

Flavobacterium enshiense sp. nov., isolated from soil, and emended descriptions of the genus *Flavobacterium* and *Flavobacterium cauense*, *Flavobacterium saliperosum* and *Flavobacterium suncheonense*

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A Gram-negative, strictly aerobic, yellow-pigmented rod, designated DK69^T, was isolated from soil collected from the waste liquid treatment facility of Bafeng Pharmaceutical Company in the city of Enshi, Hubei Province, China. Phylogenetic analysis based on 16S rRNA gene sequences placed strain DK69^T in the genus *Flavobacterium* of the family *Flavobacteriaceae*. The highest 16S rRNA gene sequence similarities were found with *Flavobacterium cauense* R2A-7^T (96.9%), *Flavobacterium saliperosum* AS 1.3801^T (96.3%) and *Flavobacterium suncheonense* GH29-5^T (95.7%). The major fatty acids ($\geq 5\%$) were iso-C_{15:0}, iso-C_{17:1 ω 9C}, C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{15:0} 3-OH. The major polar lipids were phosphatidylethanolamine, one unidentified aminolipid and one unidentified lipid. The major respiratory quinone was menaquinone-6. The genomic DNA G+C content was 34.4 mol%. Strain DK69^T represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium enshiense* sp. nov. is proposed. The type strain is DK69^T (=CCTCC AB 2011144^T =KCTC 23775^T). Emended descriptions of the genus *Flavobacterium* and *Flavobacterium cauense*, *Flavobacterium saliperosum* and *Flavobacterium suncheonense* are also proposed.

The genus *Flavobacterium*, currently consisting of over 60 species, belongs to the family *Flavobacteriaceae*, phylum *Bacteroidetes*. It was first proposed by Bergey *et al.* (1923) and an extensively emended description of the genus was given by Bernardet *et al.* (1996). Members of the genus have been found in a wide range of habitats, such as soil (Kim *et al.*, 2006; Yoon *et al.*, 2006, 2007; Weon *et al.*, 2007; Yang *et al.*, 2011), sediment, fresh water, seawater, diseased fish, glaciers and micromats (Bernardet & Bowman, 2011). The genus accommodates Gram-negative, aerobic, non-spore-forming, yellow-pigmented rods that have menaquinone-6 (MK-6) as the major respiratory quinone and are non-motile or motile by gliding. Members of the genus have a DNA G+C content in the range of 30–41 mol% (Bernardet & Bowman, 2011; Xu *et al.*, 2011), except for *Flavobacterium caeni* (52 mol%) (Liu *et al.*, 2010). Most members contain iso-C_{15:0} as the major fatty acid. Polar lipids have been analysed in only a few members of the genus; so far, phosphatidylethanolamine is the common major polar lipid (Park *et al.*, 2006, 2007; Ryu *et al.*, 2007, 2008; Sheu *et al.*, 2011).

Soil was collected from the waste liquid treatment facility of Bafeng Pharmaceutical Company (29° 52' 55.14" N 110° 03' 21.41" E) in the city of Enshi, Hubei province, China. The pH of the soil was 6.97, and the total C, N, P, S and Fe concentrations were 39.83, 3.34, 0.68, 0.36, 33.80 g kg⁻¹, respectively (determined by atomic absorption spectroscopy). For isolation, serially diluted soil samples were spread on R2A agar (Difco) and incubated at 28 °C for 7 days. A single colony, designated DK69^T, was picked and subcultivated. The isolate was routinely cultivated 28 °C for 2 days on R2A agar and preserved at -80 °C in R2A broth supplemented with 25% (v/v) glycerol.

A nearly complete (1477 bp) 16S rRNA gene sequence of strain DK69^T was amplified as described by Fan *et al.* (2008) and compared with sequences available in public databases using BLASTN. The similarities were calculated using the EzTaxon server (Chun *et al.*, 2007). Using CLUSTAL X (Thompson *et al.*, 1997), the 16S rRNA gene sequence of strain DK69^T was aligned with those of type strains of 20 members of the genus *Flavobacterium* and another member of family *Flavobacteriaceae*, *Leeuwenhoekiella marinoflava* ATCC 19326^T, which was used as an outgroup. Neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) trees were constructed using MEGA 4.0 (Tamura

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DK69^T is JN790956.

et al., 2007) and a maximum-likelihood tree was generated using the PHYL online web server (Guindon *et al.*, 2005). Bootstrap values were calculated based on 1000 replications (Felsenstein, 1985) in order to evaluate the confidence levels of the nodes. In the neighbour-joining phylogenetic tree (Fig. 1), strain DK69^T was clearly affiliated with the genus *Flavobacterium*, and formed a separate lineage supported by a high bootstrap value with *F. cauense* R2A-7^T (Qu *et al.*, 2009; 96.9% 16S rRNA gene sequence similarity), *F. saliperosum* AS 1.3801^T (Wang *et al.*, 2006; 96.3%), and *F. suncheonense* GH29-5^T (Kim *et al.*, 2006; 95.7%). Hence, the type strains of these species were obtained and used as references in this study. The 16S rRNA gene sequence similarity between strain DK69^T and the type strain of the type species, *F. aquatile* ATCC 11947^T, was 93.4%. The maximum-parsimony and maximum-likelihood trees showed essentially the same topology (Fig. 1).

For the analyses of morphological, physiological and biochemical characteristics, strain DK69^T, *F. cauense* CGMCC 1.7270^T and *F. suncheonense* DSM 17707^T were grown on R2A agar or in R2A broth at 28 °C for 2 days unless otherwise mentioned, and *F. saliperosum* CGMCC 1.3801^T was grown on modified M1 agar or in modified M1 broth as described by Wang *et al.* (2006). Cell morphology was observed using a transmission electron microscope (Hitachi 7650) as described by Nedashkovskaya *et al.* (2005). Gram-staining was tested as described by Dussault (1955) and with the KOH lysis method (Ryu, 1938). Flagellar motility was assessed in 0.3% R2A agar or modified M1 using stab inoculation while gliding motility was assessed using the hanging-drop technique according to Bernardet *et al.* (2002). Growth at 0, 4, 15, 20, 28, 32, 37 and 42 °C, with 0–5% (w/v) NaCl (at intervals of 1%) and at pH 4–10 (at intervals of one pH unit) was tested in R2A or modified

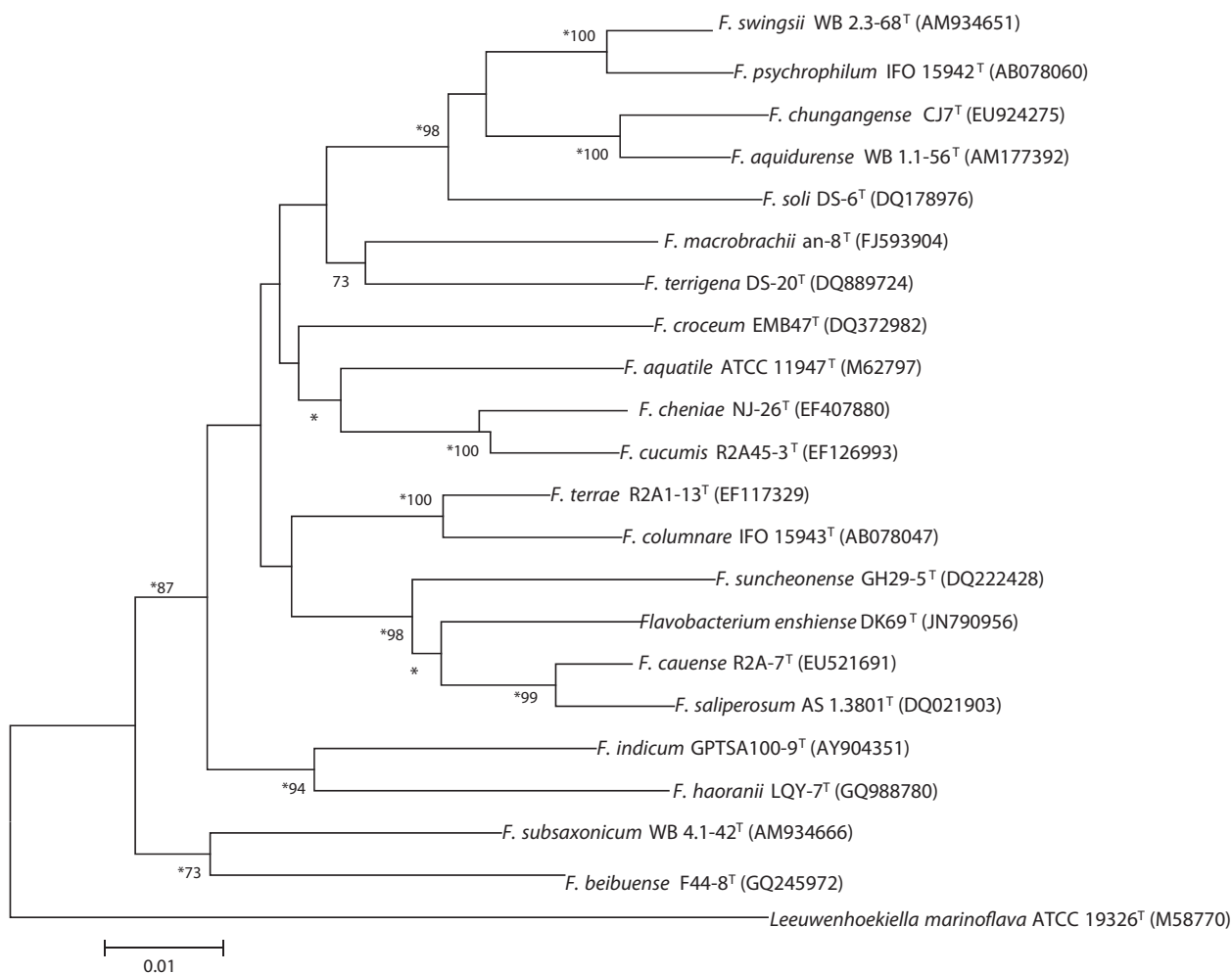


Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain DK69^T with some members of the genus *Flavobacterium*. Bootstrap values (>70%) based on 1000 replications are shown at branch nodes. Asterisks indicate that the corresponding nodes were also recovered using the maximum-parsimony and maximum-likelihood algorithms. *Leeuwenhoekiella marinoflava* ATCC 19326^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide.

M1 broth for 1 week. For the pH tests, the broth was adjusted to the initial pH value with citrate/phosphate or Tris/hydrochloride buffers (Breznak & Costilow, 1994). Anaerobic growth was evaluated by incubation in an anaerobic chamber (Mitsubishi Gas Chemical) (<1% O₂, ≥16% CO₂ and N₂) for 2 weeks. Growth was tested on nutrient agar (NA), full-strength trypticase soy agar (TSA), R2A agar and LB agar (all from Difco). Catalase activity was determined by assessing bubble production from 3% (v/v) H₂O₂, and oxidase activity was determined using 1% (w/v) tetramethyl-β-phenylenediamine (Merck). Indole production and hydrolysis of casein, aesculin, gelatin, L-tyrosine, urea, starch, Tween 80, DNA and CM-cellulose was investigated according to Smibert & Krieg (1994) and Reichenbach (1992). Nitrate reduction, glucose oxidation/fermentation and H₂S production were investigated according to the methods of Dong & Cai (2001). The production of a precipitate on egg yolk agar was tested on R2A agar supplemented with 10% egg yolk emulsion (17148; Sigma). Congo red adsorption and the production of flexirubin-type pigments were assessed according to Bernardet *et al.* (2002). Acid production from various carbohydrates was determined as described by Hugh & Leifson (1953). Antibiotic susceptibility tests were performed by the disc diffusion method (Hangzhou Microbial Reagent) on R2A agar at 28 °C for 2 days. Strains were considered susceptible when the diameter of the inhibition zone was ≥10 mm (Jorgensen & Ferraro, 2009). Other physiological/biochemical properties, enzyme activities and single carbon substrate utilization were examined at 28 °C using the API 20 NE, API ZYM and API ID 32GN systems (bioMérieux), according to the manufacturer's instructions except that the API ZYM strips after 6 h and the API 20 NE and API ID 32GN strips after 48 h. The phenotypic characteristics of strain DK69^T are given in Table 1 and the species description. Strain DK69^T could be distinguished from its closest relatives by growth on NA and LB agar, NaCl concentration for growth and *N*-acetyl-β-glucosaminidase activity (Table 1).

The DNA G+C content of strain DK69^T was determined by HPLC as described by Mesbah *et al.* (1989). For whole-cell fatty acid analysis, strain DK69^T and the reference strains were grown in R2A or modified M1 broth at 28 °C until they reached the mid-exponential phase and were analysed by GC (model 6890; Hewlett Packard) according to the instructions of the Sherlock Microbial Identification System (version 4.5, database TSBA40 4.10; MIDI) (Kroppenstedt, 1985; Sasser, 1990). The respiratory quinones of strain DK69^T were extracted and identified by HPLC as described by Xie & Yokota (2003) using methyl alcohol/isopropyl alcohol (2:1, v/v) as the solvent and a flow rate of 1.0 ml min⁻¹. The polar lipids of strain DK69^T and the reference strains were analysed by two-dimensional TLC as described by Tindall (1990) and Ventosa *et al.* (1993).

The DNA G+C content of strain DK69^T was 34.4 mol%, a value within the range reported for the genus *Flavobacterium* (Bernardet & Bowman, 2011). The major fatty acids (≥5%) were iso-C_{15:0} (38.1%), iso-C_{17:1ω9c} (18.5%), C_{15:0}

Table 1. Differential characteristics of strain DK69^T and the type strains of closely related members of the genus *Flavobacterium*

Strains: 1, *Flavobacterium enshiense* sp. nov. DK69^T; 2, *F. cauense* CGMCC 1.7270^T; 3, *F. saliperosum* CGMCC 1.3801^T; 4, *F. suncheonense* DSM 17707^T. Data were taken from this study unless otherwise stated. All strains are positive for catalase, hydrolysis of casein, gelatin and tyrosine, and formation of a precipitate on egg yolk agar. All strains are negative for hydrolysis of starch, Tween 80, urea, DNA and CM-cellulose, nitrate reduction, indole production, glucose fermentation and Congo red absorption. BY, Bright yellow; Y, yellow; YO, yellowish orange; +, positive; -, negative.

Characteristic	1	2	3	4
Colony pigmentation	BY	YO	YO	Y
Growth on/at:				
NA	-	+	+	+
R2A	+	+	-	+
LB	-	+	+	+
37 °C	-	+	-	+
pH for growth				
Range	6.0–8.0	5.5–8.0	6.5–8.5	6.0–8.0
Optimum	7.0	7.5	7.5	7.0
NaCl concentration for growth (%)	0	0–1.0	0–1.0	0–1.0
Gliding motility	-	+	-	-
Oxidase activity	+	+	-	+
Flexirubin-type pigments	+	+	+	-
Degradation of aesculin	-	-	+*	-
Production of H ₂ S	-	+	-	+
Brown pigment on tyrosine agar	-	-	+	-
Enzyme activities (API ZYM)				
Cystine arylamidase	+	+	+	-
α-Chymotrypsin	+	-	+	-
N-Acetyl-β-glucosaminidase	-	+	+	+
DNA G+C content (mol%)	34.4	37.7 ^{a†}	41.0 ^b	39.0 ^c

*Opposite data were reported by Wang *et al.* (2006).

†Data were taken from: a, Qu *et al.* (2009); b, Wang *et al.* (2006); c, Kim *et al.* (2006).

(10.0%), iso-C_{17:0} 3-OH (8.4%) and iso-C_{15:0} 3-OH (5.3%). The overall fatty acid composition of strain DK69^T was similar to those of its closest relatives with only minor differences in the respective proportions (Table 2). The predominant respiratory quinone of strain DK69^T was MK-6, which is in line with all members of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). Strain DK69^T and the reference strains all contained phosphatidylethanolamine as the major polar lipid. In addition, several aminolipids, aminophospholipids and unidentified lipids were present in all strains, but strain DK69^T contained one unidentified lipid and one aminophospholipid that were not detected in the reference strains (Fig. 2).

On the basis of phylogenetic inference and distinctive phenotypic features, it is concluded that strain DK69^T

Table 2. Fatty acid compositions of strain DK69^T and the type strains of closely related members of the genus *Flavobacterium*

Strains: 1, *Flavobacterium enshiense* sp. nov. DK69^T; 2, *F. cauense* CGMCC 1.7270^T; 3, *F. saliperosum* CGMCC 1.3801^T; 4, *F. suncheonense* DSM 17707^T. All data were taken from this study. Fatty acids amounting to <1% of the total in all strains are not shown. tr, Trace (<1%); –, not detected.

Fatty acid (%)	1	2	3	4
iso-C _{13:0}	1.0	1.4	–	1.4
iso-C _{14:0}	tr	–	tr	1.2
Unknown 13.565*	1.6	2.5	3.5	1.3
C _{15:0}	10.0	3.8	6.3	5.4
C _{15:0} 3-OH	–	tr	1.0	–
anteiso-C _{15:0}	1.8	tr	2.4	3.9
iso-C _{15:0}	38.1	37.9	27.7	29.2
iso-C _{15:0} 3-OH	5.3	12.2	6.2	7.2
iso-C _{15:1} G	1.9	5.5	6.4	3.8
iso-C _{16:0}	3.0	tr	4.6	5.7
iso-C _{16:0} 3-OH	tr	tr	1.8	1.5
iso-C _{17:0} 3-OH	8.5	16.6	12.7	13.0
iso-C _{17:1} ω9c	18.5	12.1	16.1	13.7
Summed feature 3†	2.6	1.5	1.5	3.9

*Unknown fatty acids are designated by their equivalent chain-length.

†Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C_{16:1}ω7c and/or C_{16:1}ω6c.

represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium enshiense* sp. nov. is proposed. On the basis of new data obtained in this study, emended descriptions of the genus *Flavobacterium* and *Flavobacterium cauense*, *F. saliperosum* and *F. suncheonense* are also proposed.

Emended description of the genus *Flavobacterium* (Bergey et al. 1923, emend. Bernardet et al. 1996)

3 The description is as given by Bernardet et al. (1996) with the following amendments. Most strains are positive for catalase. The major polar lipid is phosphatidylethanolamine.

Emended description of *Flavobacterium cauense* (Qu et al. 2009)

The description is as given by Qu et al. (2009) with the following amendments. Colonies on R2A agar are yellowish orange. Growth occurs on R2A agar, TSA, NA and LB agar. Aesculin is not hydrolysed. None of the substrates in the API 20 NE and API ID 32GN strips is assimilated. In the API ZYM strip, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase activities are positive, but lipase (C14), α-chymotrypsin,

α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase activities are negative. The major polar lipids are phosphatidylethanolamine, one unidentified aminolipid and one unidentified lipid; two other unidentified lipids and one aminophospholipid are also present.

Emended description of *Flavobacterium saliperosum* (Wang et al. 2006)

The description is as given by Wang et al. (2006) with the following amendments. Colonies on M1 agar are yellowish orange and about 1–2 mm in diameter. Growth occurs on NA, TSA and LB agar, but not on R2A agar. Positive for aesculin hydrolysis and negative for H₂S production. None of the substrates in the API 20 NE and API ID 32GN strips is assimilated. In the API ZYM strip, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase activities are positive, but lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase activities are negative. The major polar lipids are phosphatidylethanolamine, one unidentified aminolipid and one unidentified lipid; two other unidentified lipids and one unidentified aminolipid are also present.

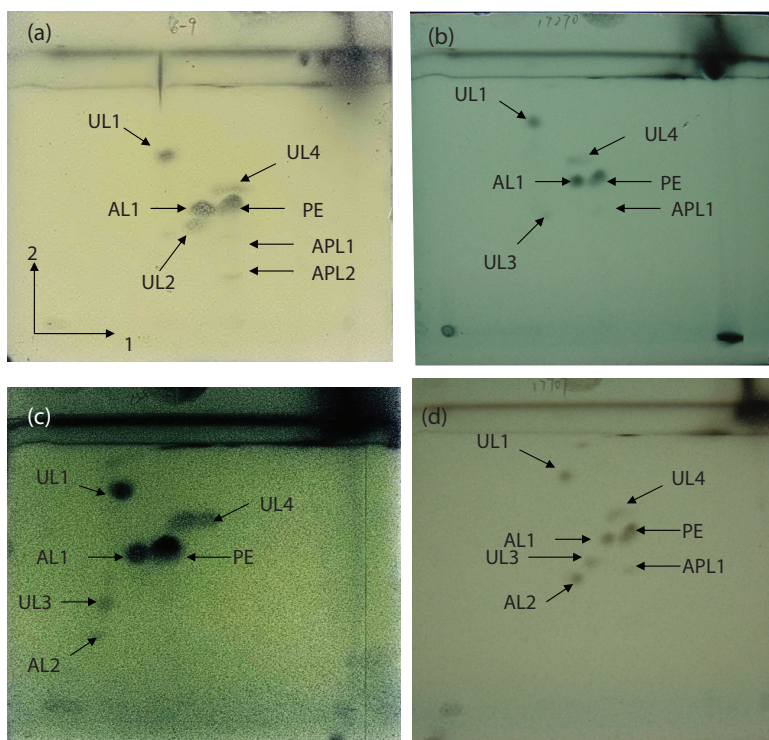
Emended description of *Flavobacterium suncheonense* (Kim et al. 2006)

The description is as given by Kim et al. (2006) with the following amendments. Colonies on R2A agar are about 1–2 mm in diameter. Optimum growth occurs at pH 7.0 and with optimum 0% NaCl. Growth occurs on R2A agar, TSA, NA and LB agar. Positive for H₂S production. None of the substrates in the API ID 32GN strips is assimilated. In the API ZYM strip, trypsin is positive. The major polar lipids are phosphatidylethanolamine, one unidentified aminolipid and one unidentified lipid; two other unidentified lipids, another unidentified aminolipid and one unidentified aminophospholipid are also present.

Description of *Flavobacterium enshiense* sp. nov.

Flavobacterium enshiense (en.shi.en'se. N.L. neut. adj. *enshiense* pertaining to the city of Enshi, where the type strain was isolated).

Cells are Gram-negative, non-flagellated, non-motile, strictly aerobic rods, approximately 0.3–0.4 μm wide and 1.1–1.7 μm in length after 48 h of incubation. Colonies grown on R2A agar are circular, bright yellow, smooth with regular edges and about 1 mm in diameter. Growth occurs at 4–32 °C (optimum 28 °C), but not at 37 °C, at pH 6.0–8.0 (optimum pH 7.0) and with 0% NaCl. Growth occurs on R2A agar and TSA, but not on NA and LB agar.



COLOUR FIGURE

Fig. 2. Two-dimensional thin-layer chromatograms of the total polar lipids of strain DK69^T and the type strains of closely related members of the genus *Flavobacterium*, detected with 10% ethanolic molybdatophosphoric acid: (a) strain DK69^T; (b) *F. cauense* CGMCC 1.7270^T; (c) *F. saliperosum* CGMCC 1.3801^T; (d) *F. suncheonense* DSM 17707^T. AL, Unidentified aminolipid; APL, unidentified aminophospholipid; PE, phosphatidylethanolamine; UL, unidentified lipid.

Oxidase- and catalase-positive. Flexirubin-type pigments are present and Congo red is not absorbed. Anaerobic growth is not observed. Nitrate is not reduced to nitrite. Casein and gelatin are hydrolysed, but aesculin, starch, Tween 80, urea, DNA and CM-cellulose are not. Negative for glucose fermentation and arginine dihydrolase activity. Forms a precipitate on egg yolk agar. Degrades tyrosine but no brown pigment is produced on tyrosine agar. Negative for H₂S and indole production. Acid is produced from sucrose and D-mannitol, but not from L-arabinose, melibiose, maltose, trehalose, D-sorbitol, melezitose, D-fructose, raffinose, D-ribose, D-glucose, glycerol, D-xylose, turanose, cellobiose, adonitol or salicin. None of the substrates in the API 20 NE and API ID 32GN strips is assimilated. In the API ZYM strip, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are positive, but lipase (C14), α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activities are negative. Resistant to (μ g per disc unless otherwise stated) trimethoprim (5), sulfamethoxazole (75), novobiocin (5), ofloxacin (5), oxacillin (1) and lincomycin (2), but sensitive to nitrofurantoin (300), teicomycin (30), cefalotin (30), chloramphenicol (30), rifampicin (5), amoxicillin (10), penicillin (10 U), ampicillin (10), carbenicillin (100), cephalosporin V (30), cephalosporin IV (30), streptomycin (10), tobramycin (10), vancomycin (30), norfloxacin (10),

nalidixic acid (30), erythromycin (15), tetracycline (30), neomycin (30), minocin (30), kanamycin (30) and cefoxitin (30). Menaquinone 6 is the major respiratory quinone. The major polar lipids are phosphatidylethanolamine, one unidentified aminolipid and one unidentified lipid; two other unidentified lipids and two unidentified aminophospholipids are also present. The predominant fatty acids ($\geq 5\%$) are iso-C_{15:0}, iso-C_{17:1 ω 9c}, C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{15:0} 3-OH.

The type strain is DK69^T (=CCTCC AB 2011144^T =KCTC 23775^T), isolated from soil taken from the waste liquid treatment facility of Bafeng Pharmaceutical Company in the city of Enshi, Hubei Province, China. The DNA G+C content of the type strain is 34.4 mol%.

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