Maribacter stanieri sp. nov., a marine bacterium of the family *Flavobacteriaceae*

Olga I. Nedashkovskaya,¹ Seung Bum Kim² and Valery V. Mikhailov¹

Correspondence

Olga I. Nedashkovskaya
olganedashkovska@piboc.dvo.ru
or olganedashkovska@gmail.com

¹Pacific Institute of Bioorganic Chemistry of the Far-Eastern Branch of the Russian Academy of Sciences, Pr. 100 Let Vladivostoku 159, 690022, Vladivostok, Russia

²Department of Microbiology, Chungnam National University, 220 Gung-dong, Yusong, Daejeon 305-764, Republic of Korea

The taxonomic status of two novel heterotrophic, Gram-negative, gliding and yellow pigmented bacterial strains was established in this study. Phylogenetic analysis based on 16S rRNA gene sequencing revealed that the strains formed a distinct lineage within the genus *Maribacter*, a member of the family *Flavobacteriaceae*, with sequence similarities of 96.3–98.5% to recognized species of the genus *Maribacter*. The maximum growth temperature of the strains was 35 °C and they required NaCl or seawater for growth. They hydrolysed aesculin and gelatin, reduced nitrates to nitrites and produced acid from carbohydrates. The DNA G + C contents of strains KMM 6025 and KMM 6046^T were 36–37 mol%. On the basis of phenotypic, genotypic and phylogenetic characteristics, it is suggested that the new isolates represent a novel species of the genus *Maribacter*, for which the name *Maribacter stanieri* sp. nov. is proposed. The type strain is KMM 6046^T (=KCTC 22023^T=LMG 22581^T).

The genus *Maribacter* was created by Nedashkovskaya *et al.* (2004) to accommodate aerobic and obligatory marine bacteria that move by gliding and form a distinct phylogenetic lineage within the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). The description of the genus was recently emended because it was found that some newly isolated strains did not require Na⁺ ions for growth (Barbeyron *et al.*, 2008). At the time of writing, the genus *Maribacter* comprised eight recognized species (Barbeyron *et al.*, 2008; Cho *et al.*, 2008; Nedashkovskaya *et al.*, 2004, 2007; Yoon *et al.*, 2005). Most species of the genus *Maribacter* are free-living organisms, while strains of two species are associated with green and red algae. Environmental clones identified as belonging to the genus *Maribacter* have been obtained from seawater, sea ice and algae collected from the subantarctic regions of Argentina, from the Inland Sea of Japan and from the north-western Mediterranean Sea (Prabakaran *et al.*, 2007; Hamasaki *et al.*, 2007; Agogué *et al.*, 2005).

In a course of a survey of the biodiversity of coastal microbial communities inhabiting the Sea of Japan (also known as the East Sea), we discovered two novel, yellow pigmented isolates. A study of their phylogenetic and phenotypic characteristics revealed that strains KMM 6025 and KMM 6046^T belonged to the family *Flavobacteriaceae* and clustered with the species of the genus *Maribacter*, amongst which they formed a distinct lineage.

Strain KMM 6025 was isolated from a seawater sample, while strain KMM 6046^T was obtained from the green alga *Ulva fenestrata* collected from the Gulf of Peter the Great of the Sea of Japan. For strain isolation, 0.1 ml seawater or algal tissue homogenate were transferred onto plates of marine agar 2216 (Difco). After primary isolation, the strains were cultivated at 28 °C on marine agar (Difco) and stored at –80 °C in marine broth (Difco) supplemented with 20% (v/v) glycerol.

The phylogenetic position of strains KMM 6025 and KMM 6046^T was determined from their almost-complete 16S rRNA gene sequences (1434 and 1432 bp, respectively). Genomic DNA extraction, PCR and sequencing of 16S rRNA genes were performed by using previously described procedures (Cho *et al.*, 2006). The sequences obtained were aligned with those of representative members of selected genera belonging to the family *Flavobacteriaceae* by using PHYDIT version 3.1 (<http://plaza.snu.ac.kr/~jchun/phydit/>). Phylogenetic trees were inferred by using appropriate programs from the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated from the two-parameter model (Kimura, 1980) and the trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1993) and maximum-parsimony (Kluge & Farris, 1969) algorithms. Bootstrap analysis was performed with 1000 resampled datasets, using the SEQBOOT and CONSENSE programs of the PHYLIP package.

Phylogenetic analysis of the almost-complete 16S rRNA gene sequences of strains KMM 6025 and KMM 6046^T

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of *Maribacter stanieri* KMM 6025 and KMM 6046^T are EF536746 and EU246691, respectively.

0.01

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of species of the genus *Maribacter* and related taxa. The tree was constructed using Kimura's two-parameter model and the neighbour-joining algorithm. Asterisks indicate branches that were also recovered in the maximum-likelihood and maximum-parsimony trees. The numbers at nodes indicate levels of the bootstrap support (%). Bar, 0.01 nucleotide substitutions per position.

revealed that the strains formed a distinct evolutionary lineage within the genus *Maribacter*, a member of the family *Flavobacteriaceae* (Fig. 1). The nearest neighbours of the novel isolates were *Maribacter aquivivus* KMM 3949^T (98.3–98.5% sequence similarity) and *Maribacter orientalis* KMM 3947^T (98.2–98.4%). Sequence similarities of strains KMM 6025 and KMM 6046^T to other species of the genus *Maribacter* were in the range 96.3–97.9%. The sequence similarity between the two strains studied was 99.6%.

For determination of the DNA base composition, DNA was isolated following the technique of Marmur (1961) and the DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962). The DNA G+C contents of KMM 6025 and KMM 6046^T were 36.1 and 36.6 mol%, respectively. DNA–DNA hybridization was performed spectrophotometrically and initial renaturation rates were recorded as described by De Ley *et al.* (1970). The level of DNA–DNA hybridization between strains KMM 6025 and KMM 6046^T was 96%, demonstrating that the two novel isolates belonged to the same species according to the proposal of Wayne *et al.* (1987). The level of DNA–DNA

reassociation between strain KMM 6046^T and recognized species of the genus *Maribacter* ranged from 34 to 51%. Based on these data, the new isolates can be affiliated to the genus *Maribacter* as a distinct and novel species.

Analysis of fatty acid methyl esters was carried out on cells grown on marine agar at 28 °C for 48 h according to the standard protocol of the Microbial Identification System (Microbial ID Inc.). The dominant fatty acids of strain KMM 6046^T were iso-C_{15:1} G, iso-C_{15:0}, C_{15:0}, iso-C_{17:1} ω9c, iso-C_{17:0} 3-OH and summed feature 3, comprising C_{16:1} ω7c and/or iso-C_{15:0} 2-OH (Table 1).

The physiological and biochemical properties of strains KMM 6025 and KMM 6046^T were tested as described by Nedashkovskaya *et al.* (2004, 2006). API 20E, API 20NE and API ZYM galleries (bioMérieux) were also used in accordance with the manufacturer's instructions.

Strains KMM 6025 and KMM 6046^T were heterotrophic, aerobic, Gram-negative, yellow pigmented organisms that were motile by gliding. They demonstrated many features that are characteristic for representatives of the genus *Maribacter* (Table 1). However, strains KMM 6025 and KMM 6046^T differed from their closest relatives, *Maribacter aquivivus* and *Maribacter orientalis*, by their maximum growth temperature, acetoin production, raffinose oxidation and utilization of mannitol and citrate (Table 2). Additional properties such as hydrolysis of Tweens 20 and 80, acid production from cellobiose, galactose, glucose, lactose, maltose, melibiose and sucrose also separated the novel isolates from *Maribacter aquivivus*. Strains KMM 6025 and KMM 6046^T could also be separated from *Maribacter orientalis* on the basis of the maximum concentration of NaCl for growth, by its ability to reduce nitrate to nitrite, to oxidize and to utilize L-arabinose and the DNA G+C contents. Other trends that may help in the differentiation of the novel strains from recognized species of the genus *Maribacter* are shown in Table 2. The novel isolates demonstrated several differences from each other. Thus, KMM 6025 and KMM 6046^T grow with 1–8 and 0.5–6% NaCl and at temperatures of up to 33 and 35 °C, respectively. In contrast to strain KMM 6025, the type strain is able to hydrolyse DNA and to oxidize L-rhamnose.

In conclusion, the phylogenetic and phenotypic distinctiveness between strains KMM 6025 and KMM 6046^T and other species of the genus *Maribacter* clearly indicated that they represent a novel species of the genus, for which the name *Maribacter stanieri* sp. nov. is proposed.

Originally, members of the genus *Maribacter* were described as bacteria that required Na⁺ ions for growth (Nedashkovskaya *et al.*, 2004). Recently, the genus description was emended because strains of a novel species, *Maribacter forsetii*, demonstrated weak growth on nutrient media containing no NaCl (Barbeyron *et al.*, 2008). The inability of *Maribacter* strains to reduce nitrate to nitrite was also included in the emended description of the genus. Production of nitrate reductase by *Maribacter forsetii* and

Table 1. Whole-cell fatty acid profiles (%) of species of the genus *Maribacter*

Species: 1, KMM 6046^T; 2, *Maribacter sedimenticola* KMM 3903^T; 3, *Maribacter aquivivus* KMM 3949^T; 4, *Maribacter dokdonensis* KCTC 12393^T; 5, *Maribacter forsetii* KT02ds18-6^T; 6, *Maribacter orientalis* KMM 3947^T; 7, *Maribacter polysiphoniae* KMM 6151^T; 8, *Maribacter ulvicola* KMM 3951^T. Only those fatty acids amounting to >1.0% in one of the strains listed are indicated. Summed feature 3 consisted of one or more of the following fatty acids which could not be separated by the Microbial Identification System: iso-C_{15:0} 2-OH and C_{16:1}ω7c. Summed feature 4 consisted of one of the following fatty acids: C_{17:1} iso I and C_{17:1} anteisoB. Data are taken from Barbeyron *et al.* (2008), Nedashkovskaya *et al.* (2004, 2007), Yoon *et al.* (2005) and this study.

Fatty acid	1	2	3	4	5	6	7	8
C _{13:1}	0.6–1.0	0.7	1.1	—	—	0.3	—	1.1
iso-C _{14:0}	—	—	—	—	0.4	—	1.4	—
C _{14:0}	0.6	0.5	1.0	0.9	0.4	0.5	—	0.9
C _{14:1} ω5	—	—	1.0	—	—	—	—	0.9
iso-C _{15:0}	10.4–14.0	20.5	12.3	19.7–20.3	11.9–12.5	10.6	15.4	13.6
anteiso-C _{15:0}	2.4–2.5	1.2	1.3	0.3–0.4	1.0	2.3	7.6	1.9
iso-C _{15:1} G	15.2–16.0	16.9	13.6	15.7–16.1	11.1–11.7	10.1	9.0	18.9
C _{15:0}	6.1–9.9	6.3	14.5	3.5–3.8	14.7–15.5	12.3	11.0	8.1
C _{15:1} ω6c	1.6–2.1	1.7	4.8	0.6	2.8	2.5	3.9	1.6
iso-C _{16:1} H	0–0.2	—	—	—	0.7–0.9	—	1.4	—
iso-C _{16:0}	0.4–0.7	1.1	0.7	—	1.6–1.7	0.3	1.8	0.3
C _{16:0}	0.6	1.0	0.5	1.3–1.4	1.0	1.2	1.0	1.0
iso-C _{17:1} ω5c	—	—	1.4	—	—	—	—	1.2
iso-C _{17:1} ω9c	7.5–8.6	2.3	2.2	2.0–2.2	4.1–5.0	4.0	5.4	2.2
C _{17:1} ω8c	0–0.3	—	—	—	0.8	—	1.2	—
C _{17:1} ω6c	0.9–1.0	0.5	1.7	0.2	1.1–1.2	1.3	1.4	0.5
iso-C _{15:0} 3-OH	3.5–3.6	5.4	3.2	5.0	3.7–3.8	2.9	5.0	4.1
C _{15:0} 3-OH	—	2.4	2.3	1.4	1.4–1.5	1.5	2.1	1.5
iso-C _{16:0} 3-OH	0.7–0.9	1.7	2.5	1.0–1.1	5.9–6.8	2.1	9.1	1.7
C _{16:0} 3-OH	0.9–1.2	2.2	2.9	5.4–5.5	1.8–2.2	3.0	0.5	3.7
iso-C _{17:0} 3-OH	11.1–11.9	20.4	11.6	28.6–29.2	18.3–19.7	18.8	7.2	14.5
C _{17:0} 2-OH	0.3–0.4	—	—	—	0–0.3	—	1.4	—
iso-C _{18:1}	—	—	—	—	—	2.4	—	—
Summed feature 3	11.4–14.8	5.8	12.9	8.4–8.6	10.1–11.0	11.4	10.1	12.2
Summed feature 4	0–1.2	—	—	—	—	—	—	—

by the type strains of other *Maribacter* species was tested only by using the API 20NE gallery but it is already known that the API system gives negative results for flavobacteria and therefore this phenotypic feature needs to be checked using other classical methods. The species *Maribacter aquivivus* and *Maribacter sedimenticola* were firstly reported as nitrate reducers (Nedashkovskaya *et al.*, 2004). The type strain of *Maribacter arcticus* also reduces nitrate to nitrite (Cho *et al.*, 2008). Strains KMM 6025 and KMM 6046^T did not show the presence of nitrate reductase when the API 20NE gallery was used, but production of this enzyme was found by the method described by Gerhardt *et al.* (1994). This information is included in an emended description of the genus as proposed below.

Emended description of the genus *Maribacter* Nedashkovskaya *et al.* 2004 emend. Barbeyron *et al.* 2008

This description is based on that given by Nedashkovskaya *et al.* (2004) and Barbeyron *et al.* (2008) except that strains can reduce nitrate to nitrite. Arginine dihydrolase, lysine-

and ornithine-decarboxylases and tryptophan deaminase activities are absent.

Description of *Maribacter stanieri* sp. nov.

Maribacter stanieri (sta'ni.er.i. N.L. gen. n. *stanieri* named in honour of the famous Canadian microbiologist Roger Y. Stanier for his great contribution to development of marine microbiology and, especially, to the investigation of the taxonomy and ecology of *Cytophaga*-like bacteria, currently the phylum *Bacteroidetes*).

Cells are strictly aerobic, heterotrophic, motile by gliding, Gram-negative, rod-shaped and 0.3–0.5 µm wide and 2.4–5.3 µm long. On marine agar, colonies are circular, yellow pigmented, shiny with entire edges and 2–4 mm in diameter. No flexirubin-type pigments are formed. Growth occurs with 0.5–8% NaCl, at 4–35 °C and at pH 5.5–10.0. Optimal growth is observed with 0.5–2% NaCl, at 23–28 °C and at pH 7.0–7.5. Oxidase, catalase, β-galactosidase and alkaline phosphatase activities are present. Decomposes aesculin, gelatin, Tweens 20 and 40.

Table 2. Phenotypic characteristics of species of the genus *Maribacter*

Species: 1, KMM 6025 and KMM 6046^T; 2, *Maribacter aquivivus*; 3, *Maribacter arcticus*; 4, *Maribacter dokdonensis*; 5, *Maribacter forsetii*; 6, *Maribacter orientalis*; 7, *Maribacter polysiphoniae*; 8, *Maribacter sedimenticola*; 9, *Maribacter ulvicola*. All strains are positive for the following characteristics: respiratory metabolism; gliding motility and oxidase, catalase and alkaline phosphatase activities. All strains are negative for the following characteristics: production of flexirubin type pigments; hydrolysis of urea, CM-cellulose and chitin, and production of indole and H₂S. Data from Barbeyron *et al.* (2008); Cho *et al.* (2008); Nedashkovskaya *et al.* (2004, 2007) and Yoon *et al.* (2005). w, Weakly positive reaction; v, variable reaction; ND, not detected; +, positive; –, negative.

Characteristic	1	2	3	4	5	6	7	8	9
Temperature range for growth (°C)	4–35	4–30	4–30	4–35	4–32	4–32	4–41	4–33	4–32
Salinity range for growth (%)	1.5–8	1–7	1–7	1–10	0–9	1–5	1–8	1–6	1–4
Requirement for Ca ²⁺ or Mg ²⁺ ions for growth	–	–	+	–	–	–	–	–	–
Nitrate reduction	+	+	+	–	–	–	–	+	–
β -Galactosidase activity	+	+	–	–	+	+	+	–	+
Acetoin production	w	–	+	–	ND	–	w	–	–
Hydrolysis of:									
Agar	–	+	–	+	–	–	–	+	+
Gelatin	+	+	–	–	–	+	+	–	–
Starch	–	–	–	–	–	–	+	+	–
DNA	v	+	ND	ND	–	–	–	–	–
Tween 20	+	–	ND	+	ND	+	+	+	+
Tween 40	+	+	–	+	+	+	+	+	+
Tween 80	–	+	–	+	+	–	+	+	+
Acid production from:									
L-Arabinose	–	–	–	+	–	+	+	+	–
Cellobiose, D-glucose, D-lactose, maltose, DL-xylose	+	–	–	+	+	+	+	–	+
L-Fucose	+	?	–	+	+	?	?	ND	?
D-Galactose	+	–	–	+	+	+	+	–	–
Melibiose	+	–	–	+	v	+	+	–	–
Raffinose	+	–	–	+	v	–	+	–	–
L-Rhamnose	v	–	–	–	–	–	–	–	+
Sucrose	+	–	–	+	+	+	+	–	+
N-acetylglucosamine	–	–	–	–	–	–	+	–	–
Mannitol	–	–	–	+	–	–	+	–	–
Utilization of:									
L-Arabinose	–	–	–	+	ND	+	+	+	–
D-Glucose, D-mannose	+	+	–	+	+	+	+	–	+
D-Lactose, sucrose	+	+	ND	+	+	+	+	–	+
Mannitol	+	–	–	+	+	–	+	–	–
Citrate	+	–	–	–	ND	–	–	–	–
DNA G + C content (mol%)	36–37	35.0	36.0	35–37	34–35	39.0	41.1	37.0	35–37

Does not hydrolyse agar, casein, starch, Tween 80, CM-cellulose, urea or chitin. Hydrolysis of DNA is strain-specific. Produces acid from cellobiose, L-fucose, D-galactose, D-glucose, D-lactose, maltose, melibiose, raffinose, sucrose, DL-xylose, but not from L-arabinose, N-acetylglucosamine, citrate, adonitol, dulcitol, glycerol, inositol or mannitol. Oxidation of L-rhamnose is strain-dependent. Utilizes arabinose and mannose, but not inositol or sorbitol. According to the API 20NE gallery, mannitol, N-acetylglucosamine, malate, gluconate, adipate and citrate are also utilized. Testing by using API ZYM gallery indicated that the type strain produces esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, α -chymotrypsin, acid phosphatase,

naphthol-AS-BI-phosphohydrolase, α -galactosidase, α - and β -glucosidases, N-acetyl- β -glucosidase and α -mannosidase activities, but lipase (C14), cystine arylamidase, β -glucuronidase and α -fucosidase are absent. Nitrate is reduced. Acetoin (Voges–Proskauer reaction) is produced weakly, but H₂S and indole are not. Susceptible to carbenicillin, chloramphenicol, erythromycin, doxycycline, lincomycin, neomycin, oleandomycin, streptomycin and tetracycline, resistant to ampicillin, benzylpenicillin, gentamicin, kanamycin and polymixin. The predominant fatty acids are iso-C_{15:1} G, iso-C_{15:0}, C_{15:0}, iso-C_{17:1} ω 9c, iso-C_{17:0} 3-OH and summed feature 3, comprising C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH. The DNA G + C content is 36–37 mol%.

The type strain, KMM 6046^T (=KCTC 22023^T=LMG 22581^T), was isolated from the green alga *Ulva fenestrata*, collected in Troitsa Bay, Gulf of Peter the Great, Sea of Japan (also known as the East Sea).

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