Mariniflexile aquimaris sp. nov., isolated from seawater, and emended description of the genus *Mariniflexile* Nedashkovskaya *et al.* 2006

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A Gram-staining-negative, non-flagellated, non-gliding and rod-shaped bacterial strain, designated HWR-17^T, was isolated from seawater of the Yellow Sea in Korea. Strain HWR-17^T grew optimally at pH 7.0–8.0, at 30 °C and in the presence of 2 % (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain HWR-17^T clustered with the two *Mariniflexile* species in the family *Flavobacteriaceae*, exhibiting 16S rRNA gene sequence similarity of 97.1–97.2 % to their type strains and less than 95.7 % sequence similarity to other members of the family *Flavobacteriaceae*. Strain HWR-17^T contained MK-6 as the predominant menaquinone and iso-C_{15:0} as the major fatty acid. The polar lipid profile of strain HWR-17^T contained phosphatidylethanolamine, an unidentified aminolipid and four unidentified lipids. The DNA G + C content of strain HWR-17^T was 35.7 mol% and it exhibited 11 and 10 % DNA–DNA relatedness, respectively, with *Mariniflexile gromovii* KCTC 12570^T and *Mariniflexile fucanivorans* DSM 18792^T. The phylogenetic and genetic distinctiveness and differential phenotypic properties revealed that strain HWR-17^T is distinguishable from the two recognized *Mariniflexile* species. On the basis of the data presented, strain HWR-17^T is considered to represent a novel species of the genus *Mariniflexile*, for which the name *Mariniflexile aquimaris* sp. nov. is proposed. The type strain is HWR-17^T (=KCTC 23346^T =CCUG 60529^T). An emended description of the genus *Mariniflexile* is also proposed.

The genus *Mariniflexile*, a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*, was proposed by Nedashkovskaya *et al.* (2006) with *Mariniflexile gromovii*, isolated from a sea urchin, as the sole recognized species. *Mariniflexile fucanivorans* was subsequently described from a water-treatment facility recycling the effluent of an alginate-extraction plant (Barbeyron *et al.*, 2008). In this study, a novel bacterial strain, designated HWR-17^T and isolated from seawater off Hwangdo, an island of the Yellow Sea in Korea, is described. Comparative 16S rRNA gene sequence analysis indicated that strain HWR-17^T was most closely related phylogenetically to members of the genus *Mariniflexile*. The aim of the present work was to determine the exact taxonomic position of strain HWR-17^T by using a polyphasic characterization that included

the determination of phenotypic properties, a detailed phylogenetic investigation based on 16S rRNA gene sequences and genetic analysis.

Strain HWR-17^T was isolated by the dilution plating technique at 25 °C on marine agar 2216 (MA; Difco) and cultivated routinely on MA at 30 °C. *M. gromovii* KCTC 12570^T and *M. fucanivorans* DSM 18792^T were used as reference strains for DNA–DNA hybridization, fatty acid and polar lipid analyses and a number of other phenotypic tests. Cell morphology was examined by using light microscopy (DP70; Olympus) and transmission electron microscopy (CM-20; Philips). The latter technique was also used to assess the presence of flagella on cells from an exponentially growing MA culture. For this purpose, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. Gliding motility was investigated as described by Bowman (2000). The Gram reaction was determined by

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HWR-17^T is HQ144198.

Table 1. Differential phenotypic characteristics of strain HWR-17^T and the type strains of the two *Mariniflexile* species

Strains: 1, *M. aquimaris* sp. nov. HWR-17^T; 2, *M. gromovii* KCTC 12570^T; 3, *M. fucanivorans* DSM 18792^T. All data were obtained in this study except for Gram-staining, optimal temperature, flagellar motility, catalase activity, susceptibility to antibiotics and DNA G+C content of reference strains; these data were taken from Nedashkovskaya *et al.* (2006) and Barbeyron *et al.* (2008). +, Positive; –, negative; w, weakly positive. All strains are positive for the following: catalase activity; hydrolysis of aesculin and Tweens 20, 40 and 60; utilization of D-glucose, D-fructose, D-galactose, D-mannose and D-xylose; acid production from D-xylose, D-fructose, D-glucose and D-galactose; and activity of alkaline phosphatase, esterase (C4), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. All strains are negative for the following: Gram staining; flagellar motility; production of flexirubin-type pigments; oxidase activity [opposite result reported by Nedashkovskaya *et al.* (2006) for *M. gromovii* KCTC 12570^T]; H₂S production; nitrate reduction; hydrolysis of urea and xanthine; utilization of trehalose, acetate, citrate, succinate, benzoate, L-malate, pyruvate, salicin, formate and L-glutamate; acid production from *myo*-inositol, D-ribose, melibiose, melezitose, trehalose, and raffinose; susceptibility to streptomycin and kanamycin; and activity of lipase (C14), trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -mannosidase and α -fucosidase.

Characteristic	1	2	3
Optimal growth temperature (°C)	30	23–25	25
Gliding motility	–	+	+
Hydrolysis of:			
Casein	+	+	–
Gelatin	–	+	–
Hypoxanthine	–	–	+
Starch	+	–	w
Tween 80	+	–	+
L-Tyrosine	+	+	w
Utilization of:			
L-Arabinose	+	–	–
Cellobiose	+	–	+
Maltose	w	–	–
Sucrose	+	+	–
Acid production from:			
L-Arabinose	+	–	–
Cellobiose	+	–	+
Lactose	–	–	+
Maltose	+	–	+
D-Mannitol	+	w	–
D-Mannose	w	+	+
L-Rhamnose	–	w	–
Sucrose	+	+	–
Susceptibility to (per disc):			
Ampicillin (10 µg)	–	+	+
Tetracycline (30 µg)	–	+	+
Enzyme activity (API ZYM)			
Esterase lipase (C8)	+	w	+
Valine arylamidase	+	w	+
Cystine arylamidase	–	w	–

Table 1. cont.

Characteristic	1	2	3
α -Glucosidase	–	–	+
β -Glucosidase	–	w	–
N-Acetyl- β -glucosaminidase	–	–	+
DNA G+C content (mol%)	35.7	35.7	34.5

using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber (Forma 1029; N₂/CO₂/H₂, 86:7:7) on MA and on MA supplemented with potassium nitrate (0.1%, w/v), both of which had been prepared anaerobically under a nitrogen atmosphere. Growth at 4, 10, 20, 25, 28, 30, 34 and 35 °C was measured on MA. The pH range for growth was investigated in marine broth 2216 (MB, Difco) adjusted to pH 4.5–10.0 (in increments of 0.5 pH units) by using sodium acetate/acetic acid and Na₂CO₃ buffers. The pH was verified after autoclaving. Growth in the absence of NaCl and in the presence of 0.5, 1.0, 2.0 and 3.0% (w/v) NaCl was investigated in trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was omitted and that 0.45% (w/v) MgCl₂·6H₂O was added. Growth in the presence of 2.0–7.0% (final concentration, w/v, in increments of 1.0%) NaCl was investigated in MB. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, starch, hypoxanthine, tyrosine and xanthine was tested on MA using the substrate concentrations described by Cowan & Steel (1965). Nitrate reduction and hydrolysis of aesculin, gelatin, Tweens 20, 40, 60 and 80 and urea were investigated as described previously (Lányi, 1987) with the modification that artificial seawater was used for the preparation of media. The artificial seawater contained (l⁻¹ distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). H₂S production was tested as described previously (Bruns *et al.*, 2001). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). Acid production from carbohydrates was tested as described by Leifson (1963). Utilization of substrates as sole carbon and energy sources was tested according to the method of Yurkov *et al.* (1994). Susceptibility to antibiotics was tested on MA plates using antibiotic discs (Advantec) containing the following amounts: ampicillin (10 µg), carbenicillin (100 µg), cephalothin (30 µg), chloramphenicol (100 µg), gentamicin (30 µg), kanamycin (30 µg), lincomycin (15 µg), neomycin (30 µg), novobiocin (5 µg), oleandomycin (15 µg), penicillin G (20 U), polymyxin B (100 U), streptomycin (50 µg) and tetracycline (30 µg). The API ZYM system (bioMérieux) incubated at 30 °C for 8 h was used to investigate enzyme activities. Morphological, cultural, physiological and biochemical properties of strain HWR-17^T are given in the species description and in Table 1.

Cell biomass of strain HWR-17^T for DNA extraction and for the analyses of isoprenoid quinones and polar lipids was obtained from cultures grown at 30 °C in MB. Cell biomass of *M. gromovii* KCTC 12570^T and *M. fucanivorans* DSM 18792^T for DNA extraction and polar lipid analysis was obtained from cultures grown at 30 °C in MB. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified using a PCR with two universal primers as described previously (Yoon *et al.*, 1998) and the PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. For cellular fatty acid analysis, cell mass of strain HWR-17^T, *M. gromovii* KCTC 12570^T and *M. fucanivorans* DSM 18792^T was harvested from MA plates after cultivation for 5 days at 30 °C. Fatty acids were saponified, methylated and extracted using the standard protocol of the Sherlock Microbial Identification System, version 4.0 (MIDI). The fatty acids were analysed by GC (Hewlett Packard 6890) and identified by using the TSBA40 database of the Microbial Identification System (Sasser, 1990). Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with the appropriate detection

reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC equipped with a YMC ODS-A (250 × 4.6 mm) column. The nucleotides were eluted with a mixture of 0.55 M NH₄H₂PO₄ (pH 4.0) and acetonitrile (40:1, v/v), at a flow rate of 1 ml min⁻¹ at room temperature, and detected by absorbance at 270 nm. DNA–DNA hybridization was performed fluorometrically at 45 °C by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample; the highest and lowest values obtained for each sample were excluded and the means of the remaining three values are quoted as DNA–DNA relatedness values.

The almost-complete 16S rRNA gene sequence of strain HWR-17^T comprised 1471 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. In the phylogenetic tree reconstructed using the neighbour-joining algorithm, strain HWR-17^T joined the cluster comprising the type strains of the two *Mariniflexile* species with a bootstrap resampling value of 99.9 % (Fig. 1). The relationships among strain HWR-17^T and the two *Mariniflexile* type strains were maintained in trees reconstructed using the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain HWR-17^T exhibited 16S rRNA gene sequence similarity of 97.1 and 97.2 % to the type strains of *M. gromovii* and *M. fucanivorans*, respectively, and less than 95.7 % similarity to those of the other species used in the phylogenetic analysis.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of *Mariniflexile aquimaris* sp. nov. HWR-17^T, other *Mariniflexile* species and representatives of some related taxa. Only bootstrap values (expressed as percentages of 1000 replications) greater than 70 % are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. The sequence of *Lutibacter litoralis* CL-TF09^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

The predominant isoprenoid quinone detected in strain HWR-17^T was menaquinone-6 (MK-6), in line with members of the genus *Mariniflexile* (Nedashkovskaya *et al.*, 2006) and all other members of the family *Flavobacteriaceae* (Bernardet, 2011). The complete cellular fatty acid profiles of strain HWR-17^T and the type strains of the two *Mariniflexile* species grown and analysed under identical conditions in this study are compared in Table 2. The major fatty acid (>10% of the total fatty acids) found in strain HWR-17^T was iso-C_{15:0} (24.8%) (Table 2). The fatty acid profiles of the three strains were similar, though there were also differences in the proportions of some fatty acids, particularly C_{15:0}, iso-C_{15:1} G and C_{15:1}ω6c. Strain HWR-17^T contained phosphatidylethanolamine, an unidentified aminolipid and an unidentified lipid (L1) as the major polar lipids; it also contained minor amounts of three additional unidentified lipids (L2–L4) (Fig. 2). This

Table 2. Cellular fatty acid compositions of strain HWR-17^T and the type strains of the two *Mariniflexile* species

Strains: 1, *M. aquimaris* sp. nov. HWR-17^T; 2, *M. gromovii* KCTC 12570^T; 3, *M. fucanivorans* DSM 18792^T. All data were obtained in this study. Values are percentages of total fatty acids; fatty acids that represented <1.0% in all strains have been omitted. tr, Trace (<1.0%); –, not detected; TBSA, tuberculostearic acid.

Fatty acid	1	2	3
Straight-chain saturated			
C _{15:0}	3.6	18.0	8.0
C _{16:0}	tr	1.1	tr
Straight-chain unsaturated			
C _{15:1} ω6c	2.4	4.3	11.0
C _{17:1} ω6c	tr	tr	3.9
Branched			
iso-C _{14:0}	1.9	1.2	1.8
iso-C _{15:0}	24.8	18.3	15.4
anteiso-C _{15:0}	7.2	5.6	6.2
iso-C _{15:1} G*	6.4	12.9	2.0
iso-C _{16:0}	1.2	tr	tr
iso-C _{16:1} H*	1.4	tr	1.3
iso-C _{17:1} ω9c	1.7	tr	tr
Hydroxy			
C _{15:0} 2-OH	1.7	1.4	2.3
C _{15:0} 3-OH	2.8	2.7	5.0
iso-C _{15:0} 3-OH	9.3	6.1	8.9
iso-C _{16:0} 3-OH	9.2	5.0	7.8
C _{17:0} 2-OH	2.3	1.3	2.9
iso-C _{17:0} 3-OH	9.6	9.1	7.5
Others			
10-Methyl C _{18:0} (TBSA)	tr	–	1.3
Summed feature 3†	7.2	4.0	6.1

*Double bond position indicated by a capital letter is unknown.

†Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

polar lipid profile was very similar to those of *M. gromovii* KCTC 12570^T and *M. fucanivorans* DSM 18792^T. The DNA G + C content of strain HWR-17^T was 35.7 mol%, a value in line with those reported for the two *Mariniflexile* species (Nedashkovskaya *et al.*, 2006; Barbeyron *et al.*, 2008). Hence, it is appropriate to classify strain HWR-17^T as a member of the genus *Mariniflexile*, as shown by phylogenetic inference and the absence of differentiating chemotaxonomic properties from the two described *Mariniflexile* species.

Strain HWR-17^T exhibited mean DNA–DNA relatedness values of 11 and 10% with *M. gromovii* KCTC 12570^T and *M. fucanivorans* DSM 18792^T, respectively. Strain HWR-17^T was distinguishable from these two strains by differences in several phenotypic characteristics, as shown in Table 1. These differences, in combination with phylogenetic and genetic distinctiveness between strain HWR-17^T and the two *Mariniflexile* species, suggest that the novel strain represents a novel species of the genus *Mariniflexile* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994), for which the name *Mariniflexile aquimaris* sp. nov. is proposed. An emended description of the genus *Mariniflexile* is also proposed on the basis of new data obtained in this study.

Emended description of the genus *Mariniflexile* Nedashkovskaya *et al.* 2006

Mariniflexile [Ma.ri.ni.fle'xi.le. L. adj. *marinus* marine; L. part. adj. *flexilis* -e pliant, pliable, flexible; N.L. neut. n. *Mariniflexile* a flexible marine (bacterium)].

The description of the genus *Mariniflexile* is as given by Nedashkovskaya *et al.* (2006) with the following amendments. Motile by gliding or non-gliding. Optimal growth temperature is 25–30 °C. Oxidase activity is negative. The major polar lipids are phosphatidylethanolamine, an unidentified aminolipid and an unidentified lipid.

Description of *Mariniflexile aquimaris* sp. nov.

Mariniflexile aquimaris (a.qui.ma'ris. L. n. *aqua* water; L. gen. n. *maris* of the sea; N.L. gen. n. *aquimaris* of the water of the sea).

Cells are Gram-staining-negative, non-flagellated, non-gliding and rod-shaped, approximately 0.2–0.5 µm in diameter and 2.0–8.0 µm long. Colonies on MA are circular, convex, smooth, glistening and yellow, 0.2–1.0 mm in diameter after incubation for 3 days at 30 °C. Optimal growth temperature is 30 °C; growth occurs at 10 and 34 °C, but not at 4 or 35 °C. Optimal growth at pH 7.0–8.0; growth occurs at pH 5.5, but not at pH 5.0. Growth occurs in the presence of 0–5% (w/v) NaCl with an optimum of approximately 2.0% (w/v) NaCl. Mg²⁺ ions are required for growth. Anaerobic growth does not occur on MA or on MA supplemented with nitrate. H₂S is not produced. Flexirubin-type pigments are not produced. Susceptible to cephalotin, gentamicin, neomycin, penicillin

Fig. 2. Thin-layer chromatograms of total polar lipids of *M. aquimaris* sp. nov. HWR-17^T (a), *M. gromovii* KCTC 12570^T (b) and *M. fucanivorans* DSM 18792^T (c). Spots were revealed using molybdotophosphoric acid, molybdenum blue, ninhydrin and α -naphthol reagents. PE, Phosphatidylethanolamine; AL, unidentified aminolipid; L1–L4, unidentified lipids.

G and polymyxin B, but not to ampicillin, carbenicillin, chloramphenicol, kanamycin, lincomycin, novobiocin, oleandomycin, streptomycin or tetracycline. The predominant menaquinone is MK-6 and the major fatty acid (>10% of the total fatty acids) is iso-C_{15:0}. In addition to the major polar lipids given in the emended genus description, three unidentified lipids are present in minor amounts. The DNA G+C content of the type strain is 35.7 mol%. Other phenotypic characteristics are given in Table 1.

The type strain, HWR-17^T (=KCTC 23346^T =CCUG 60529^T), was isolated from seawater off Hwang-do, an island of Korea.

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