Vibrio marisflavi sp. nov., isolated from seawater

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A Gram-reaction-negative, facultatively anaerobic bacterial strain, designated WH134^T, was isolated from a seawater sample collected at a depth of 10 m near the Yellow Sea Cold Water Mass (YSCWM), 31° 59.970' N 123° 0.157' E, PR China. Cells of strain WH134^T were slightly curved rods, motile by means of a polar flagellum and positive for poly- β -hydroxybutyrate (PHB) accumulation. The strain was able to grow in 1–6 % (w/v) NaCl, at pH 5–10 and 16–37 °C but not at 4 or 40 °C. The major cellular fatty acids were summed feature 3 C_{16:1 ω 7c} and/or iso-C_{15:0} 2-OH, C_{16:0}, C_{18:1 ω 7c}, C_{18:0} and C_{14:0}. The DNA G+C content was 42.5 mol%. Phylogenetic analyses based on 16S rRNA, *gyrB*, *topA*, *recA*, *ftsZ*, *mreB*, *gapA* and *rpoA* gene sequences revealed that strain WH134^T belongs to the genus *Vibrio* and showed gene sequence similarities of 96.6, 75.7, 74.6, 83.6, 78.9, 82.9, 86.0 and 89.4 %, respectively, to *Vibrio rumoiensis* S-1^T. The possession of a flagellum, activity of arginine dihydrolase and lysine decarboxylase and inability to utilize citrate, however, differentiated strain WH134^T from *V. rumoiensis* DSM 19141^T. On the basis of the phenotypic, chemotaxonomic and phylogenetic evidence, strain WH134^T represents a novel species of the genus *Vibrio*, for which the name *Vibrio marisflavi* sp. nov. is proposed. The type strain is WH134^T (=CGMCC 1.8994^T =LMG 25284^T =DSM 23086^T).

Vibrios are typically Gram-negative, motile rods, mesophilic, chemo-organotrophic and have a facultatively fermentative metabolism (Baumann *et al.*, 1984). Micro-organisms belonging to the genus *Vibrio* occur frequently in marine and estuarine waters where they are often found associated with various organisms, ranging from plankton to fish (Thompson *et al.*, 2004). Phenotypic and molecular analyses have shown that members of the genus *Vibrio* are highly heterogeneous (Thompson *et al.*, 2005). In the last few years, with improvements in the taxonomy of vibrios, there has been a large increase in the number of species assigned to the genus *Vibrio* (Chang *et al.*, 2008; Gomez-Gil *et al.*, 2008; Rameshkumar *et al.*, 2008; Beaz-Hidalgo *et al.*, 2009; Yoshizawa *et al.*, 2009; Xu *et al.*, 2009; Wang *et al.*, 2010). In the present study, a *Vibrio*-like bacterial strain, designated WH134^T, recently isolated from seawater samples, was analysed and its exact taxonomic position was determined using a polyphasic approach.

Abbreviations: MLSA, multilocus sequence analysis; O/129, 2,4-diamino-6,7-diisopropyl-pteridine phosphate salt; PHB, poly- β -hydroxybutyrate; YSCWM, Yellow Sea Cold Water Mass.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyrB*, *topA*, *recA*, *ftsZ*, *mreB*, *rpoA* and *gapA* gene sequences of strain WH134^T are FJ847833, GU366192, GU366193, HM036066, HM036067, HM036068, HM036069 and HM036070, respectively.

One supplementary table and nine supplementary figures are available with the online version of this paper.

Strain WH134^T was isolated from a seawater sample collected at a depth of about 10 m near the Yellow Sea Cold Water Mass (YSCWM), 31° 59.970' N 123° 0.157' E, China, in July 2008. The temperature of the seawater was 20.5 °C with salinity of 33.2 g l⁻¹, conductivity of 46.2 ms cm⁻¹, density of 1023.3 kg m⁻³, chlorophyll content of 0.32 p.p.b. and turbidity of 1 FTU. A semitransparent colony, strain WH134^T, was isolated on marine 2216E agar (MA; Difco) after incubation at 28 °C for 2 days and was obtained in pure culture after three successive transfers to fresh MA plates. Working cultures were maintained on MA at 28 °C and stocks were kept as cell suspensions at –80 °C in sterile 0.9 % (w/v) saline supplemented with 15 % (v/v) glycerol.

Standard protocols to determine Gram reaction, catalase and oxidase activities, endospore formation, degradation of casein, starch, gelatin, DNA, Tween 80 and urea, nitrate reduction, hydrogen sulfate production from thiosulfate and 2,4-diamino-6,7-diisopropyl-pteridine phosphate salt (O/129) sensitivity were employed (Tindall *et al.*, 2007) using suitably modified MA or marine 2216E broth (MB; Difco). Cell morphology and the presence of flagella were determined using transmission electron microscopy (JEM-1200EX; JEOL) after cells of strain WH134^T had been negatively stained with 1 % (w/v) phosphotungstic acid. Nile blue A was used as a fluorescent stain to test cells for poly- β -hydroxybutyrate (PHB) accumulation under a fluorescence microscope (BH2; Olympus) (Ostle & Holt, 1982). Growth was tested on thiosulfate-citrate-bile salts sucrose (TCBS)

agar (Difco) and tryptone soy agar (TSA; Difco) supplemented with 2 % (w/v) NaCl for 48 h at 28 °C. Swarming motility was tested on tryptone agar (1 % tryptone, 1.5 % NaCl and 0.6 % agar). Growth under anaerobic conditions was determined by incubation on MA for 5 days at 28 °C in a dry cylinder containing 10 g gallic acid and 100 ml 10 % NaOH reacting to consume oxygen. The temperature range for growth was determined on MA plates by incubating cultures at 4–40 °C for 10 days (Tindall *et al.*, 2007). Halotolerance was investigated using synthetic marine ZoBell broth comprising 5 g Bacto peptone, 1 g yeast extract and 0.1 g FePO₄ in 1 l modified artificial seawater (Lyman & Fleming, 1940) supplied with 0–15 % (w/v) NaCl. In the modified artificial seawater, all of the Na⁺ was replaced by K⁺. Growth at pH 4–11 (increments of 1 pH unit) was determined by measuring changes in OD₆₀₀ over time in MB buffered with citrate/phosphate (0.2 mol l⁻¹), Tris/HCl (0.05 mol l⁻¹) or Na₂CO₃/NaOH (0.025 mol l⁻¹) (Breznak & Costilow, 1994). Susceptibility to antibiotics was investigated on MA plates by using discs containing different antibiotics. Other phenotypic and enzymic properties of strain WH134^T were tested using API 20 E, API 20 NE, API 50 CH and API ZYM test strips (bioMérieux) and Gram-negative MicroPlates (Biolog) with incubation at 28 °C according to the manufacturer's instructions, except that sterile 1.5 % (w/v) NaCl was used to prepare the inocula. *Vibrio rumoiensis* DSM 19141^T was obtained from DSMZ (Germany) and used as a reference strain.

For fatty acid methyl ester analysis, cells of strain WH134^T and *V. rumoiensis* DSM 19141^T were harvested from MB after incubation at 28 °C for 48 h and the fatty acid profiles were determined as described previously (Xie & Yokota, 2003) using the Sherlock microbial identification system (MIDI). The G+C content of the genomic DNA was determined according to the methods described by Mesbah & Whitman (1989) using reverse-phase HPLC.

For 16S rRNA gene sequencing, DNA was extracted from strain WH134^T, purified by standard methods (Ausubel *et al.*, 1995) and amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Multilocus sequence analysis (MLSA) based on partial sequences of seven housekeeping genes encoding DNA gyrase B subunit (*gyrB*), topoisomerase I (*topA*), a recombination repair protein (*recA*), a cell division protein (*ftsZ*), rod shaping protein B subunit (*mreB*), glyceraldehyde 3-phosphate dehydrogenase (*gapA*) and RNA polymerase alpha-subunit (*rpoA*) was used for further identification and classification of strain WH134^T. PCR primer sequences were obtained from Sawabe *et al.* (2007) for the *topA*, *recA*, *ftsZ*, *mreB* and *gapA* genes, from Yamamoto *et al.* (1999) for the *gyrB* gene and from Thompson *et al.* (2005) for the *rpoA* gene. The PCR products were purified using a TaKaRa agarose gel DNA purification kit (TaKaRa Biotechnology). The purified products were sequenced by Shanghai Biosune (China) with an Applied Biosystems automatic sequencer (ABI3730).

The 16S rRNA and seven housekeeping gene sequences were aligned and compared with available sequences in the GenBank/EMBL/DDBJ databases using BLAST searches and the 16S rRNA gene sequence was also compared with those in the EzTaxon database (<http://www.eztaxon.org/>) (Chun *et al.*, 2007). Phylogenetic analyses based on the individual and concatenated sequences were performed using the software package MEGA version 4.0 (Tamura *et al.*, 2007) after the multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997). 16S rRNA gene sequence similarities between WH134^T and other species of the genus *Vibrio* were calculated using the program PHYDIT (<http://plaza.snu.ac.kr/~jchun/phydit>) and the EzTaxon public database (Chun *et al.*, 2007). Phylogenetic trees were reconstructed using the neighbour-joining and maximum-parsimony methods using the Kimura 2-state parameter model implemented in MEGA version 4.0 (Tamura *et al.*, 2007).

Detailed morphological, physiological and biochemical characteristics of strain WH134^T are given in the species description and in Table 1 and transmission electron micrographs of strain WH134^T are presented in Supplementary Fig. S1 (available in IJSEM Online). Cells of strain WH134^T were Gram-reaction-negative, slightly curved, 0.4–0.6 × 0.8–1 µm with a single polar flagellum and were facultatively anaerobic based on observations of growth in both aerobic and anaerobic conditions. Growth occurred in 1–6 % (w/v) NaCl, while no growth occurred in the absence of NaCl. Based on this phenotypic evidence, strain WH134^T was clearly assigned to the genus *Vibrio* (Baumann *et al.*, 1984) but could be differentiated from its closest relative *V. rumoiensis* DSM 19141^T by the possession of a flagellum, arginine dihydrolase and lysine decarboxylase activities and the inability to utilize citrate. Strain WH134^T could also be clearly differentiated from other closely related *Vibrio* species in tests for lysine decarboxylase activity, acid production from melibiose and D-mannitol and the utilization of gentiobiose, myo-inositol, raffinose and turanose as sole carbon and energy sources (Table 1). Other characteristics that differentiated strain WH134^T from members of closely related species of the genus *Vibrio* are shown in Table 1.

The major fatty acid composition of strain WH134^T was as follows: summed feature 3 comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (30.3 %), C_{16:0} (30.1 %), C_{18:1}ω7c (16.7 %), C_{18:0} (5.3 %), C_{14:0} (5.3 %), C_{12:0} 3-OH (2.9 %), C_{12:0} (2.6 %) and summed feature 2 comprising C_{14:0} 3-OH and/or iso-C_{16:1} I (1.2 %) (Table 2). The fatty acid profile of strain WH134^T was similar to that of other members of the genus *Vibrio* but showed a distinct pattern from type strains of the closest phylogenetically related species. The quantities of fatty acids of summed feature 3 comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH were much lower, and the quantities of fatty acids C_{16:0} and C_{18:0} were much higher, in strain WH134^T than in *V. rumoiensis* DSM 19141^T, *V. gigantis* DSM 18531^T, *V. pomeroyi* LMG 20537^T and *V. littoralis* KCTC 12520^T (Table 2). For other fatty acids, no clear

Table 1. Differential characteristics of strain WH134^T and closely related species of the genus *Vibrio*

Taxa: 1, WH134^T; 2, *V. rumoiensis* DSM 19141^T (data from this study); 3, *V. gigantis* DSM 18531^T (Le Roux *et al.*, 2005; Noguerola & Blanch, 2008); 4, *V. pomeroyi* LMG 20537^T (Thompson *et al.*, 2003; Noguerola & Blanch, 2008); 5, *V. gallaecicus* LMG 24045^T (Beaz-Hidalgo *et al.*, 2009); 6, *V. litoralis* MANO22D^T (Nam *et al.*, 2007; Noguerola & Blanch, 2008); 7, *V. proteolyticus* ATCC 15338^T (Noguerola & Blanch, 2008). From repeat experiments: +, positive for $\geq 90\%$; (+), positive for 75–89%; –, negative for $\leq 10\%$; v, variable for 26–74%; ND, no data available. All taxa were negative for luminescence and acid production from D-sorbitol and L-rhamnose and were positive for nitrate reduction and oxidase and catalase activities.

Characteristic	1	2	3	4	5	6	7
Arginine dihydrolase	+	–	+	+	–	–	+
Lysine decarboxylase	+	–	–	–	–	ND	+
Ornithine decarboxylase	–	–	–	–	–	ND	–
Gelatinase	+	–	+	+	+	–	v
Flagella	+	–	+	+	+	+	+
Swarming motility	–	–	–	–	–	ND	+
β -Galactosidase	+	–	–	+	–	–	+
Indole production	–	–	ND	+	–	–	+
Citrate utilization	–	+	ND	–	–	+	+
Voges-Proskauer	–	–	–	–	–	ND	(+)
Susceptibility to:							
O/129 (150 μ g)	–	–	–	–	+	–	v
Ampicillin	–	–	ND	+	–	ND	–
Growth at/in:							
4 °C	–	+	+	+	+	+	–
37 °C	+	–	–	–	–	+	+
1 % NaCl	+	+	+	+	+	+	+
8 % NaCl	–	+	–	+	–	+	+
Acid produced from:							
D-Mannitol	–	+	+	+	+	+	+
Melibiose	+	–	–	–	–	ND	–
L-Arabinose	–	+	–	–	–	+	–
Utilization of:							
Glycogen	–	+	+	+	ND	–	ND
L-Arabinose	–	+	–	ND	ND	+	–
D-Arabitol	–	+	–	–	ND	+	ND
myo-Inositol	+	–	–	–	ND	ND	–
D-Mannitol	–	+	+	ND	ND	+	+

differences were observed between strain WH134^T and type strains of related species of the genus *Vibrio* (Table 2). The DNA G+C content of strain WH134^T was 42.5 mol%, which was well within the range of 38–51 mol% typical of the genus *Vibrio* (Farmer *et al.*, 2005).

Searches using the almost-complete 16S rRNA gene sequence (1290 bp) of strain WH134^T in the NCBI GenBank and EzTaxon databases placed it among members of the genus *Vibrio*. However, 16S rRNA gene sequence similarities between strain WH134^T and recognized species of the genus *Vibrio* were not greater than 97%; the highest similarity being to *V. rumoiensis* S-1^T (96.6%). The 16S rRNA gene sequence-based neighbour-joining (Fig. 1) and

Table 2. Cellular fatty acid content (%) of strain WH134^T and closely related species of the genus *Vibrio*

Taxa: 1, strain WH134^T; 2, *V. rumoiensis* DSM 19141^T (data from this study); 3, *V. gigantis* DSM 18531^T (Le Roux *et al.*, 2005); 4, *V. pomeroyi* LMG 20537^T (Thompson *et al.*, 2003); 5, *V. litoralis* KCTC 12520^T (Nam *et al.*, 2007). –, Not detected or detected in trace amounts <1%.

Fatty acid	1	2	3	4	5
Straight-chain fatty acids					
C _{12:0}	2.6	3.5	4.5	8.9	3.7
C _{14:0}	5.3	3.3	4.7	10.5	–
iso-C _{15:0}	1.2	–	–	–	–
C _{16:0}	30.1	21.1	23.5	29.2	13.5
iso-C _{16:0}	–	–	2.4	–	–
iso-C _{17:0}	1.2	–	–	–	–
C _{18:0}	5.3	4.1	–	1.6	–
Unsaturated fatty acids					
C _{18:1} ω 7c	16.7	17.0	12.5	7.6	19.5
C _{18:1} ω 9c	–	1.3	–	–	–
Hydroxy fatty acids					
C _{12:0} 3-OH	2.9	–	1.9	3.9	–
Summed features*					
2	1.2	5.1	2.3	4.1	7.1
3	30.3	42.9	40.4	32.9	38.6

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 comprises C_{14:0} 3-OH and/or iso-C_{16:1} I. Summed feature 3 comprises C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH.

maximum-parsimony (Supplementary Fig. S2) trees showed that strain WH134^T formed a cluster with *V. rumoiensis* S-1^T and *V. litoralis* MANO22D^T within the radiation including members of the genus *Vibrio*.

MLSA has proven to be a valuable technique for the identification of vibrios, as well as for studying the phylogeny of the genus (Thompson *et al.*, 2005). Neighbour-joining trees based on *gyrB* (1259 bp), *recA* (500 bp), *ftsZ* (444 bp), *gapA* (683 bp) and *rpoA* (600 bp) gene sequences aligned by CLUSTAL_X showed that strain WH134^T formed a clade on its own, the tree based on *topA* (414 bp) gene sequences showed that strain WH134^T formed a cluster with *Vibrio xuii* LMG 21346^T and the tree based on *mreB* (507 bp) gene sequences showed that strain WH134^T formed a cluster with *V. rumoiensis* DSM 19141^T (Supplementary Figs S3–S9). The *gyrB*, *topA*, *recA*, *ftsZ*, *mreB*, *gapA* and *rpoA* gene sequence similarities between strain WH134^T and *V. rumoiensis* DSM 19141^T were 75.7, 74.6, 83.6, 78.9, 82.9, 86.0 and 89.4%, respectively. The neighbour-joining phylogenetic tree (Fig. 2) based on concatenated sequences of the seven housekeeping genes (*gyrB*, *topA*, *recA*, *ftsZ*, *mreB*, *gapA* and *rpoA*) confirmed that strain WH134^T had a close evolutionary distance from *V. rumoiensis* DSM 19141^T supported by an elevated bootstrap value. The GenBank accession numbers for the *gyrB*, *topA*, *recA*, *ftsZ*, *mreB*, *gapA* and *rpoA* gene sequences used for

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of strain WH134^T and related members of the genus *Vibrio* and reconstructed using the neighbour-joining method. Bootstrap values >50% (based on 1000 replicates) are given at branching points. GenBank sequence accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position.

reconstructing phylogenetic trees are listed in Supplementary Table S1.

Based on the phenotypic characterization and phylogenetic analysis, strain WH134^T represents a novel species of the genus *Vibrio*, for which the name *Vibrio marisflavi* sp. nov. is proposed.

Description of *Vibrio marisflavi* sp. nov.

Vibrio marisflavi (ma.ris fla'vi. L. n. mare -is the sea; L. adj. flavus -a -um yellow; N.L. gen. n. marisflavi of the Yellow Sea, isolated from the Yellow Sea Cold Water Mass).

Cells are Gram-reaction-negative, slightly curved rods, 0.4–0.6 × 0.8–1 µm, facultatively anaerobic and motile by means of a polar flagellum. Swarming motility and bioluminescence are not detected. Colonies on MA media are light-yellow, 1–1.5 mm in diameter, smooth and

circular. Grows on TSA and forms yellow, translucent colonies on TCBS agar. Accumulation of PHB inside the cells is observed. Voges–Proskauer reaction is negative. Catalase and oxidase activities are weakly positive. Tests for gelatinase, caseinase, DNase, lipase, arginine dihydrolase, lysine decarboxylase and β-galactosidase activities and hydrolysis of aesculin are positive but tests for amylase, urease, tryptophan deaminase and ornithine decarboxylase activities, citrate utilization and indole and hydrogen sulfide production are negative. Nitrate is reduced to nitrite. Growth occurs at 16–37 °C, but not at 4 or 40 °C, at pH 5–10 and in 1–6% (w/v) NaCl, but not in the absence of NaCl. In the API 50 CH system, acid is produced from D-glucose, sucrose, melibiose, amygdalin, glycerol, D-ribose, D-xylose, D-galactose, D-fructose, methyl α-D-glucopyranoside, N-acetylglucosamine, arbutin, D-mannose, aesculin, salicin, cellobiose, maltose, trehalose, gentiobiose and potassium gluconate but not from the other carbon sources on the test strips. In the API ZYM system, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-B1-phosphohydrolase but negative for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities. In MicroPlates (Biolog), the following substrates are utilized as sole carbon and energy sources: Tweens 40 and 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, myo-inositol, maltose, D-mannose, melibiose, D-psicose, raffinose, sucrose, trehalose, turanose, methyl pyruvate, acetic acid, cis-aconitic acid, citric acid, D-gluconic acid, succinic acid, bromosuccinic acid, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-serine, L-threonine, urocanic acid, inosine, uridine, glycerol, α-DL-glycerol phosphate and glucose 6-phosphate; other substrates on

Fig. 2. Phylogenetic tree based on concatenated sequences of seven gene loci, including *gyrB* (1259 bp), *topA* (414 bp), *recA* (500 bp), *ftsZ* (444 bp), *mreB* (507 bp), *rpoA* (600 bp) and *gapA* (683 bp), and reconstructed using the neighbour-joining method. Bootstrap values >50% (based on 1000 replicates) are given at branching points. GenBank sequence accession numbers for the individual genes are available in Supplementary Table S1. Bar, 0.02 substitutions per nucleotide position.

the test strips are not utilized. Resistant to (μg per disc unless stated otherwise) vancomycin (30), clindamycin (2), amikacin (30), gentamicin (10), kanamycin (30), neomycin (30), chloromycetin (30), minocycline (30), doxycycline (30), oxacillin sodium (1), ampicillin (10), cephalothin IV (30), cephalothin V (30) and Fortum (30), but sensitive to midecamycin (30), norfloxacin (10), ofloxacin (5), ciprofloxacin (5), polymyxin B (100 U), compound sulfamethoxazole (75), furazolidone (300), chloramphenicol (30), piperacillin (100) and cephalothin VI (30). The predominant cellular fatty acids are summed feature 3 ($\text{C}_{16:1}\omega 7\text{c}$ and/or iso- $\text{C}_{15:0}$ 2-OH), $\text{C}_{16:0}$, $\text{C}_{18:1}\omega 7\text{c}$, $\text{C}_{18:0}$, $\text{C}_{14:0}$, $\text{C}_{12:0}$ 3-OH, $\text{C}_{12:0}$, summed feature 2 ($\text{C}_{14:0}$ 3-OH and/or iso- $\text{C}_{16:1}$ 1), iso- $\text{C}_{17:0}$ and iso- $\text{C}_{15:0}$.

The type strain, WH134^T (=CGMCC 1.8994^T =LMG 25284^T =DSM 23086^T), was isolated from seawater samples collected near the Yellow Sea Cold Water Mass (31° 59.970' N 123° 0.157' E), China. The DNA G+C content of the type strain is 42.5 mol%.

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