Leptobacterium flavescens gen. nov., sp. nov., a marine member of the family *Flavobacteriaceae*, isolated from marine sponge and seawater

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Six obligately aerobic, Gram-negative, non-motile, pale-yellow-pigmented, rod-shaped bacterial strains, designated YM3-301^T, HG868, 04PA2 Co4-8B, 04PA2 Co4-99A, 04PA2 018SW-3 and 04PA2 018SW-18, were isolated from a marine sponge and seawater and were subjected to a polyphasic taxonomic investigation. Phylogenetic analyses based on 16S rRNA gene sequences revealed that the novel isolates were affiliated with the family *Flavobacteriaceae* (phylum *Bacteroidetes*) and that they showed the highest sequence similarity (90.3–90.9 %) to members of the genus *Leeuwenhoekiella* and to *Zhouia amylotica*. The 16S rRNA gene sequence similarities with respect to members of other related genera were only 86.3–89.1 %. In contrast, the six isolates shared high levels of 16S rRNA gene sequence similarity (99.7–100 %) and DNA-DNA relatedness (72–94 %) with each other. The novel isolates were phenotypically and physiologically different from members of related genera. The G+C content of the DNA was 41.8–43.5 mol%, MK-6 was the major menaquinone and i15 : 0, i15 : 1 and i17 : 0 3-OH were the major fatty acids. On the basis of the data from the taxonomic studies, it was concluded that these six novel strains represent a novel genus and species of the family *Flavobacteriaceae*, for which the name *Leptobacterium flavescens* gen. nov., sp. nov. is proposed. The type strain of *Leptobacterium flavescens* is YM3-301^T (=KCTC 22160^T=MBIC 06275^T=NBRC 104141^T).

The family *Flavobacteriaceae* belongs to the phylum *Bacteroidetes* (Ludwig & Klenk, 2001), which accounts for almost one-third of marine bacteria (Pinhassi *et al.*, 2006). Briefly outlined by Reichenbach (1989), the description of the family *Flavobacteriaceae* was completed and emended by Bernardet *et al.* (1996, 2002). Members of the family have given rise to extensive research owing to the diversity of their ecological niches and physiological characteristics and also to the fact that some of them may cause disease in humans and various animal species (Bernardet & Nakagawa, 2006).

In the present study, we attempted to elucidate the taxonomic positions of strains YM3-301^T, HG868, 04PA2 Co4-8B, 04PA2 Co4-99A, 04PA2 018SW-3 and 04PA2 018SW-18 by using a polyphasic taxonomic approach that included 16S rRNA gene sequence analysis and physiological, biochemical and chemotaxonomic investigations. On the basis of these data, we suggest that the isolates represent a novel genus and species of the family *Flavobacteriaceae*, phylum *Bacteroidetes*.

All of the strains were isolated from specimens collected in the Republic of Palau, located in the Pacific Ocean, close to the Philippines (almost 1000 km from Mindanao Island, the Philippines). The source of strain YM03-301^T was a marine sponge – identified as *Clathria* (*Microciona*) *eurypa* (de Laubenfels) – collected at Iwayama Bay on Koror Island in December 2002. All of the other strains were isolated from specimens collected in September 2004. Strain HG868 was isolated from an unidentified marine sponge collected on the shore of Angaur Island (GPS location, 06° 53′ 33.0″ N 134° 8′ 21.4″ E). The four 04PA2 strains were isolated from the hard coral *Galaxea fascicularis* Linnaeus 1767 and from seawater collected at the coral reef in the south-west of Malakal Bay on Malakal Island in the Republic of Palau (depth, 5 m; GPS location, 07° 18′ 1.6″ N 134° 27′ 21.5″ E).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains YM3-301^T, 04PA2 018SW-18, 04PA2 018SW-3, HG868, 04PA2 Co4-8B and 04PA2 Co4-99A are AB362212, AB362213, AB362214, AB362215, AB362216 and AB362217, respectively.

A transmission electron micrograph of a cell of strain YM3-301^T is presented as a supplementary figure available with the online version of this paper.

Strains 04PA2 018SW-3 and 04PA2 018SW-18 were isolated from seawater kept for 2–5 h in a 500 ml plastic bottle with the hard coral; strains 04PA2 Co4-8B and 04PA2 Co4-99A were isolated from a piece of hard coral. For strains 04PA2 018SW-3 and 04PA2 018SW-18, 50 µl seawater was spread onto marine agar 2216 (MA; Difco). The sponge and hard-coral specimens (0.5–1 cm^3) were homogenized with a glass rod in 5 ml sterile seawater. For strain YM3-301^T, 50 µl homogenate was spread onto medium P (Yoon *et al.*, 2007). For isolation of strain HG868, the 1/100-diluted homogenate (50 µl) was spread onto MA diluted to 1/100 with 70 % seawater. For strain 04PA2 Co4-8B, 50 µl homogenate was diluted to 1/10 and spread onto MA. For strain 04PA2 Co4-99A, the 1/100-diluted homogenate was spread onto MA containing 1 % $CaCO_3$. After cultivation at 25 °C for more than 30 days, colonies were picked and then re-isolated on MA.

Growth at different temperatures and pH values was tested at 17–35 °C (using increments of 5 °C) on MA and at pH 6–9 (using increments of one pH unit) in marine broth (Difco), respectively. The pH of the marine broth was adjusted with NaOH and HCl prior to autoclaving and was verified after autoclaving. The NaCl concentrations for growth were determined using a salt-tolerance test medium containing 1 % tryptone, 0.3 % yeast extract, 0.9 % $MgCl_2.6H_2O$, 0.9 % $MgSO_4.7H_2O$, 0.2 % $CaCl_2.2H_2O$, 0.06 % KCl and 1.5 % agar, with 0–10 % (w/v) NaCl. Gram-staining was performed using the BD Gram-staining kit (Becton Dickinson). Cell morphology was observed using transmission electron microscopy (see Supplementary Fig. S1, available in IJSEM Online). For transmission electron microscopy, cells were mounted on Formvar-coated copper grids and negatively stained with 1 % (w/v) aqueous uranyl acetate. Grids were observed in a JEOL 1010 transmission electron microscope operated at 100 kV. Gliding motility was observed by means of phase-contrast microscopy (BX60; Olympus) of marine broth cultures (Bernardet *et al.*, 2002). Growth under anaerobic conditions was determined after 2 weeks incubation in an AnaeroPack (Mitsubishi Gas Chemical) on MA. Catalase and oxidase activities were determined by testing bubble formation in a 3 % H_2O_2 solution and using cytochrome oxidase paper (Nissui Pharmaceutical), respectively. The presence of flexirubin pigments was investigated by observing the colour shift of the cell mass after exposure to a 20 % (w/v) KOH solution (Reichenbach, 1989; Bernardet *et al.*, 2002). API commercial strips (bioMérieux) were used to determine physiological and biochemical characteristics of the novel strains. All of the suspension media used with the API test strips were supplemented with NaCl to a final concentration of 0.85 % (w/v) NaCl. API 20E, API ZYM and API 50 CH strips were incubated for 24 h at 30 °C, 4 h at 37 °C and 48 h at 25 °C, respectively.

The respiratory quinone system and the cellular fatty acid content were determined as described by Xie & Yokota (2003). For the fatty acid analysis, strains were grown on MA for 24 h and analysed using the MIDI Microbial Identification System. The major quinone in the novel strains was MK-6 and the major cellular fatty acids were. The major fatty acids (>5.0 %) for strain YM3-301^T were i15 : 0 (22.7 %), i15 : 1 (14.5 %), i17 : 0 3-OH (11.7 %), unknown (equivalent chain-length, 13.562) (10.8 %), i16 : 0 3-OH (6.8 %) and i15 : 0 3-OH (6.6 %) (Table 1).

DNA was extracted using the method of Marmur (1961) from cells grown on MA and the DNA G+C content was determined with the HPLC method of Mesbah *et al.* (1989). The DNA G+C contents of the six isolates were in the range 41.8–43.5 mol%.

An approximately 1500 bp fragment of the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers specific to the 16S rRNA gene: primer 9F (5′-GAGTTTGATCCTGGCTCAG-3′; positions 8–27, according to the *Escherichia coli* numbering system of Brosius *et al.*, 1978) and primer 1510R (5′-GGCTACCTT-GTTACGTA-3′; positions 1510–1527). The PCR and sequencing protocols have been described previously (Xie & Yokota, 2003). DNA–DNA hybridization between the six isolates was carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989). The hybridization temperature was 42 °C.

To determine the phylogenetic positions of the strains, their 16S rRNA gene sequences were compared with sequences obtained from GenBank. Multiple alignments of the sequences were performed using CLUSTAL_X, version 1.83 (Thompson *et al.*, 1997). Alignment gaps and ambiguous bases were not taken into consideration in the comparison of 1174 bases of the 16S rRNA gene sequence. Phylogenetic relationships were analysed using the same software. Distances were calculated using the Kimura two-parameter model (Kimura, 1980). Clustering based on the neighbour-joining method (Saitou & Nei, 1987) was determined using bootstrap percentages based on 1000 replications (Felsenstein, 1985) (Fig. 1). Similarity values were calculated using MEGA3.1 (Kumar *et al.*, 2004).

The phylogenetic analysis based on 16S rRNA gene sequences revealed that strains YM3-301^T, HG868, 04PA2 Co4-8B, 04PA2 Co4-99A, 04PA2 018SW-3 and 04PA2 018SW-18 formed a distinct lineage within the family *Flavobacteriaceae*, showing the highest sequence similarity (90.3–90.5 %) with respect to members of the genus *Leeuwenhoekiella* (Nedashkovskaya *et al.*, 2005); the similarities with respect to the type strain of *Zhouia amylolytica* (Liu *et al.*, 2006) were 90.8–90.9 % and those for members of other genera in the family were 86.3–89.1 %. In contrast, the novel isolates shared high levels of 16S rRNA gene sequence similarity (99.7–100 %) and DNA–DNA relatedness (72–94 %) with each other. Hence, they represent a new genus and novel species in the family *Flavobacteriaceae*.

The phenotypic characteristics shared by strains YM3-301^T, HG868, 04PA2 Co4-8B, 04PA2 Co4-99A, 04PA2 018SW-3 and 04PA2 018SW-18 are listed in the genus and species descriptions, while those that serve to distinguish them from

Table 1. Cellular fatty acid contents (%) of the novel strains and of related taxa in the family *Flavobacteriaceae*

Taxa: 1, YM3-301^T; 2, HG868; 3, 04PA2 Co4-8B; 4, 04PA2 Co4-99A; 5, 04PA2 018SW-3; 6, 04PA2 018SW-18; 7, *Leeuwenhoekiella* (three species; Nedashkovskaya *et al.*, 2005); 8, *Croceibacter atlanticus* HTCC 2559^T (Cho & Giovannoni, 2003); 9, *Vitellibacter vladivostokensis* KMM 3516^T (Nedashkovskaya *et al.*, 2003); 10, *Aequorivita* (four species; Bowman & Nichols, 2002); 11, *Sediminicola luteus* LMG 23246^T (Khan *et al.*, 2006); 12, *Cellulophaga* (six species; Bowman, 2000). Fatty acids amounting to less than 1 % of the total fatty acids in all strains listed are not shown. Some of the taxa were not cultivated under the same conditions. tr, Trace amount (<0.5 %); –, not detected.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12
Saturated acids												
15 : 0	2.8	6	2.9	6.5	7.0	2.7	–	3.8	3.7	0.6–3.1	–	9.3–14.3
16 : 0	0.9	1.5	0.9	2.1	1.9	0.9	–	0.5	2.2	0.9–2.9	–	1.2–6.2
Branched acids												
i14 : 0	1.2	tr	0.7	tr	tr	1.2	–	–	–	tr–1.0	1	–
i15 : 0	22.7	33.7	19.3	29.9	31.2	20.2	16–18.2	13.3	68.8	7.6–16.8	12	7.5–31.3
a15 : 0	0.5	tr	0.5	tr	tr	0.5	2.7–4.5	1.5	8.4	15.7–20.7	11	1–3.3
i15 : 1	14.5	23.1	16.3	20.9	22.2	14.0	7.6–10.4	–	2.4	–	11	–
i16 : 0	4.6	2.1	4.9	1.9	1.7	3.6	1.1–3.8	1.5	3	1.4–5.3	5	tr–2.7
i16 : 1	0.9	tr	1.1	tr	tr	0.8	–	–	tr	–	3	–
i17 : 0	tr	1.1	tr	1.2	0.9	tr	–	tr	2.7	–	–	tr–0.9
Unsaturated acids												
17 : 1ω6*c*	0.6	tr	1.1	tr	tr	1.0	1.8–2.1	1.17	–	–	–	tr
Hydroxy acids												
15 : 0 2-OH	0.6	tr	0.8	0.5	tr	0.9	1.2–1.4	1.17	–	–	–	–
i14 : 0 3-OH	0.8	tr	0.6	tr	tr	0.6	–	–	–	–	–	–
15 : 0 3-OH	0.9	tr	1.0	0.6	0.5	1.4	tr–1.3	–	–	–	–	tr–0.7
i15 : 0 3-OH	6.6	3.9	6.1	4.1	3.9	7.5	2.1–3.9	4.26	1.6	1.7–5.4	4	0.8–8.6
16 : 0 3-OH	1.3	0.99	1.2	1.9	1.4	2.1	–	tr	–	–	–	tr–2.1
i16 : 0 3-OH	6.8	1.1	4.9	1.1	1.2	5.9	2.5–3.4	4.41	–	2.1–9.2	5	5–7.3
17 : 0 3-OH	0.7	0.6	0.7	0.9	0.9	0.7	–	–	–	–	–	–
i17 : 0 3-OH	11.7	10.1	14.0	12.4	12.1	14.8	12.7–22.1	28	0.8	2.0–4.5	12	4.5–20.8
Summed feature 3*	4.9	2.8	9.1	4.1	3.0	9.5	9.4–10.9	5.11	–	–	14	–
ECL 11.539†	1.9	1.1	0.9	0.7	0.7	0.9	–	–	–	–	–	–
ECL 13.562†	10.8	8.1	7.9	6.7	6.0	6.6	tr–1.3	–	–	–	–	–
ECL 16.583†	1.3	0.9	1.6	1	0.9	1.2	tr–1.2	–	–	–	–	–

*Summed features consist of one or more fatty acids that could not be separated by the Microbial Identification system. Summed feature 3 comprised 16 : 1ω7*c* and/or i15 : 0 2-OH.

†Unidentified fatty acids (ECL, equivalent chain-length).

their closest relatives are listed in Table 2. In particular, the six isolates were characterized by their inability to grow at 0–4 °C and with >4 % NaCl (although they required at least 1 % NaCl for growth), by their inability to move over surfaces by gliding and by the high G+C content of their DNA.

On the basis of the phylogenetic, physiological and biochemical data for strains YM3-301^T, HG868, 04PA2 Co4-8B, 04PA2 Co4-99A, 04PA2 018SW-3 and 04PA2 018SW-18, it is concluded that they represent a novel species in a new genus belonging to the family *Flavobacteriaceae*, for which the name *Leptobacterium flavescens* gen. nov., sp. nov. is proposed.

Description of *Leptobacterium* gen. nov.

Leptobacterium (Lep.to.bac.te′ri.um. Gr. adj. *leptos* thin, fine, narrow; L. neut. n. *bacterium* rod; N.L. neut. n. *Leptobacterium* a slender rod).

Cells are straight, non-gliding rods. Gram-negative. Strictly aerobic. Cells are devoid of flagella and endospores are not formed. Flexirubin pigments are not produced. Catalase- and oxidase-positive. Nitrate and nitrite are not reduced. The major respiratory quinone is MK-6. The DNA G+C content of the type species is 42–44 %. The major cellular fatty acids are i15 : 0, i15 : 1 and i17 : 0 3-OH. The type species is *Leptobacterium flavescens*.

Description of *Leptobacterium flavescens* sp. nov.

Leptobacterium flavescens (fla.ves′cens. L. part. adj. *flavescens* becoming golden–yellow).

The main characteristics are the same as those given for the genus. In addition, cells are approximately 0.5–0.6 μm wide and 8.5–9.0 μm long. Colonies on MA are circular,

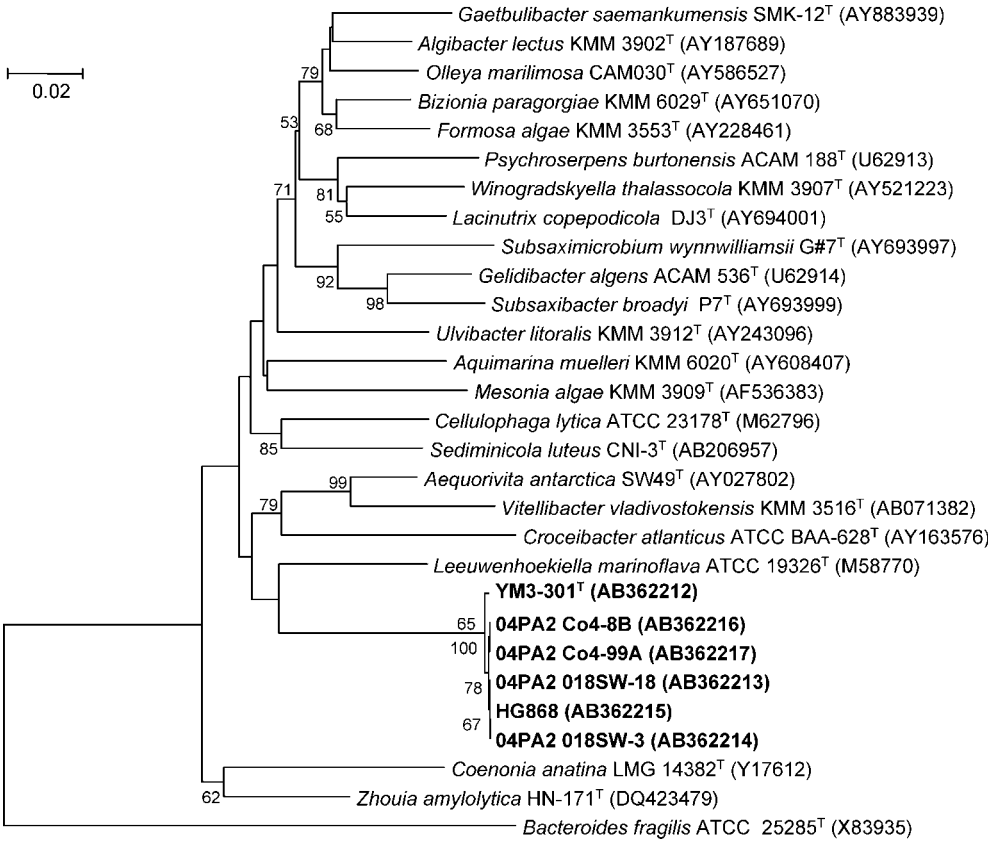

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the phylogenetic position of strains YM3-301^T, HG868, 04PA2 Co4-8B, 04PA2 Co4-99A, 04PA2 018SW-3 and 04PA2 018SW-18, representatives of closely related genera and other genera of the family *Flavobacteriaceae*. The tree was rooted using *Bacteroides fragilis* ATCC 25285^T (X83935) as an outgroup. Numbers at nodes indicate bootstrap percentages (based on 1000 trees); only values greater than 50 % are shown. Bar, 2 % sequence divergence.

flat with entire edges and pale-yellow-pigmented. Growth occurs at 17–30 °C (optimally at 25–30 °C). No growth occurs at 4 or 45 °C. The pH range for growth is 6–9 (optimum, around pH 6–7). NaCl is required for growth and can be tolerated up to 4 % (w/v). In API 20E strips, gelatin and tryptophan are hydrolysed, but tests for the Voges–Proskauer reaction, citrate utilization, urea hydrolysis and the production of ONPG, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide and indole give negative results. In API 50 CH strips, acid is produced from aesculin-ferric citrate, maltose, 5-ketogluconate and D-arabinose, but not from L-arabinose, D-xylose, L-xylose, methyl β-D-xylopyranoside, galactose, glucose, fructose, ribose, mannose, rhamnose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, sucrose, trehalose, melezitose, inulin, raffinose, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, glycerol, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, sorbitol, starch, glycogen, xylitol, D-arabitol, L-arabitol, gluconate or 2-ketogluconate. In API ZYM strips, alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol phosphohydrolase and *N*-acetyl-β-glucosaminidase activities are present, but esterase, esterase lipase, β-glucuronidase, cystine arylamidase, α-galactosidase, β-galactosidase, naphthol-AS-BI-phosphohydrolase, α-glucuronidase, α-mannosidase, α-glucosidase and β-glucosidase activities are absent. The major fatty acids are i15 : 0, i15 : 1 and i17 : 0 3-OH.

The type strain, YM3-301^T (=KCTC 22160^T=MBIC 06275^T=NBRC 104141^T), was isolated from a marine sponge *Clathria* (*Microciona*) *eurypa* collected at Iwayama Bay on Koror Island, Republic of Palau. The DNA G+C content of the type strain is 42 mol%.

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Table 2. Differential characteristics of the novel strains and related taxa in the family *Flavobacteriaceae*

Taxa: 1, *Leptobacterium flavescens* sp. nov. (six strains); 2, *Leeuwenhoekiella* (three species; Nedashkovskaya *et al.*, 2005); 3, *Croceibacter atlanticus* HTCC 2559^T (Cho & Giovannoni, 2003); 4, *V. vladivostokensis* KMM 3516^T (Nedashkovskaya *et al.*, 2003); 5, *Aequorivita* (four species; Bowman & Nichols, 2002); 6, *S. luteus* LMG 23246^T (Khan *et al.*, 2006); 7, *Cellulophaga* (six species; Johansen *et al.*, 1999; Bowman, 2000). +, Positive; −, negative; W, weakly positive; V, variable, ND, no data available; M, marine environment; MP, marine sponge; MS, marine sediment; QSS, quartz-stone subliths; SC, sea cucumber; SI, sea ice; SW, seawater; BO, bright orange; NY, non-diffusible yellow; NY–O, non-diffusible yellow–orange; O–Y, orange–yellow; Y, yellow.

Characteristic	1	2	3	4	5	6	7
Origin	MP	SW	SW	SC	SI/SW/QSS	MS	M
Gliding motility	−	+	−	−	−	−	+
Pigment	Y	NY	BO	NY–O	O–Y	Y	Y
Flexirubin	−	V	−	+	−	−	−
NaCl requirement	+	−	+	+	V	ND	+
Growth at/with:							
4 °C	−	+	−	+	+	−	−
30 °C	+	+	−	+	−	+	+
15 % NaCl	−	+	+	−	−	−	−
Oxidase	+	+	−	+	−	+	+
Catalase	+	+	+	+	+	W	+
Nitrate reduction	−	−	−	−	−	+	−
Hydrolysis of:							
Tryptophan	+	−	ND	ND	ND	−	ND
Starch	−	+	+	−	+	+	+
Acid production from:							
Aesculin	+	−	ND	ND	+	ND	ND
Maltose	+	−	−	−	ND	+	+
DNA G+C content (mol%)	41.8–43. 5	35–38	35	41.3	33–39	38–40	32–34

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