Flavobacterium haorarii sp. nov., a cypermethrin-degrading bacterium isolated from a wastewater treatment system

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A Gram-negative, non-spore-forming, yellow-pigmented bacterium, strain LQY-7^T, was isolated from activated sludge treating synthetic pyrethroid-manufacturing wastewater. The taxonomic status of the strain was determined using a polyphasic taxonomic approach. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain LQY-7^T was a member of the genus *Flavobacterium* but had low similarities with other species of this genus (95.0 % similarity with *Flavobacterium indicum* GPTSA100-9^T and <94 % similarities with other *Flavobacterium* species). On the basis of phenotypic, genetic and phylogenetic data, strain LQY-7^T should be classified as a representative of a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium haorarii* sp. nov. is proposed; the type strain is LQY-7^T (=ACCC 05409^T =KCTC 23008^T).

The genus *Flavobacterium* was proposed by Bergey *et al.* (1923) and emended by Bernardet *et al.* (1996) to include Gram-negative, aerobic, rod-shaped, yellow-pigmented bacteria that are usually motile by gliding and have a DNA G+C content of 30–41 mol% (Bernardet & Bowman, 2006; Park *et al.*, 2006). At the time of writing, the genus comprised about 60 recognized species isolated from diverse habitats such as fresh- and salt-water, diseased fish, soil, sediment and micromats.

Cypermethrin is one of the most important synthetic pyrethroid pesticides and is widely used to control pests in cotton and vegetable crops. However, cypermethrin affects the central nervous system, causes allergic skin reactions, lymph node and spleen damage, and eye irritation. In addition, cypermethrin is highly toxic to fish and other aquatic organisms, as well as to bees. It has been classified as ‘moderately hazardous’ (Class II) by the World Health Organization and considered as a possible human carcinogen by the US Environmental Protection Agency. Microbes play significant roles in degrading and detoxifying cypermethrin residues in the environment (Kaufman *et al.*, 1981; Roberts & Standen, 1981). In this paper, a cypermethrin-degrading bacterial strain, designated LQY-7^T, was isolated from activated sludge in a synthetic pyrethroid-manufacturing wastewater treatment facility

(Yangnong Chemical Group Co., Jiangsu Province, China). The taxonomic status of this strain was determined using a polyphasic taxonomic approach. The data obtained suggest that the isolate represents a novel species of the genus *Flavobacterium*.

For investigation of morphological features, strain LQY-7^T was cultivated aerobically on trypticase soy agar (TSA; Difco) at 30 °C. Cell morphology and dimensions were examined by light microscopy (BH-2; Olympus) and transmission electron microscopy (H-7650; Hitachi) using cells from an exponentially growing culture. Gram-staining was performed according to the classical Gram procedure (Buck, 1982). Gliding motility, production of flexirubin-type pigments and adsorption of Congo red by colonies were investigated by the methods of Bernardet *et al.* (2002). Growth at various temperatures (4, 10, 15, 20, 25, 30, 35, 37 and 40 °C) and pH (4.0–10.0 at intervals of 0.5 pH units) was assessed on trypticase soy broth (TSB) after 3 days of incubation. Salt tolerance was tested on TSB supplemented with 0–8 % (w/v) NaCl after 3 days of incubation. Growth on nutrient agar, R2A agar, cetrimide agar, Simmons’ citrate agar and MacConkey agar was also evaluated at 30 °C. Sensitivity to antibiotics was tested on TSA plates using discs containing the following antibiotics: erythromycin (15 µg), tetracycline (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), kanamycin (30 µg), streptomycin (10 µg), rifampicin (5 µg), ampicillin (10 µg), polymyxin B (30 µg), penicillin G (1 µg),

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain LQY-7^T is GQ988780.

cefradine (30 µg), roxithromycin (15 µg), lincomycin (2 µg), carbenicillin (100 µg), spectinomycin (100 µg), amoxicillin (10 µg), bacitracin (0.04 µg) and vancomycin (30 µg). Standard physiological tests were carried out according to the methods described by Lányi (1987). Additional physiological characteristics were determined with the API 20NE, API 50CH and API ZYM (bioMérieux) systems according to the manufacturer's instructions.

For analysis of fatty acids, the strain was grown at 30 °C on TSB. Cells were harvested during the exponential phase by centrifugation, washed with distilled water and freeze-dried. The cellular fatty acids in the freeze-dried cells were identified by the Identification Service of the DSMZ (Braunschweig, Germany) as described by Kämpfer & Kroppenstedt (1996), Kuykendall *et al.* (1988) and Miller (1982).

Extraction and purification of total DNA was carried out according to standard procedures (Sambrook & Russell, 2001). The DNA G+C content was determined by thermal denaturation (Marmur & Doty, 1962) using *Escherichia coli* K-12 as a standard. Amplification of the 16S rRNA gene was performed by PCR using a bacterial universal primer set (27F and 1492R; Lane, 1991). The PCR product was purified

using the AxyPrep PCR Purification kit (AxyGen) and sequenced using an automated sequencer (Applied Biosystems model 3730). Similarity searches were performed using the NCBI program BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) and the sequence match program of the Ribosomal Database Project (<http://rdp.cme.msu.edu/seqmatch>). A phylogenetic tree of strain LQY-7^T and closely related strains was reconstructed using the software package MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of data using CLUSTAL_X (Thompson *et al.*, 1997). Distances were calculated according to the Kimura two-parameter model (Kimura, 1980, 1983) and clustering was performed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis of 1200 resamplings was used to evaluate tree topology (Felsenstein, 1993).

An almost complete 16S rRNA gene sequence (1438 bp) was obtained for strain LQY-7^T. Similarity search results in the Ribosomal Database Project and GenBank indicated that strain LQY-7^T was a member of the genus *Flavobacterium*. It shared 95.0% similarity with *Flavobacterium indicum* GPTSA100-9^T and <94% similarities with other *Flavobacterium* species. A tree (Fig. 1) depicting the phylogenetic relationships of strain LQY-7^T with closely related strains demonstrated that the strain formed a subclade with *F.*

Fig. 1. Neighbour-joining tree showing the phylogenetic relationship between strain LQY-7^T and related species based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1200 replications) are indicated at the branch-points. Bar, evolutionary distance (K_{nuc}) of 0.01.

Table 1. Cellular fatty acid composition (%) of strain LQY-7^T and closely related species of the genus *Flavobacterium*

Strains: 1, strain LQY-7^T (this study); 2, *F. indicum* GPTSA100-9^T (Saha & Chakrabarti, 2006); 3, *F. cucumis* R2A45-3^T (Weon *et al.*, 2007); 4, *F. columnare* ATCC 23463^T (Weon *et al.*, 2007); 5, *F. terrae* R2A1-13^T (Weon *et al.*, 2007); 6, *F. suncheonense* KACC 11423^T (Kim *et al.*, 2006); 7, *F. saliperosum* S13^T (Wang *et al.*, 2006); 8, *F. cheniae* NJ-26^T (Qu *et al.*, 2008). –, <1 % or not detected; NR, not reported.

Fatty acid	1	2	3	4	5	6	7	8
C _{15:0}	1.1	NR	—	NR	—	NR	4.6	15.4
C _{16:0}	—	NR	1.2	1.1	—	1.0	NR	—
C _{15:0} 2-OH	—	NR	—	NR	—	NR	NR	—
C _{15:0} 3-OH	—	NR	1.5	1.9	2.2	NR	NR	1.4
C _{16:0} 3-OH	—	NR	1.7	NR	1.3	NR	NR	—
iso-C _{13:0}	1.0	NR	—	1.3	—	1.9	—	—
iso-C _{14:0}	—	NR	5.3	3.8	4.7	NR	1.3	—
iso-C _{15:0}	39.5	18.5	17.5	30.1	18.4	29.9	28.2	27.7
iso-C _{16:0}	—	5.1	18.8	8.7	16.6	1.0	6.9	—
iso-C _{16:1}	—	NR	4.0	4.1	3.0	NR	NR	—
iso-C _{15:1} G	26.3	18.0	9.9	14.6	10.7	12.0	6.7	7.2
iso-C _{14:0} 3-OH	—	NR	1.8	1.5	1.1	NR	NR	—
iso-C _{15:0} 3-OH	5.6	5.0	5.1	5.2	6.7	11.1	5.0	6.3
iso-C _{16:0} 3-OH	—	4.5	12.6	7.4	5.0	NR	2.0	—
iso-C _{17:0} 3-OH	7.9	9.0	5.3	6.6	10.3	17.7	8.6	7.6
C _{15:1} ω6c	—	NR	2.0	NR	1.1	NR	NR	2.7
iso-C _{17:1} ω9c	1.5	NR	1.5	3.7	6.2	7.5	19.0	15.5
anteiso-C _{15:0}	6.3	NR	4.4	2.2	2.8	2.1	3.9	2.0
Unknown 11.543	1.1	NR	NR	NR	NR	NR	NR	NR
Unknown 13.565	3.1	NR	NR	NR	NR	NR	NR	1.6
Summed feature 3*	—	16.6	2.0	NR	1.3	9.8	NR	2.5

*Summed feature 3 contains C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

indicum GPTSA100-9^T with a high bootstrap value (98 %), also indicating that this isolate probably represents a novel member of the genus *Flavobacterium*.

Chemotaxonomically, the major fatty acids (>5 %) of the strain included iso-C_{15:0} (39.5 %), iso-C_{15:1} G (26.3 %), iso-C_{17:0} 3-OH (7.9 %), anteiso-C_{15:0} (6.3 %) and iso-C_{15:0} 3-OH (5.6 %), which is in agreement with the profiles for most species of the genus *Flavobacterium*. Detailed fatty acid profiles are given in Table 1.

The novel strain was Gram-negative, non-spore-forming and rod-shaped (0.3–0.7 × 1.3–2.0 µm); gliding motility was not observed, Congo red was not absorbed and flexirubin-type pigments were not produced. Colonies on TSA were yellow, glistening, sticky, convex and circular with entire margins. Growth was observed at 15–37 °C (optimum, 30 °C), at pH 6.0–8.5 (optimum, pH 7.0) and in 0–5 % NaCl (optimum, 1 %). Good growth was observed on nutrient agar and TSA. Weak growth occurred on R2A agar. No growth occurred on cetrimide agar, Simmons' citrate agar or MacConkey agar. The G+C content of the genomic DNA was 34 mol%, which fell within the range of 30–42 mol% reported for the genus *Flavobacterium* (Bernardet & Bowman, 2006). Phenotypic characteristics that differentiate strain LQY-7^T from related *Flavobacterium* species are listed in Table 2.

Therefore, on the basis of its phenotypic and phylogenetic characteristics, strain LQY-7^T should be classified as a representative of a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium haoranii* sp. nov. is proposed.

Description of *Flavobacterium haoranii* sp. nov.

Flavobacterium haoranii (hao.ran'i.i. N.L. gen. masc. n. *haoranii* of Hao-Ran, in honour of Hao-Ran Jian, a respected Chinese microbiologist, for his enormous contributions to the establishment and development of environmental microbiology in China).

Cells are Gram-negative rods, 0.3–0.7 µm in width and 1.3–2.0 µm in length, non-spore-forming and non-motile. Congo red is not absorbed and flexirubin-type pigments are not produced. Colonies on TSA are yellow, circular and convex with entire margins. Grows at 15–37 °C, at pH 6.0–8.5 and in 0–5 % NaCl. Good growth occurs on nutrient agar and TSA; weak growth occurs on R2A agar. No growth occurs on cetrimide agar, Simmons' citrate agar or MacConkey agar. Starch, gelatin and aesculin are degraded, but casein, cellulose, agar, pectin, chitin, DNA, tyrosine and egg yolk are not. Positive for catalase and oxidase. The Voges–Proskauer test is negative. In API 20NE tests, nitrate reduction, indole production, glucose fermentation,

Table 2. Differential phenotypic characteristics of strain LQY-7^T and type strains of closely related species of the genus *Flavobacterium*

Strains: 1, strain LQY-7^T (this study); 2, *F. indicum* GPTSA100-9^T (Saha & Chakrabarti, 2006); 3, *F. cucumis* R2A45-3^T (Weon *et al.*, 2007); 4, *F. columnare* ATCC 23463^T (Bernardet & Grimont, 1989; Bernardet *et al.*, 1996); 5, *F. terrae* R2A1-13^T (Weon *et al.*, 2007); 6, *F. suncheonense* KACC 11423^T (Kim *et al.*, 2006); 7, *F. saliperosum* S13^T (Wang *et al.*, 2006); 8, *F. cheniae* NJ-26^T (Qu *et al.*, 2008). +, Positive; –, negative; w, weakly positive; NR, not reported; Y, yellow; GY, greenish yellow; YO, yellowish orange.

Characteristic	1	2	3	4	5	6	7	8
Growth on:								
TSA	+	–	W	–	W	W	W	W
Nutrient agar	+	W	+	–	+	+	+	+
Colony pigmentation	Y	YO	Y	GY	YO	Y	Y	Y
Oxidase	+	+	+	+	+	+	–	+
Catalase	+	W	+	+	–	+	+	+
Nitrate reduction	–	–	+	NR	–	+	–	–
Flexirubin reaction	–	–	–	+	+	–	+	–
Gliding motility	–	–	+	+	–	–	–	+
Glucose utilization	–	W	–	–	–	–	NR	–
Hydrolysis of:								
Starch	+	+	+	–	+	–	–	+
Aesculin	+	–	+	–	–	–	–	NR
Tyrosine	–	–	+	–	–	+	+	+
Casein	–	+	+	+	+	+	+	–
Acid production from:								
Glucose	–	+	NR	–	NR	–	–	–
D-Fructose	–	+	NR	–	NR	–	–	–
DNA G + C content (mol%)	34	31	38	32	34	39	41	40.6

arginine dihydrolase and β -galactosidase are negative, but urease and aesculin hydrolysis are positive; does not assimilate all carbohydrates tested. In API ZYM tests, alkaline phosphatase, esterase C4, esterase lipase C8, cystine arylamidase, trypsin, α -chymotrypsin, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α -glucosidase activities are present. Lipase C14, α -galactosidase, β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, β -glucosidase, α -mannosidase and α -fucosidase activities are absent. In API 50CH tests, acid is produced from aesculin, maltose, inulin and starch. Sensitive to erythromycin, chloramphenicol, rifampicin, spectinomycin, cefradine, streptomycin, ampicillin, polymyxin B, tetracycline, penicillin G, lincomycin, carbenicillin, amoxicillin and vancomycin; resistant to roxithromycin, gentamicin, kanamycin and bacitracin.

The type strain is LQY-7^T (=ACCC 05409^T =KCTC 23008^T), isolated from activated sludge in a synthetic pyrethroid-manufacturing wastewater treatment facility (Yangnong Chemical Group Co., Jiangsu Province, China).

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