

Description of *Maribacter forsetii* sp. nov., a marine *Flavobacteriaceae* isolated from North Sea water, and emended description of the genus *Maribacter*

Tristan Barbeyron,¹ Flavien Carpentier,¹ Stéphane L'Haridon,²
Margarete Schüler,³ Gurvan Michel¹ and Rudolf Amann³

Correspondence

Tristan Barbeyron
barboun@sb-roscoff.fr

Rudolf Amann
ramann@mpi-bremen.de

¹Centre National de la Recherche Scientifique; Université Pierre et Marie Curie-Paris 6, Unité Mixte de Recherche 7139 'Végétaux Marins et Biomolécules', Station Biologique, F-29682 Roscoff Cedex (Bretagne), France

²Centre National de la Recherche Scientifique; Université de Bretagne Occidentale, IFREMER, Unité Mixte de Recherche 6197 'Laboratoire de Microbiologie des Environnements Extrêmes', IFREMER, F-29280 Plouzané (Bretagne), France

³Max-Planck-Institut für Marine Mikrobiologie, D-28359 Bremen, Germany

Three rod-shaped, Gram-negative, chemo-organotrophic, heterotrophic, strictly aerobic, gliding bacterial strains, KT02ds18-4, KT02ds18-5 and KT02ds18-6^T, were isolated from North Sea surface waters near the island of Helgoland, Germany. Their taxonomic position was investigated by a polyphasic approach. The three strains were light yellow, oxidase- and catalase-positive, and grew optimally at 25 °C, at pH 7.5, and in the presence of 2.5% (w/v) NaCl. The Chargaff's coefficient was 34.2–34.4 mol%. The three strains shared >90% DNA–DNA relatedness and an identical 16S rRNA gene sequence. Comparative 16S rRNA gene sequence analysis allocated the three strains to the genus *Maribacter* in the family *Flavobacteriaceae*, with similarities of 97.0–97.4% to five of the recognized *Maribacter* species. Their low level of DNA–DNA relatedness (<20%) with these species and differentiating phenotypic characteristics demonstrated that they constitute a new *Maribacter* species for which the name *Maribacter forsetii* sp. nov. is proposed. Strain KT02ds18-6^T (=CIP 109504^T=DSM 18668^T) is the type strain. An emended description of the genus *Maribacter* is also proposed.

The *Cytophaga-Flavobacterium* cluster within the phylum *Bacteroidetes* often accounts for more than 10% of the total microbial community in coastal and offshore waters (Glöckner *et al.*, 1999; Abell & Bowman, 2005; DeLong *et al.*, 2006). Moreover, flavobacteria have been found in high abundance during natural and induced phytoplankton blooms, suggesting utilization of polymeric organic matter in marine systems (Kirchman, 2002). This characteristic was recently confirmed by whole-genome sequence analysis of the first marine *Bacteroidetes*, '*Gramella forsetii*' strain KT0803^T (Bauer *et al.*, 2006) isolated from the North Sea.

In this study, we report the polyphasic characterization of strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T isolated

from seawater sampled at the same location as '*Gramella forsetii*', as a new species of the genus *Maribacter* in the family *Flavobacteriaceae* (Bernardet *et al.*, 1996, 2002), phylum *Bacteroidetes*. The genus *Maribacter* initially contained four species: *Maribacter aquivivus*, *Maribacter orientalis*, *Maribacter ulvicola* and the type species *Maribacter sedimenticola* was isolated from marine sediment, water and algae (Nedashkovskaya *et al.*, 2004). A fifth species, *Maribacter dokdonensis*, was described from seawater (Yoon *et al.*, 2005). The comparative study of our isolates with these five *Maribacter* species allows us to also provide an emended description of the genus *Maribacter*.

Strain KT02ds18 was isolated from a North Sea water sample taken on 25 August 1999 at a depth of 1 m at station Kabeltonne, also known as Helgoland Roads (54°11'N; 7°54'E). Isolation was performed on an oligotrophic synthetic seawater medium (Schut *et al.*, 1993) containing a mixture of defined monomeric sugars (at 10 µM), amino acids (at 1 µM) and acetate, lactate, ethanol and glycerol (all at 15 µM) with reduced NH₄Cl

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Maribacter forsetii* KT02ds18-4, KT02ds18-5 and KT02ds18-6^T are AM712898, AM712899 and AM712900, respectively.

Electron micrographs of strain KT02ds18-6^T are available with the online version of this paper.

and KH_2PO_4 concentrations of 50 and 1.5 μM , respectively (Eilers, 2000). Upon subcultivation on ZoBell agar 2216E (ZoBell, 1941), strain KT02ds18 was shown to actually consist of three different strains, KT02ds18-4, KT02ds18-5 and KT02ds18-6^T, distinguished on the basis of different colony morphology. For direct comparison, *Maribacter aquivivus* KMM 3949^T, *M. orientalis* KMM 3947^T, *M. sedimenticola* KMM 3903^T, *M. ulvicola* KMM 3951^T and *M. dokdonensis* DSM 17201^T were studied in parallel with the isolates in all phenotypic tests (except growth at different temperatures, pH and NaCl concentrations) and whole-cell protein analysis. All strains were routinely cultivated in liquid or on solid (solidified with 1.5 %, w/v, agar) ZoBell medium 2216E (ZoBell, 1941). Pure cultures were stored at $-80\text{ }^\circ\text{C}$ in culture medium containing 20 % (v/v) glycerol.

Growth was monitored by the increase in optical density at 600 nm using a UV1601 spectrophotometer (Shimadzu). All growth experiments were performed in triplicate. The optimal pH value was determined in *Cytophaga* marine broth no. 172 (DSMZ) using 10 mM MES buffer at pH 5.0, 5.5 and 6.0, 10 mM PIPES buffer at pH 6.5 and 7.0, 10 mM HEPES buffer at pH 7.5, 10 mM Tris/HCl at pH 8.0 and 8.5, and no buffer at pH 9.0. The effect of NaCl on growth was determined in the same medium containing 0–9 % (w/v) NaCl at 1 % intervals. The temperature range for growth was determined at 4, 10, 15, 20, 25, 32 and 37 $^\circ\text{C}$. The effects of pH and NaCl concentration were determined at 25 $^\circ\text{C}$. Cell morphology and gliding motility were investigated on an exponential ZoBell broth culture at 20 $^\circ\text{C}$ by phase-contrast microscopy (Olympus BH2 microscope, $\times 1000$ magnification). Gram staining was carried out according to the method described by Doetsch (1981). For transmission electron microscopy, 20 μl of a bacterial suspension was dropped onto carbon-coated grids (400 mesh) and negatively stained with 2 % (w/v) uranyl acetate or 2 % (v/v) phosphotungstic acid for 1 min. Micrographs were taken on a model CM12 electron microscope (Philips) with an acceleration voltage of 100 kV. To determine the respiratory type of metabolism, the bacteria were inoculated in Veillon's tubes containing ZoBell medium solidified with 0.6 % (w/v) agar. Oxygen was removed from the medium by boiling. To determine oxidative or fermentative metabolism, the strains were inoculated in a modified Leifson O-F medium (Hugh & Leifson, 1953; Smibert & Krieg, 1981) containing 0.5 % (w/v) glucose. Cytochrome oxidase activity was assayed by vigorous shaking of cells from a 2.5 ml broth culture for 5 min in 0.5 ml natural seawater and in the presence of 20 μl dimethyl-*p*-phenylenediamineoxalate (bioMérieux). The presence of the enzyme was revealed by a blue coloration of the cell suspension. Catalase activity was assayed by mixing one colony from a ZoBell agar plate with a drop of hydrogen peroxide (10 %, v/v). Amylase activity was assayed on 0.2 % (w/v) soluble starch ZoBell agar plates. DNase activity was detected in DNA agar (Difco), prepared with seawater. Amylase and DNase activities were revealed by flooding the plates with Lugol solution or 1 M HCl, respectively. Agarase, κ -carrageenase and ι -carrageenase activities were assessed

by inoculating ZoBell media solidified with 15 g agar l^{-1} , 10 g κ -carrageenan l^{-1} or 20 g ι -carrageenan l^{-1} , respectively. Strains whose colonies were surrounded by a depression in the substrate were considered positive. Production of flexirubin-type pigments was assessed by flooding a 4-day-old plate culture with 20 % (w/v) potassium hydroxide, followed by the observation of changes in colony colour from yellow to red or brown (Reichenbach *et al.*, 1974). The ability to use simple carbohydrates as carbon sources was tested in artificial seawater supplemented with 2 g ammonium nitrate l^{-1} , 250 mg Casamino acids l^{-1} , 150 mg yeast extract l^{-1} and the carbohydrate under investigation at a concentration of 0.5 % (w/v). Other tests were performed by using the identification strips API 20 NE (with API AUX medium), API ZYM and API 50 CH (bioMérieux). The latter were used both in the acidification and assimilation modes, using API 50CHB/E medium and a medium containing 2 g ammonium sulfate l^{-1} , 100 mg yeast extract l^{-1} and 1.5 g agar l^{-1} in 40 mM phosphate buffer, pH 8, respectively. All culture media were supplemented up to 25 g NaCl l^{-1} . GN2 microplates (Biolog) were inoculated with bacterial suspensions in a medium containing 25 g NaCl l^{-1} , 8 g MgCl_2 l^{-1} and 0.5 g KCl l^{-1} , supplemented with 1.5 g ι -carrageenan l^{-1} . API galleries and GN2 microplates were incubated at 20 $^\circ\text{C}$ for 2 weeks. Sensitivity to chloramphenicol (10 $\mu\text{g ml}^{-1}$), penicillin G (200 $\mu\text{g ml}^{-1}$), streptomycin (200 $\mu\text{g ml}^{-1}$), kanamycin (200 $\mu\text{g ml}^{-1}$), ampicillin (200 $\mu\text{g ml}^{-1}$), rifampicin (10 $\mu\text{g ml}^{-1}$) and tetracycline (10 $\mu\text{g ml}^{-1}$) (all from Sigma) was tested at 20 $^\circ\text{C}$ in *Cytophaga* marine broth.

The fatty acid composition of the three isolates was determined by the DSMZ, according to the standard protocol of the MIDI Microbial Identification System (Sasser, 1990), on cells grown on TSBA40 (30 g trypticase soy broth l^{-1} and 15 g agar l^{-1}) at 28 $^\circ\text{C}$ for 24 h. Cellular proteins were extracted from cell pellets from a 2.0 ml 24 h ZoBell broth culture at 25 $^\circ\text{C}$. Cells were washed twice with artificial seawater and resuspended in 375 μl BugBuster Protein Extraction Reagent (Novagen). The cells were disrupted by vigorous shaking and kept on ice for 10 min. Proteins were denatured for 5 min at 100 $^\circ\text{C}$ in the presence of 125 μl 4 \times loading buffer (60 mM Tris/HCl, pH 6.8, 2 % SDS, 0.005 %, w/v, bromophenol blue, 1.4 M β -mercaptoethanol). The amounts of protein loaded onto an SDS-PAGE gel (20 \times 20 cm, 1.5 mm thick; 12 %, w/v, polyacrylamide in running gel, 4 % in stacking gel) were standardized with respect to the strain that had the lowest optical density. The Chargaff's coefficient of the DNA, expressed as the molar percentage of guanine + cytosine (mol% G + C), was determined by the DSMZ. Genomic DNAs were obtained by using the procedure described by Barbeyron *et al.* (1984). DNA was purified on a CsCl_2 gradient and purity was checked spectrophotometrically. The 16S rRNA gene sequence was amplified by PCR using pure genomic DNA as template and a couple of primers specific for *Bacteria*, 8F (Hicks *et al.*, 1992) and 1492R (Kane *et al.*, 1993). PCR reactions were typically carried out

in a volume of 50 μ l containing 10–100 ng template, 0.4 μ M each specific primer, 250 μ M each dNTPs, 0.1 mg BSA, 1 \times GoTaq buffer (Promega) and 1.25 U GoTaq DNA polymerase (Promega). The PCR conditions were as follows: 2 min at 95 $^{\circ}$ C, followed by 30 cycles of 0.5 min at 95 $^{\circ}$ C, 0.5 min at 50 $^{\circ}$ C, 1.5 min at 72 $^{\circ}$ C and a final polymerization step of 4 min at 72 $^{\circ}$ C. PCR products were purified with the MinElute PCR purification kit (Qiagen), cloned into vector pCRII2.1 and sequenced using BigDye-labelled dideoxy-ribonucleotides, the ThermoSequenase kit (RPN 2444; Amersham) and an ABI PRISM 3130xl Genetic Analyzer automated sequencer (Applied Biosystems/Hitachi). The 16S rRNA gene sequences of strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T were aligned manually with a representative set of sequences obtained from GenBank (Benson *et al.*, 1999). The secondary structure was used as a guide to ensure that only homologous regions were compared. Of the 1383 nt that were sequenced, 1286 were used in the phylogenetic analysis. A modified version of the neighbour-joining algorithm (Saitou & Nei 1987; Gascuel, 1997) was used with the Kimura distance correction (Kimura, 1980) and bootstrap analysis was performed to provide confidence estimates for the phylogenetic tree topologies (Felsenstein, 1985). The maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods were also applied, as implemented in the PHYLO_WIN package (Galtier *et al.*, 1996). Genetic relatedness was investigated by slot-blot DNA–DNA hybridization, using the ECF random-prime labelling and signal amplification system (ECF kit RPN 5752; Amersham Life Sciences), following the procedure described by Kristjánsson *et al.* (1994). Target DNA (50 ng) denatured in 0.4 M NaOH was slotted onto a nylon hybridization membrane (Bio-Rad) and probed with 200 ng fluorescein-labelled tracer DNA. For each duplicate of DNA–DNA association, the hybridization temperature was chosen in the optimal range of the hybridization buffer (Johnson, 1984; Ivanova *et al.*, 1988).

Strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T grew on ZoBell agar plates forming round, shiny, light yellow colonies, 1–3 mm in diameter within 3 days at 20 $^{\circ}$ C. The three strains displayed a varying degree of spreading at the margin of their colonies. Colony margins of strain KT02ds18-4 were entire with only very limited spreading (Fig. 1a); those of KT02ds18-5 showed active spreading (Fig. 1b); while the intense spreading of strain KT02ds18-6^T resulted in confluent colonies (Fig. 1c). In comparison, other species of *Maribacter*, grown under the same conditions, showed circular, non-spreading, shiny and dark yellow colonies. Phase-contrast microscopy of wet mounts revealed that all strains were round-ended rods of about 0.3–0.5 μ m in diameter and 3.0–4.0 μ m in length occurring singly or in pairs (Fig. 1d). Shorter cells (1.0 μ m long) and non-refractive spherical bodies appeared in stationary-phase cultures. Electron micrographs of phosphotungstic acid-coloured cells showed vesicle-like structures that seemed to stem from the outer membrane (see supplementary Fig. S1, available with the online version of

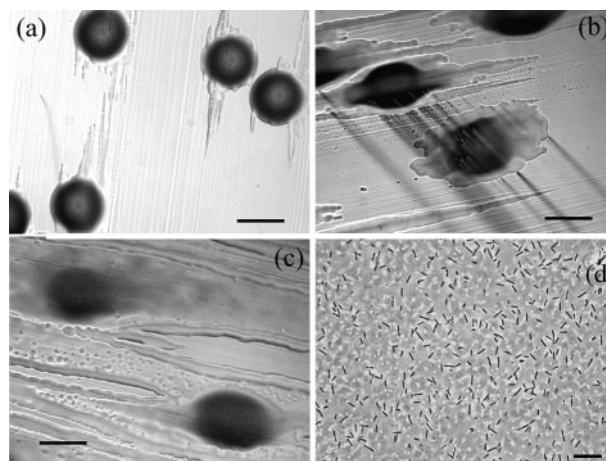


Fig. 1. Colonies of KT02ds18-4 (a), KT02ds18-5 (b) and KT02ds18-6^T (c) on ZoBell agar after 5 days incubation at 25 $^{\circ}$ C. Note the difference in the extent of spreading. (d) Phase-contrast micrograph of strain KT02ds18-6^T cultivated in ZoBell Broth for 24 h at 25 $^{\circ}$ C. Bars, (a, b, c) 1 mm; (d) 10 μ m.

this paper). The three strains were non-flagellated, adhered to the slide and to the cover glass, and displayed active gliding motility. Strain KT02ds18-6^T was the most active. All strains were Gram-negative, strictly aerobic, chemo-organotrophic and heterotrophic organisms with an oxidative metabolism that used oxygen as electron acceptor. Nitrate could not be used as electron acceptor. Cytochrome oxidase and catalase were positive and flexirubin-type pigments were not synthesized. Additional phenotypic characteristics of strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T are given in the species description and in Table 1. Overall, the results obtained in this study with five *Maribacter* species are in agreement with previously published data (Nedashkovskaya *et al.*, 2004; Yoon *et al.*, 2005). The few exceptions may have resulted from different cultivation conditions or different phenotypic tests. The major discrepancies with the original description of the genus *Maribacter* and the five *Maribacter* species in this study are: (i) agarase activity was not detected in any strain; (ii) DNA hydrolysis was detected in all species except *M. aquivivus*; and (iii) although tested several times (five experiments), none of the species was able to reduce nitrate to nitrite in API 20 NE strips (a result confirmed using the Greiss reagent and strain *Zobellia galactanivorans* Dsij^T as positive control; Barbeyron *et al.*, 2001). Moreover, in contrast with the statements of Nedashkovskaya *et al.* (2004) and Yoon *et al.* (2005), strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T were able to grow, albeit weakly, in the absence of NaCl. Consequently, we have proposed an emended description of the genus *Maribacter* (see below).

The three isolates showed very similar whole-cell protein profiles, whereas the profiles of the type strains of other *Maribacter* species were significantly different (Fig. 2). The fatty acid profiles of strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T were also very similar (Table 2), featuring

Table 1. Phenotypic characteristics of *Maribacter* species

Species: 1, *Maribacter forsetii* sp. nov. (3 strains); 2, *M. aquivivus* KMM 3949^T; 3, *M. dokdonensis* DSM 17201^T; 4, *M. orientalis* KMM 3947^T; 5, *M. sedimenticola* KMM 3903^T; 6, *M. ulvicola* KMM 3951^T. All strains are Gram-negative, non-flagellated and rod-shaped. All strains were positive for the following characteristics: respiratory metabolism; gliding motility; presence of cytochrome oxidase, catalase, alkaline and acid phosphatases, leucine, valine and cystine arylamidases and α -chymotrypsine activities; hydrolysis of aesculin and dextrin; utilization of D-xylose, cellobiose, maltose, sucrose, trehalose and aspartic acid; and acid production from glucose and sucrose. All strains were negative for the following characteristics: presence of β -glucuronidase activity; hydrolysis of agar, κ -carrageenan, glycogen and inulin; production of flexirubin pigments; nitrate reduction; utilization of D-arabinose, D-tagatose, L-xylose, D-fucose, L-sorbose, D-ribose, D-lyxose, L-arabitol, adonitol, xylitol, dulcitol, and D-sorbitol; and production of acid from D-tagatose, L-xylose, D-fucose, L-sorbose, D-ribose, D-lyxose, glycerol, L-erythritol, D- and L-arabitol, adonitol, xylitol, dulcitol, D-mannitol, D-sorbitol, inositol, glycogen and inulin. +, Positive; -, negative; w, weakly positive; v, variable; ND, not determined.

Characteristic	1*	2	3	4	5	6
Optimal growth temperature (°C)	25	22†	30‡	22†	23†	22†
Growth at/with:						
6% (w/v) NaCl	+	+†	+‡	-†	+†	-†
32 °C	+	-†	+‡	+†	+†	+†
Presence of:						
Esterase (C4)	+	-	+	-	-	-
Esterase lipase (C8)	+	-	+	+	-	-
Lipase (C14)	v (-)	-	-	-	-	-
Trypsin	v (-)	+	+	+	+	+
Naphthol-AS-BI-phosphatase	+	+	+	-	-	-
α -Galactosidase	+	-	-	-	-	-
β -Galactosidase	+	+	+	+	-	+
α -Glucosidase	+	+	+	-	-	+
α -Fucosidase	v (-)	-	-	-	-	-
Hydrolysis of:						
<i>r</i> -Carrageenan	-	+	+	+	-	+
Starch	-	-	-	-	+	-
Alginic acid	+	+	ND	+	+	+
Gelatin	-	+	-	-	+	-
DNA	-	-	+	+	+	+
Tween 40	+	-	+	-	-	+
Tween 80	+	-	+	-	-	-
Utilization of:						
D-Mannose	+	+	+	+	-	+
D-Fructose	+	+	+	-	+	+
Glycerol	-	-	+	-	-	-
D-Mannitol	+	+	+	+	-	+
D-Lactose	+	+	+	+	-	+
Amygdalin	-	+	-	-	-	-
Turanose	v (+)	+	+	+	-	+

Table 1. cont.

Characteristic	1*	2	3	4	5	6
Melezitose	v (+)	+	+	-	-	-
Methyl- β -D-glucoside	+	+	+	-	-	-
Methyl- β -D-xyloside	+	+	w	-	-	w
N-acetyl-D-glucosamine	+	+	+	+	-	+
Glucuronic acid	+	+	-	+	w	+
Lactic acid	+	-	-	+	-	+
Succinic acid	v (-)	+	-	+	-	+
L-Alanine	v (-)	-	+	+	-	+
Acid production from:						
D-Galactose	+	+	+	+	-	+
D-Mannose	+	-	+	+	-	+
D-Fructose	+	w	-	+	+	+
D-Arabinose	+	-	+	-	-	+
L-Arabinose	-	-	-	w	-	-
D-Xylose	+	+	+	+	-	+
L-Rhamnose	-	-	-	-	-	w
L-Fucose	+	-	+	-	-	-
Cellobiose	+	-	+	+	-	+
D-Lactose	+	-	+	+	-	+
Maltose	+	-	+	+	+	+
Melibiose	v (+)	-	+	+	-	+
Trehalose	v (+)	+	+	+	w	+
Gentiobiose	v (+)	-	w	-	-	-
Amygdalin	v (+)	-	+	+	-	+
Turanose	v (+)	-	+	+	-	-
Raffinose	v (+)	-	+	-	w	-
Starch	-	-	-	-	+	-
Methyl- α -D-glucoside	v (+)	-	+	+	-	+
Methyl- α -D-mannoside	v (+)	-	+	+	-	+
Methyl- β -D-xyloside	+	-	+	+	-	+
N-Acetyl-D-glucosamine	v (+)	-	-	+	-	+
Salicin	+	-	+	+	-	+
Susceptibility to antibiotics:						
Penicillin G (200 μ g ml ⁻¹)	v (+)	ND	ND	ND	ND	ND
Streptomycin (200 μ g ml ⁻¹)	v (+)	ND	ND	ND	ND	ND
Ampicillin (200 μ g ml ⁻¹)	w	-§	-§	-§	-§	+§
Kanamycin (200 μ g ml ⁻¹)	-	ND	ND	ND	ND	ND
Rifampicin (10 μ g ml ⁻¹)	v (+)	ND	ND	ND	ND	ND
Tetracycline (10 μ g ml ⁻¹)	-	-§	w‡§	-§	+§	-§
Chloramphenicol (10 μ g ml ⁻¹)	v (+)	ND	ND	ND	ND	ND
DNA G + C content (mol%)	34.4	35‡	36.1‡	39‡	37.5‡	36.7‡

*The result of the type strain is given in parentheses.

†Data from Nedashkovskaya *et al.* (2004).

‡Data from Yoon *et al.* (2005).

§Tested by the disc diffusion method; Nedashkovskaya *et al.* (2003).

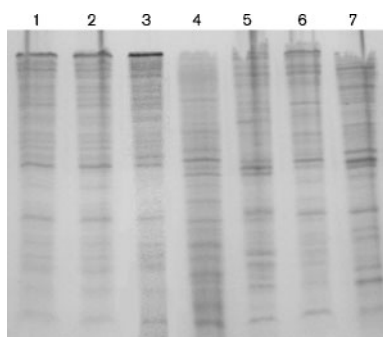


Fig. 2. SDS-PAGE of protein extracts from *Maribacter forsetii* sp. nov. strains KT02ds18-4 (lane 1), KT02ds18-5 (lane 2) and KT02ds18-6^T (lane 3), *Maribacter aquivivus* KMM 3949^T (lane 4), *M. orientalis* KMM 3947^T (lane 5), *M. sedimenticola* KMM 3903^T (lane 6) and *M. ulvicola* KMM 3951^T (lane 7). Protein bands were visualized by staining with Coomassie blue.

five major fatty acids: C_{15:0} (≈ 15%), iso-C_{15:0} (≈ 12%), iso-C_{15:1} (≈ 12%), iso-C_{17:0} 3-OH (≈ 18%) and summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; ≈ 10%). These patterns were in accordance with those published for other *Maribacter* species. However, the respective proportions of some compounds differed, probably as a result of different culture conditions.

The Chargaff's coefficients of the DNA of strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T were 34.2, 34.2 and 34.4 mol%, respectively. The 16S rRNA gene sequences of the strains were identical and shared 97.2, 97.3, 97.4, 97.0 and 97.1% similarity with those of the *M. aquivivus*, *M. dokdonensis*, *M. orientalis*, *M. sedimenticola* and *M. ulvicola* type strains (over 1404 bp of each sequence), respectively. In the neighbour-joining tree based on 16S rRNA gene sequences, the three strains fell within the cluster composed of five other *Maribacter* species (Fig. 3). Phylogenetic trees generated using the maximum-likelihood method showed essentially the same topology, whereas some differences occurred in the parsimony tree. The 16S rRNA gene sequence similarity of the three isolates with the other members of the family *Flavobacteriaceae* used in the phylogenetic analysis was <93%. The mean DNA–DNA reassociation level, as determined by slot-blot hybridization, between strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T was 90–100% when their DNAs were used individually as probes for cross-hybridization. When the DNAs of the three isolates were used to probe the DNAs of other *Maribacter* species, or when, reciprocally, the DNAs from *Maribacter* species were used as probes against the DNAs of the three new strains, DNA–DNA reassociation levels were <20–25%, significantly below the values observed at the species level (Stackebrandt & Goebel, 1994), indicating that strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T represent a novel species.

Hence, phylogenetic and fatty acid data show that strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T belong to the

Table 2. Whole-cell fatty acid profiles (%) of *Maribacter* species

Strains: 1, *Maribacter forsetii* sp. nov. KT02ds18-6^T; 2, *M. forsetii* sp. nov. KT02ds18-4; 3, *M. forsetii* sp. nov. KT02ds18-5; 4, *M. aquivivus* KMM 3949^T; 5, *M. dokdonensis* DSM 17201^T; 6, *M. orientalis* KMM 3947^T; 7, *M. sedimenticola* KMM 3903^T; 8, *M. ulvicola* KMM 3951^T. ND, Not detected; tr, trace (<1%). Fatty acids amounting to <1% are not listed; therefore, totals do not necessarily reach 100%. Some strains were cultivated under different conditions.

Fatty acid	1	2	3	4*	5†	6*	7*	8*
Straight-chain								
C _{14:0}	tr	tr	tr	tr	1	tr	tr	tr
C _{15:0}	15.5	15.5	14.7	3.8	14.5	12.3	6.3	8.1
C _{16:0}	tr	1.0	1.0	1.4	tr	1.2	1.0	1.0
Branched								
iso-C _{15:0}	12.4	12.5	11.9	19.7	12.3	10.6	20.5	13.6
iso-C _{15:1}	13.2	11.7	11.1	16.1	13.6	10.1	16.9	18.9
anteiso-C _{15:0}	tr	1.0	1.0	tr	1.3	2.3	1.2	1.9
iso-C _{16:0}	1.0	1.6	1.7	ND	tr	tr	1.1	tr
iso-C _{17:1} ω5c	ND	ND	ND	ND	1.4	ND	ND	1.2
iso-C _{17:1} ω9c	4.6	5.0	4.1	2.0	2.2	4.0	2.3	2.2
iso-C _{18:1}	ND	ND	ND	ND	ND	2.4	ND	ND
Unsaturated								
C _{13:1}	ND	ND	ND	ND	1.1	tr	tr	1.1
C _{14:1} ω5	ND	ND	ND	1.0	ND	ND	ND	tr
C _{15:1} ω6c	3.5	2.8	2.8	tr	4.8	2.5	1.7	1.6
C _{17:1} ω6c	1.1	1.1	1.2	tr	1.7	1.3	tr	tr
Hydroxy								
C _{15:0} 3-OH	1.8	1.4	1.5	1.4	2.3	1.5	2.4	1.5
iso-C _{15:0} 3-OH	3.9	3.8	3.7	5.0	3.2	2.9	5.4	4.1
C _{16:0} 3-OH	1.5	1.8	2.2	5.4	2.9	3.0	2.2	3.7
iso-C _{16:0} 3-OH	5.1	5.9	6.8	1.1	2.5	2.1	1.7	1.7
iso-C _{17:0} 3-OH	17.5	19.7	18.3	28.6	11.6	18.8	20.4	14.5
Summed feature 3‡	1.0	10.1	11.0	8.6	12.9	11.4	5.8	12.2
Unknown	3.6	2.5	2.6	2.7	10.2	5.9	7.9	10.3

*Data from Nedashkovskaya *et al.* (2004).

†Data from Yoon *et al.* (2005).

‡Summed feature 3 contained iso-C_{15:0} 2-OH and/or C_{16:1}ω7c, which could not be separated by GLC with the MIDI system.

genus *Maribacter*. Phenotypic characteristics, DNA–DNA relatedness values and protein profiles support the description of the three strains as a novel species, for which the name *Maribacter forsetii* is proposed.

Emended description of *Maribacter* Nedashkovskaya *et al.* 2004

This description is based on that given by Nedashkovskaya *et al.* (2004). Cells are rod-shaped, Gram-negative, non-flagellated and motile by gliding. Do not form endospores. Metabolism is strictly aerobic and heterotrophic. Yellow non-diffusible pigments that do not belong to the flexirubin type are produced. Cytochrome oxidase, catalase and alkaline phosphatase activities are present. Does not reduce nitrate to

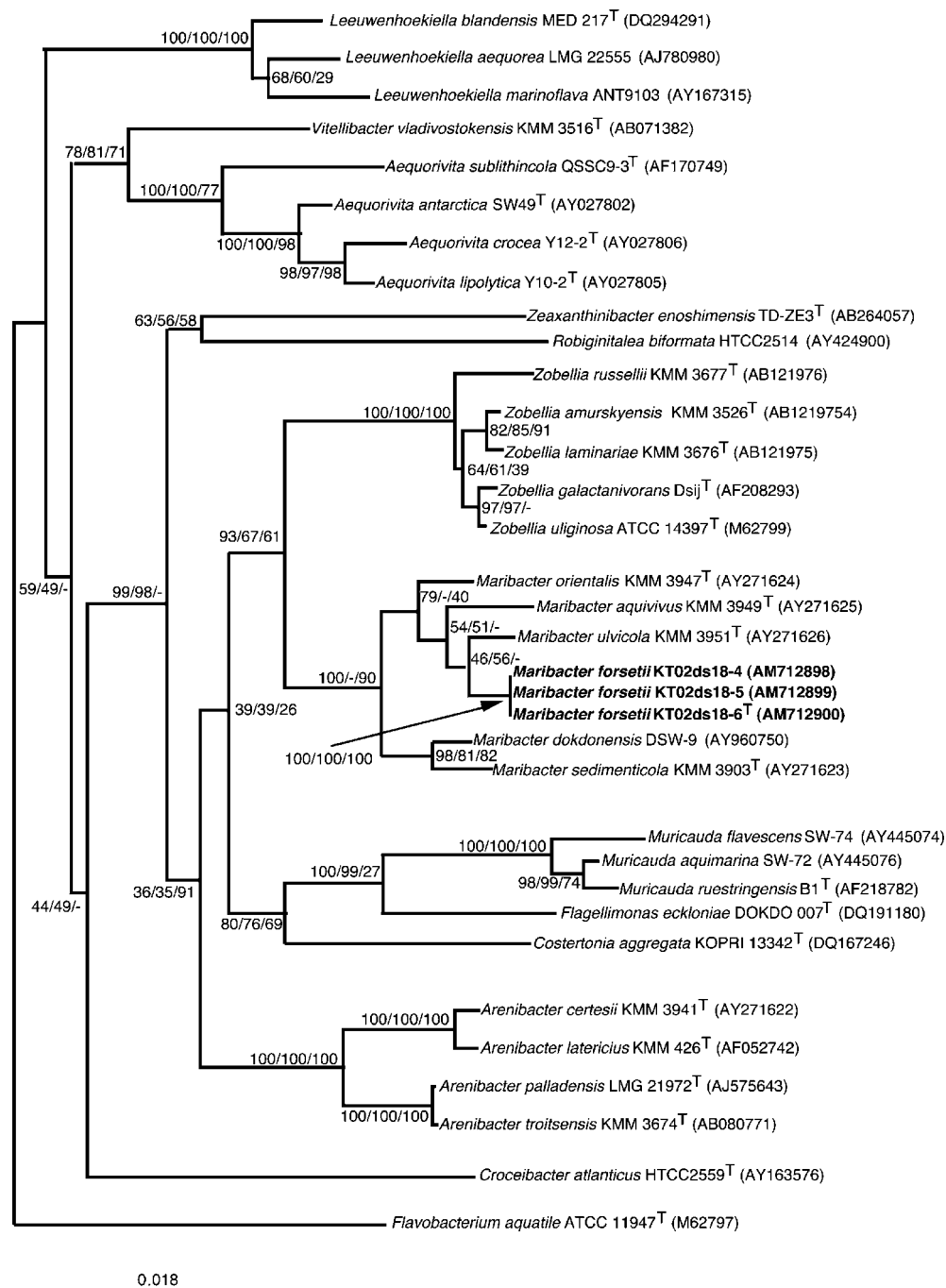


Fig. 3. Neighbour-joining tree showing the phylogenetic position of strains KT02ds18-4, KT02ds18-5 and KT02ds18-6^T, other *Maribacter* species and representatives of other genera of the family *Flavobacteriaceae*. GenBank accession numbers are given in parentheses. Numbers at the branching points refer to the bootstrap values (500 replicates) in distance, maximum-likelihood and maximum-parsimony analyses, respectively. Dashes instead of numbers indicate that the node was not observed in the corresponding analysis. *Flavobacterium aquatile* ATCC 11947^T was used as an outgroup. Bar, 0.018 substitutions per nucleotide position.

nitrite. The requirement for sodium salts is species-dependent. Predominant cellular fatty acids are straight-chain saturated, branched-chain saturated and unsaturated fatty acids iso-C_{15:0}, iso-C_{15:1}, C_{15:0} and iso-C_{17:0} 3-OH.

The main lipoquinone is MK-6. As determined by 16S rRNA gene sequence analysis, the genus *Maribacter* is a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*. The type species is *Maribacter sedimenticola*.

Description of *Maribacter forsetii* sp. nov.

Maribacter forsetii (for.set'ti.i. N.L. gen. masc. n. *forsetii*, of Forseti, a god in Scandinavian mythology that lived on Helgoland, the German island from where the bacterium was isolated).

Cells are non-flagellated, Gram-negative rods 0.3–0.5 µm wide and 3.0–4.0 µm long, occurring singly or in pairs, with rounded ends. Cells are non-motile in liquid phase, but show active gliding motility on solid surfaces. Colonies on ZoBell 2216E agar are circular, shiny, light yellow and 1.0–3.0 mm in diameter after incubation for 3 days. Spreading occurs at the margins of the colonies; the extent of spreading is strain-dependent. Optimal growth temperature is 25 °C; growth occurs between 4 and 32 °C. Optimal pH for growth is 7.5; growth occurs at pH 5.5–8.5, but not at pH 5.0 and 9.0. Optimal NaCl concentration for growth is 2.5% (w/v). Very weak growth occurs in the absence of NaCl and no growth occurs at concentrations >9% (w/v) NaCl. Under optimal growth conditions, the doubling time is about 3.0 h. Esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, alkaline and acid phosphatases, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and α-chymotrypsin activities are present, but β-glucuronidase activity is absent (API ZYM). Trypsin activity is strain-dependent (negative for the type strain). Alginate acid, Tween 40 and Tween 80 are hydrolysed, but agar, κ-carrageenan, ι-carrageenan, starch, gelatin, DNA, glycogen and inulin are not. D-Glucose, D-mannose, D-fructose, D-mannitol, D-lactose, methyl-β-D-glucoside, methyl-β-D-xyloside, N-acetyl-D-glucosamine, glucuronic acid, lactic acid, L-glutamic acid and L-ornithine are used as sole carbon sources, but D-arabinose, glycerol, amygdalin, acetic acid, citric acid, L-histidine, L-leucine, L-threonine and L-phenylalanine are not. Utilization of L-serine is strain-dependent (negative for the type strain). Acid is produced from D-glucose, D-mannose, D-fructose, D-galactose, D-arabinose, D-xylose, L-fucose, cellobiose, D-lactose, amygdalin and salicin, but not from L-arabinose, L-rhamnose and melezitose (API 50 CH). Acid production from gentiobiose is strain-dependent (positive for the type strain). Growth occurs in the presence of kanamycin (200 µg ml⁻¹) and tetracycline (10 µg ml⁻¹). Other characteristics are shown in Table 1. The DNA G + C content is 34.2–34.4 mol%.

The type strain, KT02ds18-6^T (=CIP 109504^T=DSM 18668^T), was isolated from North Sea surface water at station Kabeltonne (54°11' N; 7°54' E) near the island of Helgoland, Germany. Other reference strains are KT02ds18-4 (=CIP 109503=DSM 18666) and KT02ds18-5 (=CIP 109505=DSM 18667).

Acknowledgements

This work was performed in the frame of “Marine Genomics Europe” NoE 7 (EC contract No. GOCE-CT-2004-505403). We thank Mirjam Czjzek for helpful discussion and critical reading of the manuscript. The authors gratefully thank Olga I. Nedashkovskaya (Pacific Institute of Bioorganic Chemistry of the Far-Eastern Branch of the

Russian Academy of Sciences, Vladivostok, Russia) for providing them with the type strains of *Maribacter* spp. used in this study. We also thank Annie Cavalier and Daniel Thomas (Structure et Dynamique des Macromolécules de l'UMR Interactions Cellulaires et Moléculaires, University of Rennes, Brittany, France) for transmission electron microscopy, and Jörg Wulf for expert technical assistance.

References

- Abell, G. C. J. & Bowman, J. P. (2005). Ecological and biogeographic relationships of class *Flavobacteria* in the Southern Ocean. *FEMS Microbiol Ecol* **51**, 265–277.
- Barbeyron, T., Kean, C. & Forterre, P. (1984). DNA Adenine methylation of GATC sequences appeared recently in the *Escherichia coli* lineage. *J Bacteriol* **160**, 586–590.
- Barbeyron, T., L'Haridon, S., Corre, E., Kloareg, B. & Potin, P. (2001). *Zobellia galactanovorans* gen. nov., sp. nov., a marine species of *Flavobacteriaceae* isolated from a red alga, and classification of [*Cytophaga*] *uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Zobellia uliginosa* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **51**, 985–997.
- Bauer, M., Kube, M., Teeling, H., Richter, M., Lombardot, T., Allers, E., Würdemann, C. A., Quast, C., Kuhl, H. & other authors (2006). Whole genome analysis of the marine *Bacteroidetes* ‘*Gramella forsetii*’ reveals adaptations to degradation of polymeric organic matter. *Environ Microbiol* **8**, 2201–2213.
- Benson, D. A., Boguski, M. S., Lipman, D. J., Ostell, J., Ouellette, B. F., Rapp, B. A. & Wheeler, D. L. (1999). GenBank. *Nucleic Acids Res* **27**, 12–17.
- Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996). Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatidis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128–148.
- Bernardet, J.-F., Nakagawa, Y. & Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Bacteriol* **52**, 1049–1070.
- DeLong, E. F., Preston, C. M., Mincer, T., Rich, V., Hallam, S. J., Frigaard, N. U., Martinez, A., Sullivan, M. B., Edwards, R. & other authors (2006). Community genomics among stratified microbial assemblages in the ocean's interior. *Science* **311**, 496–503.
- Doetsch, R. N. (1981). Determinative methods of light microscopy. In *Manual of Methods for General Bacteriology*, pp. 21–33. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Eilers, H. (2000). *Pelagische Bakteriengesellschaften in der Nordsee: Kultivierung, Diversität und in situ Dynamik*. PhD thesis, Universität Bremen, Bremen, Germany.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Galtier, N., Gouy, M. & Gautier, C. (1996). SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *Comput Appl Biosci* **12**, 543–548.

- Gascuel, O. (1997).** BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Mol Biol Evol* **14**, 685–695.
- Glöckner, F.-O., Fuchs, B. M. & Amann, R. (1999).** Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. *Appl Environ Microbiol* **65**, 3721–3726.
- Hicks, R. E., Amann, R. I. & Stahl, D. A. (1992).** Dual staining of natural bacterioplankton with 4',6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. *Appl Environ Microbiol* **58**, 2158–2163.
- Hugh, R. & Leifson, E. (1953).** The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various negative bacteria. *J Bacteriol* **66**, 24–26.
- Ivanova, T. L., Turova, T. P. & Antonov, A. S. (1988).** DNA–DNA hybridization studies on some purple non sulfur bacteria. *Syst Appl Microbiol* **10**, 259–263.
- Johnson, J. L. (1984).** DNA reassociation and RNA Hybridization of bacterial nucleic acids. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 8–11. Edited by N. R. Krieg & J. G. Holt. Baltimore, USA: Williams & Wilkins.
- Kane, M. D., Poulsen, L. K. & Stahl, D. A. (1993).** Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. *Appl Environ Microbiol* **59**, 682–686.
- Kimura, M. (1980).** A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kirchman, D. L. (2002).** The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* **39**, 91–100.
- Kristjánsson, J. K., Hjörleifsdóttir, S., Marteinson, V. T. & Alfredsson, G. A. (1994).** *Thermus scotoductus*, sp. nov., a pigment-producing thermophilic bacterium from hot tap water in Iceland and including *Thermus* sp. X-1. *Syst Appl Microbiol* **17**, 44–50.
- Nedashkovskaya, O. I., Suzuki, M., Vysotskii, M. V. & Mikhailov, V. V. (2003).** *Arenibacter troitsensis* sp. nov., isolated from marine bottom sediment. *Int J Syst Evol Microbiol* **53**, 1287–1290.
- Nedashkovskaya, O. I., Kim, S. B., Han, S. K., Lysenko, A. M., Rohde, M., Rhee, M.-S., Frolova, G. M., Falsen, E., Mikhailov, V. V. & Bae, K. S. (2004).** *Maribacter* gen. nov., a new member of the family *Flavobacteriaceae*, isolated from marine habitats, containing the species *Maribacter sedimenticola* sp. nov., *Maribacter aquivivus* sp. nov., *Maribacter orientalis* sp. nov. and *Maribacter ulvicola* sp. nov. *Int J Syst Evol Microbiol* **54**, 1017–1023.
- Reichenbach, H., Kleinig, H. & Achenbach, H. (1974).** The pigment of *Flexibacter elegans*: novel and chemosystematically useful compounds. *Arch Microbiol* **101**, 131–144.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990).** *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Schut, F., De Vries, E. J., Gottschal, J. C., Robertson, B. R., Harder, W., Prins, R. A. & Button, D. K. (1993).** Isolation of typical marine bacteria by dilution culture growth maintenance and characteristics of isolates under laboratory conditions. *Appl Environ Microbiol* **59**, 2150–2159.
- Smibert, R. M. & Krieg, N. R. (1981).** General characterization. In *Manual of Methods for General Microbiology*, pp. 409–443. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Yoon, J.-H., Kang, S.-J., Lee, S.-Y., Lee, C.-H. & Oh, T.-K. (2005).** *Maribacter dokdonensis* sp. nov., isolated from sea water off a Korean island, Dokdo. *Int J Syst Evol Microbiol* **55**, 2051–2055.
- ZoBell, C. E. (1941).** Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *J Mar Res* **4**, 41–75.