Galbibacter mesophilus gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*

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A Gram-negative, yellow-pigmented, rod-shaped bacterial strain (Mok-17^T) was isolated from marine sediment sampled in Okinawa Island, Japan. Based on analysis of the almost complete sequence of its 16S rRNA gene, strain Mok-17^T was found to belong to the family *Flavobacteriaceae*. Strain Mok-17^T showed highest 16S rRNA gene sequence similarity (91 %) to *Leeuwenhoekiella marinoflava* and *Robiginitalea biformata*. In a phylogenetic tree based on the 16S rRNA gene, strain Mok-17^T formed a deep branch distinct from all other organisms in the family *Flavobacteriaceae*. The major quinone was MK-6 and the major fatty acids were iso-15:0, iso-15:1, iso-17:0 3-OH and summed feature 3 (16:1 ω 7c and/or iso-15:0 2-OH). The DNA G+C content was 37 mol%. The phylogenetic distance to the type strains of all recognized species in the family *Flavobacteriaceae* and the phenotypic properties of strain Mok-17^T supported its classification as representing a novel species in a new genus, for which the name *Galbibacter mesophilus* gen. nov., sp. nov. is proposed. The type strain is Mok-17^T (=NBRC 101624^T=CIP 109219^T).

Bacteria belonging to the phylum *Bacteroidetes* are present in high numbers in marine environments (Glöckner *et al.*, 1999; Brown & Bowman, 2001) where they perform different functions of ecological importance. Their possible roles in the carbon cycle (Abell & Bowman, 2005), degradation of dissolved and particulate organic matter (Cottrell & Kirchman, 2000; Davey *et al.*, 2001), fish pathology (Bernardet, 1998) and algicidal activity (Maeda *et al.*, 1998) have been discussed. Comprising more than 50 genera at the time of writing, the family *Flavobacteriaceae* is one of the major branches of the phylum *Bacteroidetes*. Recently, new members of the family have been isolated from various marine environments (Nedashkovskaya *et al.*, 2003, 2004, 2005). Our group has also characterized several novel species in the family, mostly from sandy sediments collected from the Pacific coasts of Japan (Khan *et al.*, 2006a, b, c, 2007).

Here we describe the isolation of a new strain, designated Mok-17^T, from a marine sediment sample collected in Okinawa Island, Japan. The sample was diluted in artificial seawater (ASW; Naigai Chemicals) and aliquots (0.1 ml) of serial dilutions were plated on marine agar 2216 (MA; Difco). Strain Mok-17^T was one of the yellow colonies that grew on the plates. Unless mentioned otherwise, plates or

slants of half-strength MA (HSMA) diluted with ASW were used for routine cultivation at 30 °C. For long-term preservation at –80 °C, bacterial cells were suspended in ASW with 20 % (v/v) glycerol.

Template DNA for 16S rRNA gene amplification was prepared by using Prepman Ultra (Applied Biosystems). The pair of universal primers 27f and 1492r was used to amplify the portion of the 16S rRNA gene corresponding to positions 8–1492 in the *Escherichia coli* 16S rRNA gene sequence (Brosius *et al.*, 1978). The amplified fragment was sequenced directly by using a BigDye Terminator v3.1 cycle sequencing kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The ATGC program (Genetyx) was used for sequence editing and assembly. The assembled sequence was then compared with the 16S rRNA gene sequences in the DNA Database of Japan (DDBJ) using BLAST searches (Altschul *et al.*, 1990) and was aligned with related sequences by using CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic trees were inferred by using the neighbour-joining (NJ; Saitou & Nei, 1987), maximum-parsimony (MP; Swofford, 2000) and maximum-likelihood (ML; Adachi & Hasegawa, 1996) algorithms. The topology of the trees was evaluated by bootstrap resampling analysis (Felsenstein, 1985) of 1000 replicates for NJ and 100 replicates for MP and ML. The BLAST search result clearly indicated that strain Mok-17^T represented a member of the family *Flavobacteriaceae*. It shared highest 16S rRNA gene sequence similarity (92 %) with an unclassified bacterium, '*Flavobacterium*' sp. (DDBJ accession no. AJ244702). Strain Mok-17^T showed highest

Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Mok-17^T is AB255367.

16S rRNA gene sequence similarity to recognized species of 91 % with both *Leeuwenhoekiella marinoflava* (DDBJ accession no. AY167315) and *Robiginitalea biformata* (DDBJ accession no. AY424900). Pairwise 16S rRNA gene sequence comparison of strain Mok-17^T with other recognized members of the family *Flavobacteriaceae* revealed similarities of only 80–91 %. In an NJ tree based on 16S rRNA gene sequences, strain Mok-17^T formed a novel lineage in the family *Flavobacteriaceae*, although the branching order of the lineage was not strongly supported by bootstrap

analysis (Fig. 1). Similar results were obtained with MP and ML analyses (data not shown).

Standard methods were used for the characterization of strain Mok-17^T as described below and elsewhere (Khan *et al.*, 2006a). Cell morphology was observed under an Olympus light microscope (CX41LF) without staining and after Gram-staining performed as described by Cowan & Steel (1993). Gliding motility was tested by observing the spread of colony edges on MA plates (Perry, 1973) and by

Fig. 1. NJ tree based on 16S rRNA gene sequences of strain Mok-17^T and related taxa in the family *Flavobacteriaceae*. Numbers indicate bootstrap values (calculated from 1000 resampled units) of greater than 500. *Flexibacter flexilis* NBRC 16028 (DDBJ accession no. AB078054; not shown) was used as the outgroup. Closed circles indicate nodes that were also recovered in MP and ML trees. Bar, 0.02 K_{nuc} .

microscopic observation of hanging drops of a marine broth 2216 (MB; Difco) culture (Bernardet *et al.*, 2002) under a 1000 × oil-immersion objective (Olympus CX41LF). The absorption spectrum (260–700 nm) of an acetone extract of the cells was obtained with a Shimadzu UV–visible spectrophotometer (UV-1650 PC) to assess the presence of carotenoid pigment(s). The bathochromic shift test with 20 % KOH (w/v) was performed for the detection of flexirubin-type pigments (Fautz & Reichenbach, 1980). Growth at different temperatures (4, 10, 20, 30, 35, 37, 40, 42 and 45 °C) was assessed on HSMA plates. Salt tolerance was tested in MB with final NaCl concentrations adjusted to 3, 5, 6, 7, 8, 9, 10 and 15 % (w/v). Growth was also tested in one-fifth-strength LB [2 g Bacto tryptone (Difco) and 1 g Bacto yeast extract (Difco) dissolved in a final volume of 1000 ml] containing 0, 30, 50 and 70 % (v/v) ASW. Cells from a 2-day-old HSMA culture were spotted on a glass slide and were flooded with 3 % hydrogen peroxide (v/v) to test for the presence of catalase. The presence of oxidase was tested by spotting a cell suspension (in sterile water) on a cytochrome oxidase strip (Nissui Pharmaceuticals). MB solidified with 1.5 % carrageenan (Type I; Sigma) or agar was used to test the ability of the strain to degrade these molecules. Degradation of cellulose was tested by immersing cellulose strips (Whatman No. 1 paper) in a culture of strain Mok-17^T in one-fifth-strength LBM (one-fifth-strength LB prepared with ASW) at 30 °C for 1 month. Degradation of carboxymethylcellulose (CM-cellulose) was tested by observing the liquefaction of one-fifth-strength LBM solidified with 3 % (w/v) CM-cellulose (High Viscosity; Sigma), while the methods described by Cowan & Steel (1993) were used to test for the degradation of starch, gelatin, chitin, casein and DNA. The recommendations of the manufacturer were followed for the API 20NE tests (bioMérieux) except that inocula were prepared by suspending cells in sterile ASW. The GN2 MicroPlate system (Biolog) was used to test for the utilization of 95 different carbon sources. Inocula were prepared as suggested by Rüger & Krambeck (1994) and the microplate was incubated at 30 °C for 26–30 h after inoculation.

The Sherlock Microbial Identification system (MIDI) was used for the analysis of fatty acid methyl esters of strain Mok-17^T cultivated on MA for 82 h at 20 °C. The predominant cellular fatty acids were iso-17:0 3-OH, iso-15:0, iso-15:1 and summed feature 3 (16:1 ω 7c and/or iso-15:0 2-OH). The detailed fatty acid profiles of strain Mok-17^T and related organisms are given in Table 1. Isoprenoid quinones were extracted and analysed according to the protocol of Nakagawa & Yamasato (1993).

Genomic DNA was prepared according to the protocol of Minamisawa (1990). The HPLC method of Mesbah *et al.* (1989) was used to determine the G+C content of the chromosomal DNA.

Table 2 lists the phenotypic characteristics that can be used to differentiate strain Mok-17^T from the three recognized *Leeuwenhoekiella* species and *R. biformata*.

Table 1. Fatty acid profiles (% of total) of strain Mok-17^T, the three recognized *Leeuwenhoekiella* species and *R. biformata*

Taxa: 1, strain Mok-17^T; 2, *Leeuwenhoekiella* (data from Pinhassi *et al.*, 2006); 3, *R. biformata* HTCC2501^T (Cho & Giovannoni, 2004). Abbreviations: tr, trace (fatty acids amounting to less than 1 %); ND, not detected.

Fatty acid	1	2	3
15:0	ND	9–12	5–6
15:0 3-OH	ND	2–3	tr
anteiso-15:0	tr	1–4	3–4
iso-15:0	16	17–19	24–28
iso-15:1	17	11–14*	14–21
15:1 ω 6c	3	1	tr
iso-15:0 3-OH	7	2–3	4
16:0	tr	1–2	1
iso-16:0	tr	1	tr
iso-16:0 3-OH	tr	4–7	1
iso-16:1	1	2*	tr
iso-17:0	ND	ND	1
17:0 2-OH	tr	ND	1
iso-17:0 3-OH	19	13–21	25–27
anteiso-17:0 3-OH	ND	5–7	ND
17:1 ω 6c	2	ND	tr
iso-17:1 ω 7c	ND	2–3	ND
iso-17:1 ω 9c	9	ND	tr
Summed feature 3†	15	10–13*	4
Summed feature 4†	2	ND	ND

*Some of the fatty acids have been identified more precisely by Pinhassi *et al.* (2006), such as iso-15:1 (as iso-15:1 ω 10c), iso-16:1 (as iso-16:1 ω 6c) and summed feature 3 (as 16:1 ω 7c).

†Summed features consist of one or more fatty acids that could not be separated by the MIDI system. Summed feature 3 comprised 16:1 ω 7c and/or iso-15:0 2-OH. Summed feature 4 comprised iso-17:1 I and/or anteiso-17:1 B.

Other phenotypic characteristics of strain Mok-17^T are given in the genus and species descriptions. Based on the large phylogenetic distances and differential phenotypic characteristics, it is proposed that strain Mok-17^T be classified as representing a novel species of a new genus in the family *Flavobacteriaceae*, for which the name *Galbibacter mesophilus* gen. nov., sp. nov. is proposed.

Description of *Galbibacter* gen. nov.

Galbibacter (Gal.bi.bac'ter. L. adj. *galbus* yellow; N.L. masc. n. *bacter* rod; N.L. masc. n. *Galbibacter* a yellow bacterium).

Cells are Gram-negative rods. Catalase- and oxidase-positive. The main respiratory quinone is menaquinone-6. Major fatty acids are iso-17:0 3-OH, iso-15:0, iso-15:1 and summed feature 3 (16:1 ω 7c and/or iso-15:0 2-OH). Member of the family *Flavobacteriaceae*. The type species is *Galbibacter mesophilus*.

Table 2. Differential phenotypic characteristics of strain Mok-17^T, members of the genus *Leeuwenhoekiella* and *R. biformata*

Taxa: 1, strain Mok-17^T; 2, *Leeuwenhoekiella* (data from Pinhassi *et al.*, 2006); 3, *R. biformata* (Cho & Giovannoni, 2004). Symbols: +, positive reaction; (+), weakly positive reaction; –, negative reaction.

Characteristic	1	2	3
Gliding motility	–*	+	–
Growth:			
At 42 °C	+	–	+
With 15 % NaCl	–	+	–
Degradation of:			
Gelatin	+	+	–
Casein	(+)	+	–
Nitrate reduction	+	–	–
DNA G+C content (mol%)	37	35–43	55–56

*Although gliding motility was not observed in hanging-drop preparations of strain Mok-17^T, the edge of some colonies showed swarming growth after prolonged incubation on MA.

Description of *Galbibacter mesophilus* sp. nov.

Galbibacter mesophilus [me.so.phi'lus. Gr. adj. *mesos* middle; Gr. adj. *philos* loving; N.L. masc. adj. *mesophilus* middle (temperature)-loving, i.e. mesophilic].

The description is as for the genus with the following additional properties. Cells are 0.5–0.7 µm in width and 1.0–3.0 µm in length. Colonies on MA are yellow with irregular margins after 3 days incubation at 30 °C. The edge of some colonies may show swarming growth; however, gliding motility is not observed in hanging-drop preparations. Carotenoid-type pigments are produced but flexirubin-type pigments are not. Growth occurs at temperatures between 10 and 42 °C, with optimal growth at 25–30 °C. No growth is observed at 4 or 45 °C. Growth occurs in the presence of 30–70 % (v/v) ASW but not in the absence of seawater. Growth occurs in the presence of 3–7 % NaCl (w/v) and optimally with 3–5 % NaCl (w/v). Positive for the degradation of starch, aesculin and gelatin, reduction of nitrate to nitrite, and production of β-galactosidase. Negative for arginine dihydrolase activity and for the degradation of agar, carrageenan, urea, arginine, DNA, chitin, cellulose and CM-cellulose. Weakly positive for the production of indole from tryptophan and for casein degradation. Acid is not produced from glucose. The following compounds are utilized: α-cyclodextrin, dextrin, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannose, D-melibiose, methyl β-D-glucoside, D-raffinose, sucrose, D-trehalose, turanose, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, L-proline, L-serine and L-threonine. The detailed fatty acid composition is given in Table 1. The DNA G+C content of the type strain is 37 mol%.

The type strain, Mok-17^T (=NBRC 101624^T=CIP 109219^T), was isolated from a marine sediment sample collected in Okinawa Island, Japan.

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