

## *Kriegella aquimaris* gen. nov., sp. nov., isolated from marine environments

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Two novel marine, heterotrophic, gliding, Gram-negative, aerobic and orange-pigmented strains were investigated by using a polyphasic approach. 16S rRNA gene sequence analysis indicated an affiliation of the novel isolates to the family *Flavobacteriaceae* of the phylum *Bacteroidetes*. The level of sequence similarity between the strains studied and their closest relatives, the recognized *Zobellia* species, ranged from 93.2 to 93.9%. Phylogenetic evidence, supported by several differences in phenotypic characteristics between the novel isolates and *Zobellia* species such as the absence of nitrate reductase, agarase and gelatinase activities together with the ability to produce acid from galactose, lactose and melibiose, allowed their assignment to a new genus and species, for which the name *Kriegella aquimaris* gen. nov., sp. nov. is proposed. The type strain of *Kriegella aquimaris* is KMM 3665<sup>T</sup> (=KCTC 22188<sup>T</sup> =DSM 19886<sup>T</sup>).

Members of the family *Flavobacteriaceae* are often found in coastal marine environments (Agogué *et al.*, 2005; Bowman, 2006). In the course of a study of microbial communities of oligotrophic and urban polluted waters of the Gulf of Peter the Great, Sea of Japan (also known as the East Sea), novel bacteria belonging to known genera such as *Cellulophaga*, *Formosa*, *Gillisia*, *Polaribacter*, *Salegentibacter* and *Zobellia* have been isolated (Nedashkovskaya *et al.*, 2004a, c, 2005a, b, 2006a, b, 2007). Herein, we report the isolation and identification of strains KMM 3665<sup>T</sup> and KMM 3942 using a polyphasic approach. 16S rRNA gene sequencing indicated that the novel isolates form a cluster with members of the genera *Zobellia*, *Costertonia*, *Flagellimonas* and *Muricauda*. Because of significant molecular and phenotypic differences between the novel bacteria and their closest relatives, strains KMM 3665<sup>T</sup> and KMM 3942 were classified as representatives of a novel genus.

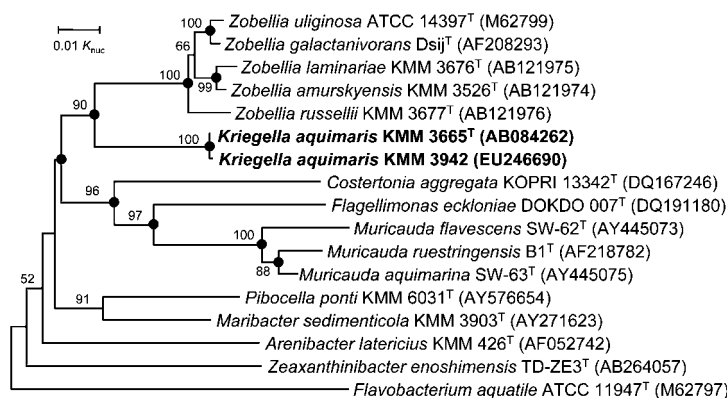
Strains KMM 3665<sup>T</sup> and KMM 3942 were isolated from seawater in Amursky Bay and from the green alga *Ulva fenestrata* collected from Troitsa Bay of the Sea of Japan. For strain isolation, 0.1 ml seawater or alga tissue homogenate was plated on marine agar 2216 (MA;

Difco). After primary isolation and purification, strains were cultivated at 28 °C on the same medium and stored at –80 °C in marine broth (Difco) supplemented with 20% (v/v) glycerol.

The almost-complete 16S rRNA gene sequences of isolates KMM 3665<sup>T</sup> and KMM 3942 were determined by PCR amplification and direct sequencing (Hiraishi, 1992). The conditions and reagents used for PCR amplification and sequencing of the 16S rRNA gene were as described previously (Suzuki *et al.*, 2001). The sequences were aligned on the secondary-structure model, maintained by the SSU rRNA database (Van de Peer *et al.*, 2000), using the profile-alignment program of the CLUSTAL W software (Thompson *et al.*, 1994). Evolutionary distances were then computed with the DNADIST program in the PHYLIP 3.572 package (Felsenstein, 1995) with the two-parameter model (Kimura, 1980) and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). To evaluate the topology of the phylogenetic tree, a bootstrap analysis with 1000 sample replications was performed with the SEQBOOT and CONSENSE programs in PHYLIP 3.572.

The 16S rRNA gene-based analysis revealed that strains KMM 3665<sup>T</sup> and KMM 3942 clustered with members of the genera *Zobellia*, *Costertonia*, *Flagellimonas*, *Muricauda* and *Maribacter* in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002) (Fig. 1). The recognized *Zobellia* species were

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KMM 3665<sup>T</sup> and KMM 3942 are AB084262 and EU246690, respectively.



**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strains KMM 3665<sup>T</sup> and KMM 3942 with the type strains of recognized *Zobellia* species and other members of the family *Flavobacteriaceae*. The 16S rRNA gene sequence of *Flavobacterium aquatile* ATCC 11947<sup>T</sup> was used as an outgroup. Bootstrap values 50 % or greater are given at nodes. Bar, genetic distance of 0.01 ( $K_{nuc}$ ).

the nearest relatives of the novel isolates, with sequence similarities to the type strains of 93.2–93.9 %. The level of 16S rRNA gene sequence similarity between strains KMM 3665<sup>T</sup> and KMM 3942 and other close relatives was 89.7–92.8 %. Hence, the two strains could be assigned to the family *Flavobacteriaceae* as members of a separate genus according to Stackebrandt & Goebel (1994). This conclusion was supported by the topology of the phylogenetic tree, in which the new genus formed a distinct lineage among members of the family *Flavobacteriaceae* (Fig. 1). The 16S rRNA gene sequence similarity between strains KMM 3665<sup>T</sup> and KMM 3942 was 99.9 %.

DNA was isolated following the method 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 strains KMM 3665<sup>T</sup> and KMM 3942 were 39.2 and 40.7 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 level of DNA–DNA reassociation between strains KMM 3665<sup>T</sup> and KMM 3942 was 83 %, demonstrating that they belong to the same species according to the proposal of Wayne *et al.* (1987).

To obtain the whole-cell fatty acid profile, strain KMM 3665<sup>T</sup> was grown at 28 °C for 24 h on MA. Analysis of fatty acid methyl esters was carried out according to the standard protocol of the Microbial Identification System (Microbial ID).

The predominant cellular fatty acids (i.e.  $\geq 5\%$  of the total) of strain KMM 3665<sup>T</sup> were the straight-chain and branched-chain saturated and unsaturated fatty acids iso-C<sub>15:1</sub> (19.7 %), iso-C<sub>17:0</sub> 3-OH (12.8 %), iso-C<sub>15:0</sub> (12.3 %), C<sub>15:0</sub> (11.1 %), summed feature 3 (iso-C<sub>15:0</sub> 2-OH and/or C<sub>16:1</sub>  $\omega 7c$ ; 9.4 %) and iso-C<sub>17:1</sub>  $\omega 9c$  (6.4 %) (Table 1). Isoprenoid quinones were extracted and analysed by the method of Nakagawa & Yamasato (1993). The major isoprenoid quinone was menaquinone 6 (MK-6).

Physiological and biochemical properties of strains KMM 3665<sup>T</sup> and KMM 3942 were tested as described by Nedashkovskaya *et al.* (2004c) and using API 20E, API 20NE and API ZYM galleries (bioMérieux) according to the manufacturer's instructions.

The physiological, morphological and biochemical characteristics of the strains studied are listed in the genus and species descriptions and in Table 2. Strains KMM 3665<sup>T</sup> and KMM 3942 share many features with members of the genera *Costertonia*, *Flagellimonas*, *Maribacter*, *Muricauda* and *Zobellia*, e.g. they are aerobic and oxidase-positive organisms, requiring NaCl or seawater for growth, and utilizing carbohydrates (Table 2). However, the novel isolates clearly differ from recognized *Zobellia* species, their closest relatives, by the absence of nitrate reductase, agarase and gelatinase activities. Production of flexirubin-type pigments separates the strains studied from representatives of the genera *Costertonia*, *Flagellimonas*, *Maribacter* and *Muricauda*. A set of traits such as absence of flagella, requirement for Ca<sup>2+</sup> ions and acetoin production taken together with the inability to hydrolyse casein and gelatin differentiate the novel isolates from *Flagellimonas eckloniae*. Additional phenotypic features that differentiate strains KMM 3665<sup>T</sup> and KMM 3942 from their close phylogenetic relatives are shown in Table 2. Strains KMM 3665<sup>T</sup> and KMM 3942 differ from each other only in their maximum growth temperature (36 and 37 °C, respectively) and in hydrolysis of DNA (negative for the type strain) and Tweens 20 and 80 (positive for the type strain).

Consequently, the phylogenetic relatedness and biochemical and physiological characteristics of the novel isolates support their placement in the family *Flavobacteriaceae* within a distinct new genus and species, for which the name *Kriegella aquimaris* gen. nov., sp. nov. is proposed.

### Description of *Kriegella* gen. nov.

*Kriegella* (Krie.gel'la. N.L. fem. dim. n. *Kriegella* named in honour of Noel R. Krieg, a famous American microbiologist, who has made a great contribution to the taxonomy of bacteria belonging to the phylum *Bacteroidetes*).

**Table 1.** Whole-cell fatty acid compositions of strain KMM 3665<sup>T</sup> and closely related members of the family *Flavobacteriaceae*

Taxa: 1, strain KMM 3665<sup>T</sup>; 2, *Costertonia aggregata* KOPRI 13342<sup>T</sup>; 3, *Flagellimonas eckloniae* DOKDO 007<sup>T</sup>; 4, *Maribacter* (range for six species); 5, *Muricauda* (two species); 6, *Zobellia* (five species). Values are percentages of total fatty acids; those fatty acids for which the mean amount in all taxa was less than 1 % are not given. tr, Trace (<1 %); –, not detected/not reported. Some strains were grown under different culture conditions. Data from Bae *et al.* (2007), Kwon *et al.* (2006), Nedashkovskaya *et al.* (2004b, 2007), Yoon *et al.* (2005a, b) and this study.

Fatty acid	1	2	3	4	5	6
<b>Straight-chain</b>						
C <sub>13:0</sub>	–	2.0	–	tr	–	–
C <sub>13:1</sub>	tr	–	–	0–1.1	–	–
C <sub>14:0</sub>	tr	1.2	–	0.5–1.0	–	0–1.0
C <sub>14:1</sub> ω5c	tr	–	–	0–1.0	–	–
C <sub>15:0</sub>	11.1	7.8	7.0	3.5–14.5	5.1–13.2	7.5–14.4
C <sub>15:0</sub> 3-OH	–	–	–	1.4–2.4	1.0–1.8	tr
C <sub>15:1</sub> ω6c	2.5	–	–	0.6–4.8	0.8–0.9	1.1–3.2
C <sub>16:0</sub>	tr	2.7	1.4	0.3–1.4	tr	1.1–2.6
C <sub>16:0</sub> 3-OH	tr	–	–	0.5–5.5	–	2.4–4.9
C <sub>17:1</sub> ω6c	tr	–	–	0.2–1.7	–	0–1.2
C <sub>17:1</sub> ω8c	tr	–	–	0–1.2	–	–
C <sub>17:0</sub> 2-OH	tr	–	–	0–1.4	0.7–1.4	–
C <sub>17:0</sub> 3-OH	tr	2.2	–	tr	0.3–1.3	–
C <sub>18:0</sub>	tr	4.0	–	tr	–	–
C <sub>18:1</sub>	tr	–	–	tr	–	0–1.1
<b>Methyl-branched</b>						
10-Methyl C <sub>16:0</sub>	–	1.6	9.2	–	–	–
<b>Branched</b>						
iso-C <sub>13:0</sub>	tr	–	1.3	tr	–	–
iso-C <sub>14:0</sub>	–	–	1.3	1.4	tr	–
anteiso-C <sub>15:0</sub>	tr	–	2.5	0.3–7.8	1.1–2.5	0–1.8
iso-C <sub>15:0</sub>	12.3	39.7	41.8	10.6–20.5	15.7–23.8	16.8–22.5
iso-C <sub>15:0</sub> 3-OH	3.2	–	–	2.9–5.4	4.6–5.5	4.6–8.3
iso-C <sub>15:1</sub> G	19.7	22.4	11.4	9–18.9	19.5–21.6	8.8–14.9
iso-C <sub>16:0</sub>	tr	–	1.5	0–1.8	tr	–
iso-C <sub>16:0</sub> 3-OH	tr	–	–	1.1–9.1	1.7–4.6	–
iso-C <sub>16:1</sub>	–	–	–	0–1.4	–	–
iso-C <sub>17:0</sub> 3-OH	12.8	–	–	7.9–29.2	17.3–20.9	15.1–25.9
iso-C <sub>17:1</sub> ω5c	–	–	–	0–1.4	–	–
iso-C <sub>17:1</sub> ω9c	6.4	–	–	2.0–5.4	1.3–1.5	2.4–5.1
iso-C <sub>18:1</sub>	–	–	–	0–2.4	–	–
iso-C <sub>19:0</sub>	–	–	–	0–1.4	–	–
<b>Summed features*</b>						
Summed feature 3	9.4	4.6	6.0	5.8–12.9	2.3–4.2	9.9–15.5
Summed feature 4	1.0	–	–	–	–	–

\*Summed features consist of two or more fatty acids that could not be separated by the Microbial Identification System. Summed feature 3: one or more of C<sub>16:1</sub>ω7c, C<sub>16:1</sub>ω7t and iso-C<sub>15:0</sub> 2-OH. Summed feature 4: iso-C<sub>17:1</sub> B. I and/or anteiso-C<sub>17:1</sub> B.

Cells are rod-shaped and strictly aerobic. Gram-negative. Do not form endospores. Non-diffusible pigments are produced. Chemo-organotrophs. Cytochrome oxidase-, catalase- and alkaline phosphatase-positive. The predominant cellular fatty acids are branched-chain saturated and unsaturated and straight-chain unsaturated fatty acids iso-C<sub>15:1</sub>, iso-C<sub>17:0</sub> 3-OH, iso-C<sub>15:0</sub>, C<sub>15:0</sub>, summed

feature 3 (iso-C<sub>15:0</sub> 2-OH and/or C<sub>16:1</sub>ω7c) and iso-C<sub>17:1</sub>ω9c. The main respiratory quinone is MK-6. The DNA G + C content of the type species is 39–41 mol%. 16S rRNA gene sequence analysis indicates that the genus *Kriegella* is a member of the family *Flavobacteriaceae* in the phylum *Bacteroidetes*. The type species is *Kriegella aquimaris*.

**Table 2.** Differential phenotypic characteristics of strains KMM 3665<sup>T</sup> and KMM 3942 and closely related members of the family Flavobacteriaceae

Taxa: 1, strains KMM 3665<sup>T</sup> and KMM 3942; 2, *Costertonia aggregata* KOPRI 13342<sup>T</sup>; 3, *Flagellimonas eckloniae* DOKDO 007<sup>T</sup>; 4, *Maribacter* (data for six species); 5, *Muricauda* (three species); 6, *Zobellia* (five species). All taxa are positive for oxidase and alkaline phosphatase activities and for carbohydrate utilization. All taxa are negative for requirement for organic growth factors and for H<sub>2</sub>S and indole production. v, Variable reaction; ND, no data available. Data were taken from Bae *et al.* (2007), Barbeyron *et al.* (2001), Bruns *et al.* (2001), Kwon *et al.* (2006), Nedashkovskaya *et al.* (2004b, c, 2007), Yoon *et al.* (2005a, b) and this study.

Characteristic	1	2	3	4	5	6
Gliding motility	+	—	—	+	v	+
Flagellation	—	—	+	—	—	—
Ca <sup>2+</sup> requirement for growth	—	+	+	—	—	—
K <sup>+</sup> requirement for growth	—	+	—	—	—	—
Salinity range for growth (%)	0.5–6	1.2–9.3	1.9–5.4	1–10	0.5–9	1–10
Temperature range for growth (°C)	4–37	10–35	17–36	4–41	8–44	4–42
Flexirubin-type pigments	+	—	—	—	—	+
Acid production from carbohydrates	+	—	—	+	+	+
Oxidase production	+	+	—	+	v	+
Nitrate reduction	—	+	—	v	—	+
Acetoin production	—	ND	+	v	ND	—
Degradation of:						
Agar	—	—	—	v	—	+
Casein	—	—	+	—	—	v
Gelatin	—	+	+	v	—	+
Urea	—	+	—	—	—	—
Tween 40	+	+	—	+	+	v
DNA G + C content (mol%)	39–41	35.8	56.3	35–42	41–46	36–44

### Description of *Kriegella aquimaris* sp. nov.

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

Displays the following properties in addition to those given in the genus description. Cells are motile by gliding, 0.4–0.5 µm wide and 1.2–1.4 µm long. On marine agar, colonies are 1–2 mm in diameter, circular, shiny with entire edges and dark-orange pigmented. Growth occurs at 4–37 °C (optimum 25–28 °C) and with 1–6 % NaCl (optimum 1–2 %). Produces β-galactosidase. Decomposes aesculin and Tween 40. Hydrolysis of DNA and Tweens 20 and 80 is strain-dependent. Does not hydrolyse agar, casein, gelatin, starch, alginate, cellulose (CM-cellulose or filter paper) or chitin. Forms acid from L-arabinose, cellobiose, D-galactose, D-glucose, L-fucose, D-lactose, maltose, melibiose, raffinose, L-rhamnose, sucrose and D- and L-xylose, but not from L-sorbose, N-acetylglucosamine, citrate, adonitol, dulcitol, glycerol, inositol or mannitol. Utilizes D-mannose, but not inositol, mannitol, sorbitol, malonate or citrate. According to the API ZYM gallery, activities of esterase (C4), esterase lipase (C8), leucine and valine arylamidases, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α- and β-galactosidases, α- and β-glucosidases, N-acetyl-β-glucosaminidase and α-mannosidase are present, but lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase and α-fucosidase activities are absent. Nitrate is not reduced. H<sub>2</sub>S, indole and

acetoin (Voges–Proskauer reaction) are not produced. Susceptible to ampicillin, lincomycin and oleandomycin and resistant to benzylpenicillin, carbenicillin, gentamicin, kanamycin, neomycin, polymyxin B, streptomycin and tetracycline.

The type strain, KMM 3665<sup>T</sup> (=KCTC 22188<sup>T</sup> =DSM 19886<sup>T</sup>), was isolated from a seawater sample from Amursky Bay of the Sea of Japan, also known as the East Sea. A reference strain, KMM 3942 (=DSM 15364 =KCTC 22189), was isolated from the green alga *Ulva fenestrata* in Troitsa Bay, Sea of Japan.

### Acknowledgements

This research was supported by grants from the Russian Foundation for Basic Research (RFBR) no. 08-04-00099, the Presidium of the Far-Eastern Branch of the Russian Academy of Sciences no. 06-III-A-06-183 and the Presidium of the Russian Academy of Sciences 'Molecular and Cell Biology'.

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