of Flavobacterium johnsoniae (Stanier 1947) Bernardet et al. 1996**

The description is as given by Bernardet et al. (1996) with the following amendment. The major polar lipid of the type strain is phosphatidylethanolamine.

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**References**


Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996). The description is as given by Cousin et al. (2007) with the following amendments. The major polar lipid of the type strain is phosphatidylethanolamine. CM-cellulose is weakly degraded. Reduces nitrate to nitrogen. The DNA G+C content of the type strain is 33.0 mol%.

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