Flavobacterium anhuiense sp. nov., isolated from field soil

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A novel strain, D3^T, isolated from a field-soil sample obtained from Anhui Province, PR China, was characterized taxonomically by using a polyphasic approach. The cells were Gram-negative, yellow-pigmented rods devoid of flagella, but showing gliding motility. The organism was able to grow at 5–37 °C and at pH 4.0–10.0. A comparative 16S rRNA gene sequence analysis indicated that strain D3^T is a member of the genus *Flavobacterium*, sharing highest sequence similarity with the type strain of *Flavobacterium defluvii* (96.7%). The major isoprenoid quinone was MK-6 and the predominant fatty acids were iso-C_{15:0}, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and C_{16:0}. The DNA G+C content was 31.4 mol%. On the basis of phylogenetic and phenotypic data, strain D3^T represents a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium anhuiense* sp. nov. is proposed. The type strain is D3^T (=KCTC 22128^T=CGMCC 1.6859^T).

The genus *Flavobacterium* (Bergey *et al.*, 1923) was emended by Bernardet *et al.* (1996) to include Gram-negative, aerobic, predominantly gliding, yellow-pigmented bacteria with DNA G+C contents in the range 32–37 mol% and MK-6 as the major respiratory quinone. *Flavobacterium* is the type genus of the family *Flavobacteriaceae*, phylum *Bacteroidetes* (previously, the *Cytophaga–Flavobacterium–Bacteroides* group) (Ludwig & Klenk, 2001; Bernardet *et al.*, 2002). *Flavobacterium* strains occur in soil, fresh water and marine habitats. Many species are cold-adapted, whilst others are pathogenic to freshwater fish (Bernardet & Bowman, 2006). The genus *Flavobacterium* currently comprises 49 recognized species, including the recently described species *Flavobacterium defluvii* (Park *et al.*, 2007), *Flavobacterium aquidurens* and *Flavobacterium hercynium* (Cousin *et al.*, 2007), *Flavobacterium terrigena* (Yoon *et al.*, 2007), *Flavobacterium terrae* and *Flavobacterium cucumis* (Weon *et al.*, 2007).

A yellow-pigmented bacterial strain, designated D3^T, was isolated from a field-soil sample from Anhui Province, PR China. Strain D3^T was isolated by using the dilution-planting

technique on Luria–Bertani (LB) agar (1^{–1}: 5 g yeast extract, 10 g tryptone, 10 g NaCl; pH 7.0) incubated at 30 °C and was cultivated routinely in LB agar or in LB broth at the same temperature.

The primers used for PCR amplification of the 16S rRNA gene were 5'-AGAGTTTGATCCTGGCTCAG-3' (forward) and 5'-AAGGAGGTGATCCAAGCCGCA-3' (reverse), corresponding to positions 8–27 and 1521–1540, respectively, in the 16S rRNA gene sequence of *Escherichia coli* (Brosius *et al.*, 1978). The 1398 bp sequence of strain D3^T was compared with available sequences retrieved from GenBank by using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>) to determine an approximate phylogenetic affiliation. A phylogenetic analysis was performed by using the software packages PHYLIP (Felsenstein, 1993) and MEGA, version 3.1 (Kumar *et al.*, 2001), after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (with distance options according to the Kimura two-parameter model; Kimura, 1980, 1983) and clustering were based on the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap analysis (1000 replications) was used to evaluate the topology of the neighbour-joining tree (Felsenstein, 1985). This tree (Fig. 1) showed that strain D3^T grouped with *Flavobacterium* species. It shared 96.7, 96.4, 95.1, 94.8 and 94.8% 16S rRNA gene sequence similarity with *F. defluvii*,

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain D3^T is EU046269.

Transmission electron micrographs of strain D3^T are available with the online version of this paper.

Fig. 1. Comparative analysis of the 16S rRNA gene sequences of strain D3^T and representative strains of the genus *Flavobacterium*, using the neighbour-joining method. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at branching points. The maximum-likelihood tree showed essentially the same topology (data not shown). Bar, 0.01 substitutions per nucleotide position.

Flavobacterium denitrificans, *Flavobacterium johnsoniae*, *Flavobacterium daejeonense* and *Flavobacterium soli*, respectively, and <94.0 % similarity with all other *Flavobacterium* species. 16S rRNA gene sequence similarity values <97 % have been proposed by Stackebrandt & Goebel (1994) as a criterion for differentiating bacteria at the species level. The levels of 16S rRNA gene sequence similarity for strain D3^T demonstrate clearly that this isolate should be regarded as a novel species.

Morphological features were examined by using light microscopy and transmission electron microscopy (7650; Hitachi) as described by Nedashkovskaya *et al.* (2005). Growth was assessed at 4, 5, 10, 25, 30, 35, 37 and 40 °C on LB agar. NaCl and pH tolerance were tested by using LB broth containing 0, 1, 2, 3 and 5 % NaCl or adjusted to pH 2.0–12.0 (using increments of 1 pH unit) according to Zhou *et al.* (2007). Growth was evaluated on cetrimide agar, Simmons' citrate agar, trypticase soy agar, MacConkey agar and nutrient agar. All tests were incubated at 30 °C for 2 days. Gram staining, endospore staining and flagellum staining were performed according to the methods described by Gerhardt *et al.* (1994). Oxidase activity was tested by determining the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated by determining the production of oxygen bubbles in a 5 % (v/v) aqueous hydrogen peroxide solution. Gliding motility, the production of

flexirubin-type pigments and the adsorption of Congo red by colonies were investigated by following the minimal standards for the description of novel taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). Acid production from carbohydrates was determined by using the medium and method described by Yamaguchi & Yokoe (2000). Carbon-source utilization was assessed by using a slightly modified ammonium salt medium [0.2 % (NH₄)₂SO₄, 0.05 % NaH₂PO₄·H₂O, 0.02 % MgSO₄·7H₂O, 0.01 % CaCl₂·2H₂O and 0.05 % K₂HPO₄] containing 1 % carbon source. Hydrolysis of agar, Tween 80, Tween 20 and CM-cellulose was investigated on LB agar after 2 days incubation according to Lanyi (1987) and Gerhardt *et al.* (1994). Urease activity and hydrolysis of casein, gelatin, aesculin, tyrosine and starch were determined as described by Cowan & Steel (1965). Hydrolysis of chitin and pectin was tested according to Reichenbach & Dworkin (1981) and Hildebrand (1971), respectively. Nitrate reduction was determined according to the method of Lanyi (1987). Additional enzymic activities and biochemical features were tested by using API ZYM and API 20E kits (bioMérieux) incubated at 30 °C for 2 days. Antibiotic-sensitivity tests were performed by using the diffusion method, as described by Park *et al.* (2007), on LB agar at 30 °C with filter-paper discs (8 mm diameter; Sanofi Pasteur) containing the following antibiotics (µg, unless indicated otherwise): streptomycin (10), penicillin G (10 IU), ampicillin (10), chloramphenicol (30), erythro-

mycin (15), tetracycline (30), ofloxacin (5), amoxicillin (10), ceftazolin (30), rifampicin (5), gentamicin (10), ceftazidime (30), vancomycin (30), kanamycin (30), carbenicillin (100) and polymyxin B (30). After 2 days incubation, the diameters of the inhibition zones were measured. Phenotypic characteristics of strain D3^T are given in the species description and in Table 1. Transmission electron micrographs of cells are shown in Supplementary Fig. S1 (available in IJSEM Online). Staining with India ink suggested that the layer surrounding the bacterial cell shown in Supplementary Fig. S1(a) represents capsular material.

Isoprenoid quinone analysis was performed according to Komagata & Suzuki (1987), using cells harvested from an LB broth culture incubated at 30 °C for 2 days. The predominant isoprenoid quinone was MK-6, which is in line with all other members of the family *Flavobacteriaceae*. For quantitative analysis of the cellular fatty acid composition, a loopful of cell mass was harvested from an LB agar plate incubated at 30 °C for 2 days and fatty acid methyl esters were prepared and identified by following the instructions of the Microbial Identification system (MIDI), as described by Sasser (1990). The major fatty acids found are listed in Table 2 and compared with those of the type strains of phylogenetically related *Flavobacterium* species. The cellular fatty acids mainly comprised iso-C_{15:0}, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and C_{16:0}, in agreement with the profiles for most *Flavobacterium* species.

Genomic DNA was extracted and purified according to Yoon *et al.* (1996) and the DNA G+C content was determined by using the thermal-denaturation method (Mandel & Marmur, 1968). The G+C content of the DNA was found to be 31.4 mol%. The description of novel *Flavobacterium* species has progressively expanded the range of DNA G+C contents for the genus from 32–37 mol% (Bernardet *et al.*, 1996) to 30–41 mol%.

Hence, phenotypic and phylogenetic data support the description of strain D3^T as the type strain of a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium anhuiense* sp. nov. is proposed.

Description of *Flavobacterium anhuiense* sp. nov.

Flavobacterium anhuiense (an.hui.en'se. N.L. neut. adj. *anhuiense* pertaining to Anhui, the province where the type strain was isolated).

Cells are Gram-negative, non-spore-forming, aerobic rods, 1.0–2.5 µm in length and 0.2–0.4 µm in width and motile by gliding. Each cell is surrounded by a layer of capsular material. Colonies are yellow, circular and smooth with entire margins. Grows at 5–37 °C (optimum, 25–30 °C), at pH 4.0–10.0 (optimum, pH 6.0–8.0) and with 0–2 % NaCl (optimum, 0–1 %). Growth occurs on trypticase-soy agar, nutrient agar and cetrimide agar, but not on Simmons' citrate agar or MacConkey agar. Congo red is not adsorbed by colonies. Flexirubin-type pigments are produced. Catalase, ornithine decarboxylase, lysine decarboxylase

Table 1. Differential characteristics of strain D3^T and related *Flavobacterium* species

Strains: 1, D3^T; 2, *F. defluvii* DSM 17963^T (Park *et al.*, 2007); 3, *F. denitrificans* DSM 15936^T (Horn *et al.*, 2005); 4, *F. johnsoniae* DSM 2064^T (Bernardet *et al.*, 1996); 5, *F. daejeonense* DSM 17708^T (Kim *et al.*, 2006); 6, *F. soli* KCTC 12542^T (Yoon *et al.*, 2006). +, Positive result; (+), weak or delayed reaction; –, negative result; v, results vary between references; ND, no data available.

Characteristic	1	2	3	4	5	6
Maximum growth temperature (°C)	37	40	30	<37	35	33
Tolerance of 2 % NaCl	+	–	+	–	+	+
pH range for growth	4–10	5–10	5.5–8.2	ND	6–8	6.5–8.5
Gliding motility	+	+	+	+	+	–
Flexirubin pigments	+	+	+	+	–	–
Acid from carbohydrates	+	+	–	+	+	–
Degradation of:						
Pectin	–	ND	+	+	–	ND
Gelatin	–	+	+	+	–	+
Casein	+	+	+	+	–	–
Starch	+	–	+	+	+	–
CM-cellulose	–	+	ND	+	–	–
Aesculin	+	+	ND	+	+	–
Tyrosine	+	+	+	+	–	–
DNA	–	ND	ND	+	–	–
β-Galactosidase activity	+	(+)	ND	+	+	–
Oxidase activity	–	–	–	+	–	+
Nitrate reduction	–	–	+	v	+	–
DNA G+C content (mol%)	31.4	33.5	34.6	35.2	35	36.9

Table 2. Fatty acid contents (%) of strain D3^T and the type strains of phylogenetically related species of the genus *Flavobacterium*

Strains: 1, D3^T; 2, *F. defluvii* DSM 17963^T (Park *et al.*, 2007); 3, *F. denitrificans* DSM 15936^T (Cousin *et al.*, 2007); 4, *F. johnsoniae* DSM 2064^T (Bernardet *et al.*, 1996); 5, *F. daejeonense* DSM 17708^T (Kim *et al.*, 2006); 6, *F. soli* KCTC 12542^T (Yoon *et al.*, 2006). Fatty acids amounting to <1% in all strains tested are not listed. tr, Trace amount (<1.0%); ND, not detected.

Fatty acid	1	2	3	4	5	6
iso-C _{14:0}	tr	tr	tr	1.5	tr	ND
C _{14:0}	1.6	ND	tr	ND	1.2	ND
C _{15:1ω6c}	tr	tr	3.8	1.3	2.7	1.9
iso-C _{15:1G}	2.2	7.6	5.4	4.9	3.9	6.3
iso-C _{15:0}	28.2	19.8	21.8	24.9	23.0	26.6
anteiso-C _{15:0}	3.8	4.4	2.1	3.4	6.3	2.7
C _{15:0}	3.4	6.4	7.1	6.1	ND	8.2
iso-C _{16:0}	tr	2.3	tr	3.4	1.1	4.1
C _{16:0}	11.3	1.9	1.4	2.7	5.4	2.0
C _{17:1ω8c}	tr	tr	1.3	ND	1.0	1.1
C _{17:1ω6c}	tr	2.4	4.2	2.2	1.9	1.4
iso-C _{17:1ω9c}	3.0	2.3	13.5	2.4	1.5	9.6
iso-C _{15:0} 3-OH	6.0	11.2	6.8	6.6	6.2	6.0
iso-C _{16:0} 3-OH	1.6	5.6	1.1	4.4	2.1	2.5
C _{16:0} 3-OH	5.1	4.5	0.9	4.7	5.1	ND
iso-C _{17:0} 3-OH	8.0	13.1	11.3	9.4	10.7	11.5
C _{17:0} 3-OH	tr	1.0	tr	ND	tr	ND
Summed feature 3*	11.9	10.1	10.9	12.8	20.5	6.0

*Summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1ω7c} that could not be separated by GLC with the MIDI system.

and arginine dihydrolase activities are present. Oxidase, methyl α-D-glucosidase, phenylalanine deaminase, arginine decarboxylase, DNase and urease activities are absent. Starch and casein are hydrolysed, but gelatin, pectin, chitin, Tween 20, Tween 80, agar and CM-cellulose are not. Acid is produced from D-glucose, L-fucose, L-rhamnose and amygdalin, but not from adonitol, D-arabinose, cellobiose, dulcitol, D-galactose, inositol, D-lactose, D-mannitol, melezitose, melibiose, raffinose, D-sorbitol or sucrose. Utilizes D-xylose, maltose, D-fructose, D-glucose, D-lactose, sucrose, L-sorbose, D-arabinose, salicin, raffinose, cellobiose, xylitol, melibiose, D-galactose and dextrin, but does not utilize erythritol, L-arabitol, D-ribose, D-sorbitol, D-mannose, inositol, adonitol, D-mannitol, inulin, turanose, citrate, acetate, D-erythrose, malonate, melezitose or tartrate. Nitrate is not reduced. Negative for indole and H₂S production and in the methyl red and Voges-Proskauer reactions (API 20E). In the API ZYM system, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-galactosidase activities are present, but lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase activities are

absent. Weak enzymic activities are observed for esterase (C4), N-acetyl-β-glucosaminidase and α-glucosidase. Resistant to gentamicin, ceftazidime, vancomycin, kanamycin, carbenicillin and polymyxin B, but sensitive to streptomycin, penicillin G, ampicillin, chloramphenicol, erythromycin, tetracycline, ofloxacin, amoxicillin, cefazolin and rifampicin. MK-6 is the major isoprenoid quinone. The major fatty acids are iso-C_{15:0} (28.2%), summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH, 11.9%) and C_{16:0} (11.3%). The DNA G+C content is 31.4 mol%.

The type strain, D3^T (=KCTC 22128^T=CGMCC 1.6859^T), was isolated from a soil sample from Anhui Province, PR China.

Acknowledgements

We are grateful for the financial support of the Chinese National Natural Science Foundation (30600001) and the Jiangsu Natural Sciences Foundation (BK2005422). We are also grateful to Che Ok Jeon for technical assistance.

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