Pseudozobellia thermophila gen. nov., sp. nov., a bacterium of the family *Flavobacteriaceae*, isolated from the green alga *Ulva fenestrata*

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Two novel aerobic, dark-orange-pigmented, Gram-negative bacterial strains, designated KMM 3531^T and KMM 3953, were isolated from the green alga *Ulva fenestrata*. Analysis of the 16S rRNA gene sequences showed that the strains represented a novel lineage within the family *Flavobacteriaceae*. The most closely related genera with validly published names were *Costertonia*, *Flagellimonas*, *Kriegella*, *Maribacter*, *Muricauda* and *Zobellia*, with 16S rRNA gene sequence similarities of 93.3–91.8%. Cells of strains KMM 3531^T and KMM 3953 were rod-shaped, motile by gliding and grew at temperatures up to 49 °C. They produced acid from carbohydrates and possessed oxidase, catalase, β -galactosidase and agarase activities. The predominant cellular fatty acids were iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{15:1} G, summed feature 3 (comprising C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH), iso-C_{17:1} ω 9c and iso-C_{15:0} 3-OH. The DNA G + C content was 47–49 mol%. On the basis of phenotypic and genotypic characteristics, strains KMM 3531^T and KMM 3953 represent a novel genus and species, for which the name *Pseudozobellia thermophila* gen. nov., sp. nov. is proposed. The type strain is KMM 3531^T (=DSM 19858^T=JCM 11733^T=KCTC 22016^T).

Members of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002) (www.bacterio.cict.fr/f/flavobacteriaceae.html) are commonly recovered from the surfaces of various algae inhabiting coastal waters. The green algae belonging to the genus *Ulva* harbour strains affiliated with bacterial taxa such as *Cellulophaga*, *Flavobacterium*, *Gelidibacter* and *Salegentibacter* (Lam & Harder, 2007; Marshall *et al.*, 2006; Nakanishi *et al.*, 1996).

During a study of the taxonomic diversity of the bacterial epiphytes of *Ulva fenestrata* collected from Posiet Bay of the Sea of Japan (also known as the East Sea), two novel strains were isolated and their taxonomic positions were analysed using a polyphasic approach.

For strain isolation, 0.1 ml algal tissue homogenate was plated on marine agar 2216 (Difco). After primary isolation and purification, the strains were cultivated at 28 °C on the same medium and stored at –80 °C in marine broth 2216 (Difco) supplemented with 20% (v/v) glycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of strains KMM 3531^T and KMM 3953 are AB084261 and EU616814, respectively.

An almost-complete 16S rRNA gene sequence of isolate KMM 3531^T was determined using PCR amplification and direct sequencing (Hiraishi, 1992). The conditions and reagents used for the PCR amplification and sequencing of the 16S rRNA gene were as described previously (Suzuki *et al.*, 2001). The sequence was aligned on the secondary-structure model maintained by the European small subunit ribosomal RNA database (Van de Peer *et al.*, 2000), using the profile-alignment program of CLUSTAL W (Thompson *et al.*, 1994). Evolutionary distances were then computed with MEGA, version 3.1 (Kumar *et al.*, 2004), using the two-parameter model (Kimura, 1980). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. A maximum-likelihood analysis was also performed, using fastDNAm1 (Felsenstein, 1981). To evaluate the topology of the neighbour-joining phylogenetic tree, a bootstrap analysis based on 1000 replicates was performed using the SEQBOOT and CONSENSE programs in the PHYLIP 3.572 package (<http://plaza.snu.ac.kr/~jchun/phydit/>). Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene of strain KMM 3953 and sequencing of purified

PCR products were carried out as described by Rainey *et al.* (1996). The 16S rRNA gene sequence was aligned with published sequences retrieved from EMBL, using CLUSTAL_X (Thompson *et al.*, 1997) and edited using BioEdit (Hall, 1999).

Analysis of the 16S rRNA gene sequences of strains KMM 3531^T and KMM 3953 (1401 and 1403 nt, respectively) revealed that the strains belong to the family *Flavobacteriaceae*, forming a phylogenetic cluster with the genera *Costertonia*, *Flagellimonas*, *Muricauda*, *Kriegella*, *Zobellia* and *Maribacter* (Fig. 1). The 16S rRNA gene sequence similarity between strains KMM 3531^T and KMM 3953 was 100 %. *Kriegella aquimaris* KMM 3665^T was the nearest neighbour of the novel isolates, with a sequence similarity of 93.3 %. The 16S rRNA gene sequence similarities between strain KMM 3531^T and other close relatives were in the range 91.4–92.5 %. However, according to the topology of the neighbour-joining tree, *Costertonia aggregata* KOPRI 13342^T, *Flagellimonas eckloniae* KCCM 42307^T and type strains of *Muricauda* species were more closely related to strain KMM 3531^T than was *K. aquimaris* KMM 3665^T. The maximum-likelihood phylogenetic tree showed essentially the same topology (data not shown). Therefore, the two novel strains can be assigned to the family *Flavobacteriaceae*, in which they form a distinct lineage representing a separate genus, according to the criteria outlined by Stackebrandt & Goebel (1994).

DNA was isolated by following the method of Marmur (1961) and the DNA G+C content was determined by means of the thermal denaturation method (Marmur & Doty, 1962). The DNA G+C contents of strains KMM

3531^T and KMM 3953 were 49.0 and 47.9 mol%, respectively.

The DNA–DNA relatedness between the two strains was determined spectrophotometrically and initial renaturation rates were recorded as described by De Ley *et al.* (1970). The DNA–DNA relatedness between strains KMM 3531^T and KMM 3953 was 93 %: on the basis of the criteria of Wayne *et al.* (1987), this value confirms that the two strains represent the same species.

To compare the whole-cell fatty acid profiles of the novel strains and their nearest neighbours, strains KMM 3531^T, KMM 3953, *Costertonia aggregata* KOPRI 13342^T and *Flagellimonas eckloniae* DOKDO 007^T were grown under the same conditions [i.e. on marine agar 2216 (Difco) at 28 °C for 48 h] and their fatty acid compositions were analysed according to the standard protocol of the Microbial Identification System (Microbial ID). The fatty analysis of *K. aquimaris* KMM 3665^T and the type strains of species of the genus *Zobellia* had been performed using the same procedure, except that the bacteria had been grown for 24 h (Nedashkovskaya *et al.*, 2004b, 2008).

The predominant cellular fatty acids (i.e. constituting ≥5 % of total fatty acids) of strains KMM 3531^T and KMM 3953 were the straight-chain and branched-chain saturated and unsaturated fatty acids iso-C_{15:0} (30.8 %), iso-C_{17:0} 3-OH (22.6 %), iso-C_{15:1} G (14.8 %), summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 6.9 %), iso-C_{17:1}ω9c (5.4 %) and iso-C_{15:0} 3-OH (5.2 %) (Table 1). Isoprenoid quinones were extracted and analysed using the method of Nakagawa & Yamasato (1993). The major isoprenoid quinone in both strains was

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain KMM 3531^T and type strains of recognized members of the family *Flavobacteriaceae*. The tree was generated using the neighbour-joining method (Saitou & Nei, 1987). The 16S rRNA gene sequence of *Flavobacterium aquatile* ATCC 11947^T (M62797) was used as an outgroup. Numbers at nodes indicate bootstrap percentages (based on 1000 replicates); only values >50 % are shown. Filled circles indicate nodes that were also recovered in the tree generated using the maximum-parsimony method (Fitch, 1971). Bar, genetic distance of 0.01 K_{nuc}.

Table 1. Whole-cell fatty acid compositions (%) of strains KMM 3531^T and KMM 3953 (*Pseudozobellia thermophila* gen. nov., sp. nov.) and closely related members of the family *Flavobacteriaceae*

Strains: 1, KMM 3531^T; 2, KMM 3953; 3, *K. aquimaris* KMM 3665^T; 4, *Costertonia aggregata* KOPRI 13342^T; 5, *Flagellimonas eckloniae* DOKDO 007^T; 6, the type strains of the genus *Zobellia* (*Z. amurskyensis* KMM 3526^T, *Z. galactanivorans* Dsij^T, *Z. laminariae* KMM 3676^T, *Z. russellii* KMM 3677^T and *Z. uliginosa* CIP 104808^T). Fatty acids constituting <1% (mean) of the total in all taxa are not shown. Data are from Bae *et al.* (2007), Kwon *et al.* (2006), Nedashkovskaya *et al.* (2004b, 2008) and this study. All strains were analysed using the same procedure and grown under the same conditions, except that strain KMM 3665^T and strains of the genus *Zobellia* were grown for 24 h. Members of the genus *Muricauda* have not been included in the table because they were grown under different culture conditions. tr, Trace amount (<1%); –, not detected.

Fatty acid	1	2	3	4	5	6
Straight-chain fatty acids						
C _{14:0}	–	–	–	–	–	0–1.0
C _{15:0}	3.0	2.8	11.1	7.8	5.6	7.5–14.4
C _{15:1ω6c}	tr	tr	2.5	tr	tr	1.1–3.2
C _{16:0}	tr	tr	tr	4.1	1.4	0.7–2.6
C _{16:0} 3-OH	tr	tr	tr	2.5	1.0	2.4–4.9
C _{17:1ω6c}	–	–	–	–	–	0–1.2
C _{18:0}	–	–	tr	–	1.2	–
C _{18:1ω6c}	–	–	–	–	–	0–1.1
Branched fatty acids						
anteiso-C _{15:0}	tr	tr	tr	1.2	tr	0–1.8
iso-C _{15:0}	30.8	33.4	12.3	17.0	26.0	16.8–22.5
iso-C _{15:0} 3-OH	5.2	4.4	3.2	4.5	4.3	4.6–8.3
iso-C _{15:1} G	14.8	16.1	19.7	11.2	18.3	8.8–14.9
iso-C _{16:0} 3-OH	tr	tr	tr	1.8	1.4	–
iso-C _{17:0}	–	–	–	1.5	–	–
iso-C _{17:0} 3-OH	22.6	20.7	12.8	22.7	25.1	15.1–25.9
iso-C _{17:1ω9c}	5.4	4.8	6.4	6.1	2.9	2.4–5.1
Summed features*						
3	6.9	6.4	9.4	12.0	4.5	9.9–15.5
4	–	tr	1.0	–	–	–

*Summed features are fatty acids that could not be separated using the MIDI system. Summed feature 3 consists of C_{16:1ω7c} and/or iso-C_{15:0} 2-OH. Summed feature 4 consists of iso-C_{17:1} I and/or anteiso-C_{17:1} B.

MK-6, in line with all members of the family *Flavobacteriaceae*.

Physiological and biochemical features of strains KMM 3531^T and KMM 3953 were tested as described by Nedashkovskaya *et al.* (2004a, b) and by using API 20E, API 20NE and API ZYM galleries (bioMérieux) according to the manufacturer's instructions (except that the incubation temperature was 28 °C).

The novel isolates were heterotrophic, aerobic, dark-orange-coloured, Gram-negative organisms. Their main physiological and biochemical characteristics are given in Table 2 and in the genus and species descriptions. Cells were motile by gliding, grew at temperatures of up to 49 °C and decomposed agar, gelatin and DNA. In addition, strains KMM 3531^T and KMM 3953 were characterized by the production of flexirubin-type pigments. The two strains differed from each other in several respects: in contrast to strain KMM 3531^T, strain KMM 3953 was able to produce

acid from galactose, glucose, lactose, maltose, sucrose and cellobiose and was resistant to ampicillin. The differential properties of the two strains and related members of the family *Flavobacteriaceae* are shown in Table 2.

Therefore, the molecular data and phenotypic characteristics of strains KMM 3531^T and KMM 3953 strongly support their affiliation to the family *Flavobacteriaceae* of the phylum *Bacteroidetes*. However, the phylogenetic distances (>5%) and differences in the fatty acid, physiological and biochemical data between the novel isolates and related bacteria justify the description of a new genus and novel species, for which the name *Pseudozobellia thermophila* gen. nov., sp. nov. is proposed.

Description of *Pseudozobellia* gen. nov.

Pseudozobellia (Pseu.do.zo.bel'li.a. Gr. adj. *pseudes* false; N.L. fem. n. *Zobellia* the name of a bacterial genus; N.L. fem. n. *Pseudozobellia* the false *Zobellia*).

Table 2. Characteristics that differentiate strains KMM 3531^T and KMM 3953 (*Pseudozobellia thermophila* gen. nov., sp. nov.) from their closest relatives in the family *Flavobacteriaceae*

Taxa (n =number of species): 1, strains KMM 3531^T and KMM 3953 ($n=1$); 2, *Costertonia* ($n=1$); 3, *Kriegella* ($n=1$); 4, *Flagellimonas* ($n=1$); 5, *Muricauda* ($n=3$); 6, *Zobellia* ($n=5$). Data are from Bae *et al.* (2007), Barbeyron *et al.* (2001), Kwon *et al.* (2006), Nedashkovskaya *et al.* (2004b, 2008), Yoon *et al.* (2005) and this study. –, Negative; +, positive.

Characteristic	1	2	3	4	5	6
Cell size (µm)	0.2–0.4 × 1.0–3.2	0.3–0.4 × 0.5–4.2	0.4–0.5 × 1.2–1.4	0.3–0.8 × 1.2–2.3	0.2–0.6 × 1.1–6.0	0.2–0.5 × 1.2–8.0
Flagellation	–	–	–	+	–	–
Gliding motility	+	–	+	–	–	+
Ca ²⁺ requirement for growth	–	+	–	+	–	–
K ⁺ requirement for growth	–	+	–	–	–	–
Flexirubin-type pigments	+	–	+	–	–	+
Nitrate reduction	–	+	–	–	–	+
Degradation of:						
Agar	+	–	–	–	–	+
Gelatin	+	+	–	+	–	+
Urea	–	+	–	–	–	–
Salinity range (%)	0.5–8	1.5–12	0.5–6	1.9–5.4	0.5–9	0.5–10
Temperature range (°C)	4–49	10–35	4–37	17–36	8–44	4–45
DNA G + C content (mol%)	47–49	35–36	39–41	56.3	41–46	36–44

Cells are strictly aerobic. Gram-negative. Cells do not form endospores. Non-diffusible pigments are produced. Chemo-organotrophic. Positive for cytochrome oxidase, catalase and alkaline phosphatase. The predominant cellular fatty acids are branched-chain saturated and unsaturated and straight-chain unsaturated fatty acids iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{15:1} G, summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), iso-C_{17:1}ω9c and iso-C_{15:0} 3-OH. The main respiratory quinone is MK-6. The DNA G + C content of the type species is 47–49 mol%. 16S rRNA gene sequence analysis indicates that the genus *Pseudozobellia* is a member of the family *Flavobacteriaceae* in the phylum *Bacteroidetes*. The type species is *Pseudozobellia thermophila*.

Description of *Pseudozobellia thermophila* sp. nov.

Pseudozobellia thermophila (ther.mo'phi.la. Gr. adj. *thermos* hot; Gr. adj. *philos* loving or having affinity for; N.L. fem. adj. *thermophila* heat-loving).

Exhibits the following properties in addition to those given in the genus description. Cells are regular rods, motile by gliding, and 0.2–0.4 µm in width and 1.0–3.2 µm in length. On marine agar 2216, colonies are 2–3 mm in diameter, circular, shiny with entire edges, dark-orange-pigmented and sunken into the agar. Flexirubin-type pigments are produced. Growth occurs at 4–49 °C and with 0.5–8 % NaCl (optimum, 4–5 % NaCl). Positive for β-galactosidase. Hydrolyses aesculin, agar, gelatin, alginate, DNA, tyrosine and Tweens 20, 40 and 80. Does not hydrolyse casein, starch, urea, cellulose (carboxymethylcellulose and filter paper) or chitin. Produces acid from L-arabinose, L-rhamnose and L-fucose but not from melibiose, raffinose,

L-sorbose, DL-xylose, N-acetylglucosamine, citrate, malate, fumarate, adonitol, dulcitol, glycerol, inositol or mannitol. Acid production from D-galactose, D-glucose, D-lactose, maltose, sucrose and cellobiose is strain-dependent. D-Glucose, D-lactose, D-mannose, sucrose and mannitol are utilized but inositol, sorbitol, malonate and citrate are not. With the API ZYM gallery, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid and alkaline phosphatases, naphthol-AS-BI-phosphohydrolase, α- and β-galactosidases, α- and β-glucosidases, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are present, but lipase (C14) and β-glucuronidase activities are absent. Nitrate is not reduced. H₂S, indole and acetoin (Voges-Proskauer reaction) are not produced. Susceptible to carbenicillin (100 µg), chloramphenicol (30 µg), lincomycin (15 µg), oleandomycin (15 µg) and erythromycin (15 µg), but resistant to benzyl penicillin (10 U), gentamicin (10 µg), doxycycline (10 µg), kanamycin (30 µg), neomycin (30 µg), polymyxin (300 U), streptomycin (30 µg) and tetracycline (30 µg). Susceptibility to ampicillin (10 µg) is strain-dependent. The DNA G + C content of the type strain is 49.0 mol%.

The type strain, KMM 3531^T (=DSM 19858^T=JCM 11733^T=KCTC 22016^T), was isolated from the green alga *Ulva fenestrata* in Posiet Bay, Sea of Japan (also known as the East Sea). A reference strain, KMM 3953 (=KCTC 22017), was isolated from the same source.

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