Flavobacterium macrobrachii sp. nov., isolated from a freshwater shrimp culture pond

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A bacterial strain, designated an-8^T, was isolated from a freshwater shrimp culture pond in Taiwan and characterized using a polyphasic taxonomic approach. Cells of strain an-8^T were Gram-reaction-negative, aerobic, rod-shaped and non-motile, formed yellow-pigmented colonies and grew at 15–30 °C (optimum 25 °C), pH 7–8 (optimum pH 8.0) and in 0–1 % (w/v) NaCl (optimum 0%). Phylogenetic analyses based on 16S rRNA gene sequences showed that strain an-8^T belonged to the genus *Flavobacterium* and its most closely related neighbours were *Flavobacterium terrigena* DS-20^T and *Flavobacterium terrae* R2A1-13^T with sequence similarities of 95.1 and 94.9 %, respectively. Strain an-8^T contained iso-C_{15:0}, summed feature 3 (C_{16:1}ω6c and/or C_{16:1}ω7c), iso-C_{16:0} 3-OH, iso-C_{15:0} 3-OH, iso-C_{17:0} 3-OH and iso-C_{15:1} as the major fatty acids. The major isoprenoid quinone was MK-6. The polar lipid profile consisted of a mixture of phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylserine and several unidentified polar lipids. The G + C content of the genomic DNA was 39.8 mol%. On the basis of the phylogenetic and phenotypic data, strain an-8^T represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium macrobrachii* sp. nov. is proposed. The type strain is an-8^T (=BCRC 17965^T =LMG 25203^T).

The genus *Flavobacterium* was first established by Frankland in 1889 (Bergey *et al.*, 1923) as part of the family *Flavobacteriaceae* in the phylum *Bacteroidetes* (Ludwig *et al.*, 2008). Due to the complex and heterogeneous characteristics of members of the genus, its description has been emended repeatedly over the years (Bernardet *et al.* 1996, 2002). Members of the genus *Flavobacterium* are typically Gram-negative, rod-shaped bacteria that display gliding motility and form yellow colonies. Metabolically, members of the genus *Flavobacterium* are aerobic chemo-organotrophs that can decompose several polysaccharides with the exception of cellulose. Chemotaxonomically, members of the genus possess MK-6 as the major respiratory quinone, C_{15:0},

iso-C_{15:0}, iso-C_{15:1} G, iso-C_{15:0} 3-OH, summed feature 4 (iso-C_{15:0} 2-OH, C_{16:1}ω7c and/or C_{16:1}ω7t), iso-C_{16:0} 3-OH, iso-C_{17:1}ω9c and iso-C_{17:0} 3-OH as the predominant fatty acids and DNA G + C contents of between 32 and 37 mol% (Bernardet *et al.* 1996). Members of this genus are physiologically diverse, some showing psychrophilic, psychrotolerant or mesophilic properties and some of which are halophilic, halotolerant or display a sensitivity to salts. Species of the genus *Flavobacterium* have been isolated from a number of diverse habitats such as freshwater and marine environments, sediments, soils, a glacier and Antarctic lakes (Bernardet *et al.*, 1996; Tamaki *et al.*, 2003; Van Trappen *et al.*, 2003; Zhu *et al.*, 2003; Yi *et al.*, 2005; Wang *et al.*, 2006; Cousin *et al.*, 2007; Weon *et al.*, 2007; Yoon *et al.*, 2007, 2009; Qu *et al.*, 2009).

During the course of a study characterizing microorganisms present in a freshwater pond in the Pingtung countryside, southern Taiwan (22° 35' 27.0" N 120° 36' 42.5" E), that was used for culturing giant river shrimp

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain an-8^T is FJ593904.

One supplementary figure and one supplementary table are available with the online version of this paper.

(*Macrobrachium rosenbergii*), a novel bacterium, designated an-8^T, which formed yellow-pigmented colonies was isolated and subjected to detailed taxonomic analysis. Strain an-8^T was isolated on R2A agar (BD Difco) after incubation at 25 °C for 3 days. The strain was maintained on R2A agar at 25 °C for 48–72 h by subculturing. On this medium, strain an-8^T was able to grow at 15–30 °C but not at 10 or 37 °C. Cultures were stored at –80 °C in R2A broth with 20% (v/v) glycerol or by lyophilization. *Flavobacterium terrigena* DS-20^T and *Flavobacterium terrae* R2A1-13^T were obtained from the DSMZ and KCTC culture collections, respectively, and used as reference strains for phenotypic and genotypic tests.

The 16S rRNA gene sequence of strain an-8^T was obtained as described previously by Chen *et al.* (2001) and analysed by using BioEdit (Hall, 1999) and MEGA version 3.1 software (Kumar *et al.*, 2004), after multiple sequence alignments were performed by using CLUSTAL_X (Thompson *et al.*, 1997). Distances (corrected according to Kimura's two-parameter model; Kimura, 1983) were calculated and clustering was performed using the neighbour-joining

method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were generated by using the treeing algorithms contained in the PHYLIP software package (Felsenstein, 1993). In each case, bootstrap values were calculated based on 1000 replications. The almost-complete 16S rRNA gene sequence (1405 bp) of strain an-8^T was compared against 16S rRNA gene sequences available from the EzTaxon server (Chun *et al.*, 2007), the Ribosomal Database Project (Maidak *et al.*, 2001) and the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST>). 16S rRNA gene sequence analysis indicated that strain an-8^T was a member of the family *Flavobacteriaceae* of the class *Flavobacteria* of the phylum *Bacteroidetes* and formed a distinct subline within the genus *Flavobacterium* in the neighbour-joining tree (Fig. 1). The phylogenetic trees obtained with the maximum-likelihood and maximum-parsimony methods had a similar topology. Sequence similarity calculations (comparing over 1400 bp) indicated that strain an-8^T was most closely related to *F. terrigena* DS-20^T (Yoon *et al.*, 2007) (95.1% 16S rRNA gene sequence similarity) and *F. terrae* R2A1-13^T (Weon *et al.*, 2007)

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain an-8^T and closely related species of the genus *Flavobacterium*. Numbers at nodes are bootstrap percentages (>50%) based on the neighbour-joining (above nodes) and maximum-parsimony (below nodes) algorithms. Filled circles indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

(94.9%). Sequence similarities <94.6% were observed between strain an-8^T and representative members of other species of the genus *Flavobacterium* (Fig. 1).

Cell morphology was observed using phase-contrast microscopy (DM 2000; Leica) at lag, exponential and stationary phases of growth. Motility was determined by using the hanging-drop method and Spot Test Flagella Stain (BD Difco) was used for flagellum staining. The presence or absence of gliding motility was determined by using phase-contrast microscopy as described by Bernardet *et al.* (2002). A Gram Stain Set S kit (BD Difco) and the Ryu non-staining KOH method (Powers, 1995) were used to test the Gram reaction. Colony morphology was observed on R2A agar by using a stereoscopic microscope (SMZ 800; Nikon).

The pH range for growth was determined by measuring OD₆₀₀ of cultures grown in R2A broth adjusted to pH 3–10 (intervals of 1 pH unit) prior to sterilization using appropriate biological buffers (Chung *et al.*, 1995). Verification after autoclaving revealed only minor changes in pH. The temperature range for growth was determined on R2A agar at 4, 10, 15, 20, 25, 30, 37, 40, 45 and 50 °C. Tolerance of NaCl was determined by preparing R2A broth according to the formula of the BD Difco medium and adjusting the NaCl concentration to 0, 0.5 or 1.0–10.0% (w/v) (intervals of 1%). Growth under anaerobic conditions was determined by incubating strain an-8^T in an Oxoid AnaeroGen system.

Strain an-8^T and the two reference strains were examined for activities of catalase, oxidase, DNase, urease and lipase (hydrolysis of corn oil) and for the hydrolysis of starch, casein, chitin, gelatin, agar, urea and Tweens 20, 40, 60 and 80 using standard methods (Gerhardt *et al.*, 1994). Hydrolysis of CM-cellulose was tested as described by Bowman (2000) using R2A agar as the basal medium. The presence of flexirubin-type pigments was determined by using 20% KOH (Fautz & Reichenbach, 1980). Congo red adsorption was determined according to the method of Bernardet *et al.* (2002). Additional biochemical tests were performed using API ZYM, API 20 E and API 20 NE kits (bioMérieux) and carbon-source utilization was evaluated using a GN2 microplate (Biolog). All commercial phenotypic tests were performed according to the manufacturers' recommendations.

Antibiotic sensitivity was tested by using the disc diffusion method after spreading cell suspensions (0.5 McFarland) on R2A agar (BD Difco). The discs (Oxoid) contained the following antibiotics (µg per disc): ampicillin (10), chloramphenicol (30), gentamicin (10), kanamycin (30), nalidixic acid (30), novobiocin (30), penicillin G (10), rifampicin (5), streptomycin (10), sulfamethoxazole plus trimethoprim (23.75:1.25) and tetracycline (30). The diameter of the antibiotic discs used was 8 mm. The effect of antibiotics on cell growth was assessed after 3 days of incubation at 25 °C. A strain was considered susceptible when the diameter of the inhibition zone was >13 mm,

intermediate at 10–12 mm and resistant at <10 mm as described by Nokhal & Schlegel (1983).

Colonies of strain an-8^T were yellow, round, smooth and convex with entire margins when grown on R2A agar. Strain an-8^T grew well on R2A agar but showed only weak growth when subcultured on nutrient agar (NA; Difco) and did not grow on tryptic soy agar (TSA; Difco). The physiological, biochemical and morphological characteristics of strain an-8^T are given in the species description and Table 1 and in Supplementary Table S1, available in IJSEM Online.

The fatty acid profiles of strain an-8^T, *F. terrigena* DS-20^T and *F. terrae* R2A1-13^T were analysed using cells grown on R2A agar at 25 °C for 3 days. The fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification System (MIDI; Sasser, 1990). Although the fatty acid profiles of strains an-8^T, *F. terrigena* DS-20^T and *F. terrae* R2A1-13^T were similar, a few differences were observed in the proportions of some fatty acids (Table 2). The major fatty acids (>7% total fatty acids) of strain an-8^T were iso-C_{15:0}, summed feature 3 (C_{16:1}ω6c and/or C_{16:1}ω7c), iso-C_{16:0} 3-OH, iso-C_{15:0} 3-OH, iso-C_{17:0} 3-OH and iso-C_{15:1}. Isoprenoid quinones were extracted and purified according to the method of Collins (1985) and were analysed using HPLC. The major respiratory quinone of strain an-8^T was MK-6. The DNA G+C content of strain an-8^T, determined by HPLC according to Mesbah *et al.* (1989), was 39.8±1.0 mol%.

Polar lipids were extracted and analysed by two-dimensional TLC as described by Ventosa *et al.* (1993). Strain an-8^T exhibited a highly similar polar lipid profile to *F. terrigena* DS-20^T and *F. terrae* R2A1-13^T, consisting of phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylserine, and several uncharacterized polar lipids (Supplementary Fig. S1).

Phenotypic examination revealed several common traits between the novel strain and its closest relatives, *F. terrigena* DS-20^T and *F. terrae* R2A1-13^T; however, strain an-8^T could be clearly differentiated from these two species by its colony pigmentation, weak growth on NA, absence of flexirubin-type pigments, ability to hydrolyse Tween 80, inability to hydrolyse urea and the presence of naphthol-AS-B1-phosphohydrolase activity (Table 1). Strain an-8^T could also be differentiated from *F. terrigena* DS-20^T by its ability to hydrolyse starch and Tweens 40 and 60 and by the absence of cystine arylamidase and trypsin activities. Certain features also distinguish the novel strain from *F. terrae* R2A1-13^T, such as the maximum temperature and optimum NaCl concentration for growth, the inability to grow on TSA and the absence of α-glucosidase activity.

Cells of strain an-8^T were Gram-reaction-negative, aerobic, chemo-organotrophic, rod-shaped, non-flagellated and formed yellow-pigmented colonies. Strain an-8^T was

Table 1. Differential characteristics of strain an-8^T and closely related species of the genus *Flavobacterium*

Taxa: 1, strain an-8^T; 2, *F. terrigena* DS-20^T; 3, *F. terrae* R2A1-13^T. Data for all strains were from this study except for the G+C contents of *F. terrigena* DS-20^T (Yoon *et al.*, 2007) and *F. terrae* R2A1-13^T (Weon *et al.*, 2007). +, Positive; –, negative; w, weakly positive. All strains were Gram-reaction-negative and positive for oxidase, catalase, alkaline phosphatase, C8 esterase lipase, leucine arylamidase and valine arylamidase activities and hydrolysis of casein, gelatin and DNA. All strains were negative for Congo red adsorption, nitrate reduction, indole production, D-glucose acidification, aesculin hydrolysis, arginine dihydrolase, C4 esterase, C14 lipase, α -chymotrypsin, acid phosphatase, α - and β -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, lipase (hydrolysis of corn oil) activities and assimilation of glucose, arabinose, mannose, mannitol, N-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate, and phenylacetate. All strains were sensitive to ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, novobiocin, penicillin G, rifampicin, streptomycin and tetracycline and resistant to sulfamethoxazole/trimethoprim.

Characteristic	1	2	3
Colony pigmentation	Yellow	Dark yellow	Yellowish orange
Maximum temperature for growth (°C)	30	30	40
Optimum NaCl concentration for growth (% w/v)	0	0	0–1
Growth on:			
Nutrient agar	w	+	+
Tryptic soy agar	–	–	+
Flexirubin-type pigments	–	+	+
Hydrolysis of:			
Casein	w	+	+
Starch	w	–	+
Tweens 40 and 60	+	–	+
Tween 80	+	–	–
DNA	w	+	+
Urea	–	w	+
Enzymic activities (API ZYM)			
Cystine arylamidase	–	+	–
Trypsin	–	+	–
Naphthol-AS-B1-phosphohydrolase	+	–	–
α -Glucosidase	–	–	+
DNA G+C content (mol%)	39.8	38.2	34.0

catalase- and oxidase positive, could decompose gelatin, casein, starch, Tweens 40, 60 and 80 and could not produce indole. The predominant cellular fatty acids were iso-C_{15:0}, summed feature 3 (C_{16:1} ω 6c and/or C_{16:1} ω 7c), iso-C_{16:0} 3-OH, iso-C_{15:0} 3-OH, iso-C_{17:0} 3-OH and iso-C_{15:1} and the major respiratory quinone was MK-6. On the basis of these characteristics, which were consistent with the description of the genus *Flavobacterium* (Bernardet *et al.* 1996), and the data obtained from 16S rRNA gene sequence comparisons, strain an-8^T occupied a distinct position within the genus

Table 2. Cellular fatty acid compositions of strain an-8^T and closely related species of the genus *Flavobacterium*

Taxa: 1, strain an-8^T; 2, *F. terrigena* DS-20^T; 3, *F. terrae* R2A1-13^T. All data obtained in this study. Strains were grown on R2A agar at 25 °C for 3 days. Values are percentages of the total fatty acids. –, Not detected. Fatty acids representing <1% are not shown. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain.

Fatty acid	1	2	3
iso-C _{10:0}	1.0	–	–
anteiso-C _{11:0}	–	1.5	1.1
anteiso-C _{12:0}	–	1.4	1.0
C _{12:0}	–	–	1.2
iso-C _{13:0}	–	1.0	1.6
iso-C _{14:0}	1.4	1.0	5.5
C _{14:0}	2.1	1.1	1.1
iso-C _{14:0} 3-OH	–	–	1.3
iso-C _{15:1}	7.6	10.7	10.6
iso-C _{15:0}	18.1	28.8	18.1
anteiso-C _{15:0}	2.1	2.4	4.1
iso-C _{15:0} 3-OH	8.8	7.1	6.3
C _{15:0} 2-OH	1.6	–	–
C _{15:0} 3-OH	2.6	–	1.3
C _{15:1} ω 6c	3.7	3.4	–
iso-C _{16:1}	1.1	–	–
iso-C _{16:0}	4.5	3.4	17.2
C _{16:0}	5.5	2.9	2.7
iso-C _{16:0} 3-OH	9.1	1.5	6.4
C _{16:0} 3-OH	4.2	2.3	1.4
iso-C _{17:1} ω 9c	1.3	6.2	4.4
iso-C _{17:0} 3-OH	7.9	11.1	8.4
C _{17:0} 3-OH	1.3	–	–
C _{18:1} ω 9c	–	4.1	–
C _{18:0}	1.7	1.0	1.3
Summed feature 3*	9.6	5.1	1.3

*Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 comprises C_{16:1} ω 6c and/or C_{16:1} ω 7c.

Flavobacterium. Based on its phylogenetic position and phenotypic characteristics along with the unique combination of chemotaxonomic and biochemical properties (Table 1 and Table 2), strain an-8^T represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium macrobrachii* sp. nov., is proposed.

Description of *Flavobacterium macrobrachii* sp. nov.

Flavobacterium macrobrachii (ma.cro.brach'i.i. N.L. gen. n. *macrobrachii* of *Macrobrachium*, a genus of freshwater shrimp, isolated from the surrounding environment of shrimp belonging to the genus *Macrobrachium*.)

Cells are Gram-reaction-negative, aerobic, non-flagellated, non-motile and rod-shaped. After 48 h of incubation at

25 °C on R2A agar, cell size is 0.3–0.5 × 0.8–2.0. Colonies on R2A agar are yellow, round, smooth and convex, have entire margins and are ~0.8–1.5 mm in diameter after 72 h of incubation at 25 °C. Growth occurs at 15–30 °C (optimum 25 °C), at pH 7–8 (optimum pH 8) and in 0–1 % (w/v) NaCl (optimum 0%). Oxidase- and catalase positive. Positive for hydrolysis of casein, starch, DNA and Tweens 40, 60 and 80. Negative for lipase and urease activities and hydrolysis of chitin, CM-cellulose and agar. Congo red adsorption is negative and flexirubin-type pigments are not present. In API 20 NE tests, positive for gelatin hydrolysis but negative for nitrate reduction, indole production, D-glucose acidification, aesculin hydrolysis, arginine dihydrolase, urease and β -galactosidase activities and assimilation of all carbohydrates tested. In API 20 E tests, positive for gelatin hydrolysis but negative for hydrogen sulfide production, ONPG hydrolysis, nitrate reduction, indole production, urea hydrolysis, acetoin production, citrate and malonate utilization, glucose, mannitol, xylose, *myo*-inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose and salicin fermentation and lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and arginine dihydrolase activities. In the API ZYM system, positive for alkaline phosphatase, C8 lipase, leucine arylamidase, valine arylamidase and naphthol-AS-B1-phosphohydrolase activities but negative for C4 esterase, C14 lipase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, α - and β -galactosidase, β -glucuronidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activities. The following compounds are utilized as sole carbon sources in the GN2 microplate system: α -cyclodextrin, dextrin, glycogen, Tweens 40 and 80, α -D-glucose, α -lactose, maltose, D-mannose, sucrose, acetic acid, L-alanyl glycine, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-proline, L-threonine, urocanic acid, inosine and uridine. All other substrates in the GN2 microplate system are not utilized. Sensitive to ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, novobiocin, penicillin G, rifampicin, streptomycin and tetracycline but resistant to sulfamethoxazole/trimethoprim. The major fatty acids are iso-C_{15:0}, summed feature 3 (C_{16:1} ω 6c and/or C_{16:1} ω 7c), iso-C_{16:0} 3-OH, iso-C_{15:0} 3-OH, iso-C_{17:0} 3-OH and iso-C_{15:1}. The major respiratory quinone is MK-6. The polar lipid profile comprises phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylserine and several uncharacterized polar lipids.

The type strain, an-8^T (=BCRC 17965^T=LMG 25203^T), was isolated from a freshwater culture pond in the Pingtung countryside, southern Taiwan, which was used for culturing giant river shrimp. The DNA G+C content of the type strain is 39.8 mol%.

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