Flavobacterium resistens sp. nov., isolated from stream sediment

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An aerobic, yellow-pigmented, Gram-negative bacterium, designated strain BD-b365^T, was isolated from sediment of the Hakjang stream in Busan, South Korea. Growth was observed at 15–40 °C (optimum 20–30 °C) and at pH 6.0–9.5 (optimum pH 7.0–8.0). Cells were non-spore-forming rods that showed gliding motility and contained branched and hydroxy fatty acids. The G+C content of the genomic DNA was 35.4 mol%. The major respiratory quinone was menaquinone-6 (MK-6). The major polar lipid of strain BD-b365^T was phosphatidylethanolamine. Comparative 16S rRNA gene sequence analysis showed that strain BD-b365^T formed a distinct phyletic line within the genus *Flavobacterium*. Based on levels of 16S rRNA gene sequence similarity, the novel strain was related most closely to *Flavobacterium aquidurens* WB 1.1-56^T, but the level of DNA–DNA relatedness between these two strains was only 9.6 %. On the basis of phenotypic and genotypic data, it is clear that strain BD-b365^T represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium resistens* sp. nov. is proposed. The type strain is BD-b365^T (=KCTC 22078^T =DSM 19382^T).

The genus *Flavobacterium*, which belongs to the phylum *Bacteroidetes*, was established in 1923, since when many amendments to its description have been made. Bernardet *et al.* (1996) defined *Flavobacterium* species as Gram-negative rods usually motile by means of gliding, containing menaquinone-6 (MK-6) as the sole respiratory quinone and having DNA G+C contents in the range 32–37 mol% (among other properties). *Flavobacterium* species have been isolated from diverse habitats such as micromats, fresh water and seawater, Antarctic lakes, soil, sediments and the gut of an earthworm (McCammon & Bowman, 2000; Zhu *et al.*, 2003; Van Trappen *et al.*, 2004, 2005; Cousin *et al.*, 2007). The physiological characteristics of members of the genus *Flavobacterium* are also diverse: they can be psychrophilic, psychrotolerant or mesophilic and

halotolerant, halophilic or sensitive to salts, and they produce a variety of enzymes (Humphry *et al.*, 2001; Tamaki *et al.*, 2003; Aslam *et al.*, 2005; Zhang *et al.*, 2006), which suggests that they may have important roles in environmental habitats. Therefore, efforts have been made in our laboratory to isolate and characterize members of the genus *Flavobacterium* from various environmental habitats such as municipal wastewater treatment plants and seawater (Park *et al.*, 2006, 2007; Ryu *et al.*, 2007). Here we describe the taxonomic characterization of a novel species belonging to the genus *Flavobacterium*.

Strain BD-b365^T was isolated from sediment of the Hakjang stream in Busan, South Korea; this stream receives wastewater from many industrial plants. The sediment sample was diluted serially with 1 % (w/v) saline solution, spread on R2A agar (Difco) and incubated at 20 °C for 5 days. Subcultivation was routinely performed on R2A agar at 30 °C for 3 days under aerobic conditions.

Sequencing of the 16S rRNA gene of strain BD-b365^T was carried out as described by Lane (1991). The resulting 16S rRNA gene sequence (1479 nt) was compared with available 16S rRNA gene sequences from GenBank by

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BD-b365^T is EF575563.

A transmission electron micrograph of cells of strain BD-b365^T, an extended neighbour-joining tree and a table comparing the fatty acid compositions of strain BD-b365^T and its closest neighbours are available as supplementary material with the online version of this paper.

using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine an approximate phylogenetic affiliation, and then aligned with sequences of closely related members of the family *Flavobacteriaceae* by using the CLUSTAL W software program (Thompson *et al.*, 1994). Phylogenetic trees were constructed by using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms available in the PHYLIP software, version 3.6 (Felsenstein, 2002). Resulting tree topologies were evaluated by bootstrap analysis based on 1000 resamplings. Sequence similarity values were computed by using three tools: Ribosomal Database Project II (<http://35.8.164.52/html/>; Cole *et al.*, 2003), the FASTA nucleotide similarity search program (<http://www.ebi.ac.uk/fasta33/nucleotide.html>; Pearson & Lipman, 1988) and the EzTaxon Server (<http://210.218.222.43:8080/>; Chun *et al.*, 2007). DNA–DNA hybridization experiments were carried out to evaluate the level of genomic DNA relatedness between strain BD-b365^T and *Flavobacterium aquidurens* WB 1.1-56^T by using the fluorometric microplate method (Ezaki *et al.*, 1989). Fluorometric data recorded after 30 min incubation were used for the calculation of a DNA–DNA hybridization value. Experiments were repeated five times and the DNA relatedness value is the mean of five values. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain BD-b365^T formed a distinct monophyletic clade within the genus *Flavobacterium* (Fig. 1 and Supplementary Fig. S1), although the bootstrap value

was low (23.0 %). The overall topologies of the maximum-likelihood and maximum-parsimony trees were essentially the same as that of the neighbour-joining tree (data not shown). 16S rRNA gene sequence comparisons via the Ribosomal Database Project II, FASTA nucleotide similarity search and the EzTaxon Server showed that strain BD-b365^T was most closely related to *F. aquidurens* WB 1.1-56^T, with similarities of 97.0, 97.5 and 97.6 %, respectively. However, the level of DNA–DNA relatedness between strain BD-b365^T and *F. aquidurens* WB 1.1-56^T was only 9.6 %, which is clearly below the threshold generally accepted for species delineation (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002). On the basis of phylogenetic analysis and levels of DNA–DNA relatedness, it is evident that strain BD-b365^T is a member of the genus *Flavobacterium* and represents a novel genomic species.

Growth was tested on several bacteriological media at 30 °C: R2A agar (Difco), laboratory-prepared Luria–Bertani (LB) agar, trypticase soy agar (TSA; Difco) and nutrient agar (NA; Difco). Physiological characteristics of strain BD-b365^T were examined by growing the isolate on R2A agar at different temperatures (5–50 °C at 5 °C intervals) and in R2A broth adjusted to different pH values (pH 5.0–10.0 at 0.5 pH unit intervals). The pH was adjusted as described by Gomori (1955). Gram staining was performed by using a bioMérieux Gram stain kit according to the manufacturer's instructions. Cell morphology and flagellar and gliding motility were studied by using phase-contrast and transmission electron microscopy (JEM-1010; JEOL) as described by Bernardet *et al.* (2002) and Jeon *et al.* (2004). Salt tolerance was tested on R2A agar supplemented with 0–3 % (w/v, 0.5 % intervals) NaCl for 5 days at 30 °C. Antibiotic susceptibility tests were performed in duplicate as described by Reva *et al.* (1995) by using filter-paper discs (diameter 8 mm; Whatman) containing the following antibiotics: ampicillin (10 µg), polymyxin B (100 U), streptomycin (50 µg), penicillin G (10 U), chloramphenicol (100 µg), gentamicin (30 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), neomycin (30 µg), carbenicillin (100 µg) and novobiocin (50 µg). Oxidase activity was tested based on oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated by production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. The production of flexirubin-type pigments and of extracellular glycans was investigated by using the KOH and Congo red tests, respectively, following the minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). Hydrolysis of casein, Tweens 80 and 20, aesculin, urea, tyrosine, starch and CM-cellulose was investigated on R2A agar after 7 days incubation at 30 °C according to previously described methods (Lányi, 1987; Smibert & Krieg, 1994). Tests for nitrate reduction were performed according to the method of Lányi (1987) and acid production from carbohydrates was tested as described by Leifson (1963). Additional enzyme activities

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain BD-b365^T and related taxa. Bootstrap values are shown as percentages of 1000 replicates (only values >50 % are shown). The sequence from *Capnocytophaga granulosa* LMG 16022^T (GenBank accession no. U41347) was used as an outgroup (not shown). Bar, 0.01 changes per nucleotide position. An extended version of this tree is available as Supplementary Fig. S1.

and biochemical features were determined by using API ZYM and API 20NE kits at 30 °C as recommended by the manufacturer (bioMérieux). Anaerobic growth was assessed under anaerobic (with 4–10 % CO₂) conditions by using the GasPak Plus system (BBL) at 30 °C for 20 days.

Cells of strain BD-b365^T were Gram-negative, straight or slightly curved rods, 0.4–0.6 µm wide and 1.6–3.2 µm long (Supplementary Fig. S2). The isolate grew optimally on R2A medium without the addition of NaCl and growth was severely inhibited on R2A medium containing more than 1 % (w/v) NaCl. Phenotypic features of strain BD-b365^T are given in Table 1 and in the species description. Some of these characteristics were in accordance with those of members of the genus *Flavobacterium*, whereas others allowed the differentiation of strain BD-b365^T from closely related *Flavobacterium* species (Table 1).

Analysis of fatty acid methyl esters was performed according to the instructions of the Microbial Identification System (MIDI, Inc.) on cells grown for 3 days on R2A agar at 30 °C. Analyses of polar lipids and isoprenoid quinones were carried out by using the methods described by Komagata & Suzuki (1987) on cells harvested from R2A broth after 3 days incubation at 30 °C. The DNA G + C content was determined by HPLC with a reversed-phase column (GROM-SIL 100 ODS-2FE; GROM) according to the method of Tamaoka & Komagata (1984). The major respiratory lipoquinone of strain BD-b365^T was menaquinone-6 (MK-6). The major cellular fatty acids were iso-C_{15:0} (35.7 %), C_{15:0} (11.4 %), iso-C_{15:0} 3-OH (8.3 %), C_{15:1}ω6c (6.0 %), summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 5.8 %),

iso-C_{17:0} 3-OH (5.6 %), iso-C_{17:1}ω9c (4.1 %), anteiso-C_{15:0} (2.8 %), iso-C_{15:1} G (2.4 %), C_{15:0} 3-OH (2.1 %), C_{16:0} (1.7 %), C_{17:1}ω6c (1.5 %) and unknown ECL 13.565 (1.2 %). Traces (i.e. <1 % of the total) of the following fatty acids were also present: C_{16:0} 3-OH, unknown ECL 11.543, iso-C_{16:0} 3-OH, iso-C_{16:0}, summed feature 1 (comprising C_{13:0} 3-OH and/or iso-C_{15:1} H), iso-C_{16:1} H, unknown ECL 16.582, iso-C_{14:0}, iso-C_{17:0}, C_{17:0} 2-OH, iso-C_{14:0} 3-OH, C_{15:0} 2-OH, C_{14:0}, C_{17:1}ω8c, C_{17:0} 3-OH, summed feature 6 (comprising C_{19:1}ω11c and/or C_{19:1}ω9c), C_{18:0} 10-methyl, iso-C_{12:0}, C_{16:1}ω5c, C_{12:1} AT 12–13, C_{13:0}, C_{18:1}ω5c, C_{18:0}, C_{15:1}ω8c and C_{18:1} 2-OH. This profile is similar to those of phylogenetically related species, but differs from them in the respective proportions of some components (Bernardet *et al.*, 1996; McCammon & Bowman, 2000) (Supplementary Table S1). The predominant polar lipid was phosphatidylethanolamine. The G + C content of the genomic DNA was 35.4 mol%. The fatty acid composition, major lipoquinone, major polar lipid and G + C content of strain BD-b365^T are in accordance with those of members of the genus *Flavobacterium* (Bernardet *et al.*, 1996; McCammon & Bowman, 2000; Zhang *et al.*, 2006; Cousin *et al.*, 2007). The data obtained from this polyphasic study support the description of strain BD-b365^T as representing a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium resistens* sp. nov. is proposed.

Description of *Flavobacterium resistens* sp. nov.

Flavobacterium resistens (re.sis'tens. L. part. adj. *resistens* resisting, based on the type strain being resistant to all antibiotics tested).

Table 1. Differential characteristics between strain BD-b365^T and related *Flavobacterium* species

Strains: 1, BD-b365^T (data from the present study); 2, *F. aquidurens* WB 1.1-56^T (Cousin *et al.*, 2007); 3, *F. flevense* DSM 1076^T (Bernardet *et al.*, 1996; McCammon & Bowman, 2000); 4, *F. johnsoniae* (seven strains; Bernardet *et al.*, 1996; McCammon & Bowman, 2000); 5, *F. glaciei* 0499^T (Zhang *et al.*, 2006); 6, *F. hydatis* DSM 2063^T (Bernardet *et al.*, 1996); 7, *F. psychrolimnae* LMG 22018^T (Van Trappen *et al.*, 2005); 8, *F. saccharophilum* NCIMB 2072^T (Bernardet *et al.*, 1996). All strains were positive for growth on NA and TSA and at 25 °C and degradation of aesculin. +, Positive; –, negative; d, variable among strains; v, variable among references; (+), weakly positive; NA, no data available.

Characteristic	1	2	3	4	5	6	7	8
Gliding motility	+	–	+	+	–	+	–	+
Glucose utilization	–	+	+	+	+	+	+	+
Acid from glucose	–	NA	+	+	–	+	–	+
Flexirubin-type pigments	+	–	–	+	–	+	–	+
Degradation of:								
Gelatin	–	–	–	+	+	+	–	+
Casein	+	+	–	+	+	+	+	+
Starch	+	+	v	+	+	+	+	+
CM-cellulose	–	–	–	+	+	v	–	+
Tyrosine	–	+	–	+	–	+	–	+
β-Galactosidase activity	+	(+)	+	+	–	+	+	+
Nitrate reduction	–	–	v	d	+	+	–	+
DNA G + C content (mol%)	35.4	33.5	35	34	36.5	34	33.8–34.5*	35.7

*Range of values for four strains including the type strain (G + C content of the type strain is 34.5 mol%) (Van Trappen *et al.*, 2005).

Cells are Gram-negative rods, 0.4–0.6 µm wide and 1.6–3.2 µm long. Motile by gliding; no flagella are observed. Colonies on R2A agar are yellow, translucent, slightly raised, irregular and spreading with lobate margins. Grows on R2A agar, LB agar, TSA and NA. Growth occurs at 15–40 °C (optimum 20–30 °C), at pH 6.0–9.5 (optimum 7.0–8.0) and in the presence of 0–2.0 % (w/v) NaCl (optimum 0–0.5 %). Anaerobic growth is not observed after 20 days at 30 °C on R2A agar. Catalase- and oxidase-positive. Nitrate is not reduced to nitrite. Casein, aesculin, starch and Tween 20 are hydrolysed, but Tween 80, urea, gelatin, tyrosine and CM-cellulose are not. Flexirubin-type pigments are produced, but Congo red is not absorbed by colonies. Acid is produced from raffinose, *myo*-inositol, lactose, L-arabinose, melibiose, D-fructose, D-mannose, D-glucose, D-mannitol, arbutin and salicin, but not from D-galactose. Negative for glucose fermentation, indole production and arginine dihydrolase activity. Positive for assimilation of maltose, but negative for assimilation of D-glucose, D-mannitol, *N*-acetylglucosamine, D-mannose, L-arabinose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid (API 20NE). Alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -glucosidase, β -galactosidase and *N*-acetyl- β -glucosaminidase activities are present, but trypsin, β -glucuronidase, α -mannosidase and α -fucosidase activities are absent; weak enzyme activities are observed for esterase lipase (C8), lipase (C14), cystine arylamidase, α -chymotrypsin, α -galactosidase and β -glucosidase (API ZYM). Resistant to polymyxin B, gentamicin, kanamycin, oleandomycin, neomycin, ampicillin, streptomycin, penicillin G, lincomycin, chloramphenicol, tetracycline, carbenicillin and novobiocin. Phosphatidylethanolamine is the major polar lipid. The major (>5 % of the total fatty acids) cellular fatty acids are iso-C_{15:0}, C_{15:0}, iso-C_{15:0} 3-OH, C_{15:1}ω6c, iso-C_{17:0} 3-OH, summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and iso-C_{17:0} 3-OH. The major isoprenoid quinone is MK-6. The DNA G + C content is 35.4 mol% (HPLC).

The type strain, BD-b365^T (=KCTC 22078^T =DSM 19382^T), was isolated from Hakjang stream sediment in Busan, South Korea.

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