Gramella marina sp. nov., isolated from the sea urchin *Strongylocentrotus intermedius*

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The taxonomic position of a novel Gram-stain-negative, aerobic, heterotrophic, gliding, yellow–orange-pigmented bacterium, isolated from the sea urchin *Strongylocentrotus intermedius* and designated strain KMM 6048^T, was established. Phylogenetic analysis based on 16S rRNA gene sequencing revealed that the isolate was a member of the family *Flavobacteriaceae* affiliated with recognized species of the genus *Gramella*, forming a distinct lineage within the genus. 16S rRNA gene sequence similarities between strain KMM 6048^T and the type strains of species of the genus *Gramella* were 97.4–98.4 %. In line with representative members of the genus *Gramella*, strain KMM 6048^T was oxidase- and catalase-positive, hydrolysed gelatin and starch, utilized carbohydrates and possessed a DNA G + C content of 40.0 mol%. However, differentiating phenotypic traits and phylogenetic distinctiveness clearly indicated that the strain represented a novel species within the genus *Gramella*, for which the name *Gramella marina* sp. nov. is proposed; the type strain is KMM 6048^T (=KCTC 12366^T=LMG 25418^T).

The genus *Gramella*, a member of the family *Flavobacteriaceae* (phylum 'Bacteroidetes'), and the species *Gramella echinicola* were created to accommodate a gliding, strictly aerobic, yellow–orange-pigmented, Gram-stain-negative, and oxidase- and catalase-positive bacterial strain associated with the sea urchin *Strongylocentrotus intermedius* from the Gulf of Peter the Great (Nedashkovskaya *et al.*, 2005). A further species in the genus, *Gramella portivictoriae*, isolated from a sediment sample from the South China Sea (Lau *et al.*, 2005), was subsequently described.

In this work, a novel strain, designated KMM 6048^T, was isolated and identified using a polyphasic approach. As shown by phylogenetic analysis, strain KMM 6048^T belonged to the family *Flavobacteriaceae*, phylum 'Bacteroidetes', and formed a distinct lineage within the genus *Gramella*. On the basis of phenotypic and molecular phylogenetic evidence, the isolate represents a novel species of the genus *Gramella*.

Strain KMM 6048^T was isolated from a sea urchin, *Strongylocentrotus intermedius*, collected at a depth of 3 m in Troitsa Bay, Gulf of Peter the Great, Sea of Japan (also known as the East Sea), Pacific Ocean, in September 2002. For strain isolation, 0.1 ml sea-urchin tissue homogenate

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

DNA extraction, PCRs and sequencing of the 16S rRNA gene were carried out as described previously (Kim *et al.*, 1998). The sequence obtained (1438 bp) was aligned with those of representative members of selected genera of the family *Flavobacteriaceae* by using PHYDIT version 3.2 (<http://plaza.snu.ac.kr/~jchun/phydit/>). Phylogenetic trees were inferred by using suitable programs of the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated from the Jukes–Cantor model (Jukes & Cantor, 1969) and trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1993) algorithms. A bootstrap analysis was performed with 1000 resampled datasets by using the SEQBOOT and CONSENSE programs of the PHYLIP package.

Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain KMM 6048^T was a member of the family *Flavobacteriaceae* and formed a coherent cluster with species of the genus *Gramella* (Fig. 1). The 16S rRNA gene sequence similarities between the isolate and the type strains of *G. echinicola* and *G. portivictoriae* were 97.5 and 98.4 %, respectively. According to Stackebrandt & Ebers

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Gramella marina* KMM 6048^T is AY753911.

Fig. 1. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of KMM 6048^T and related members of the family *Flavobacteriaceae*. The same tree topology was recovered using the maximum-likelihood algorithm. Numbers at nodes are the levels of bootstrap support (%) from 1000 resampled datasets. *Stenothermobacter spongiae* UST030701-156^T (DQ064789), *Persicivirga dokdonensis* DSW-6^T (DQ017065) and *Sandarakinotalea sediminis* CKA-5^T (AB206954) were used as the outgroups. Bar, 0.01 substitutions per nucleotide position.

(2006), DNA–DNA reassociation experiments between two bacterial strains are required to demonstrate whether or not they belong to the same species only when they share more than 98.7–99.0% 16S rRNA gene sequence similarity. For this reason, DNA–DNA hybridization experiments were not performed.

DNA was isolated following the method of Marmur (1961) and its G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962). The DNA G+C content of strain KMM 6048^T was 40.0 mol%, a value similar to those reported for the two other recognized species of the genus *Gramella* (Lau *et al.*, 2005; Nedashkovskaya *et al.*, 2005).

Analysis of fatty acid methyl esters was performed according to the standard protocol of the Microbial Identification System (Microbial ID). Strain KMM 6048^T was cultivated on marine agar 2216E at 25 °C for 48 h. The fatty acids of strain KMM 6048^T accounting for greater than 5% of the total fatty acids were: iso-C_{15:0}, 17.9%; iso-C_{17:0} 3-OH, 12.4%; summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH fatty acids), 11.6%; anteiso-C_{15:0}, 8.6%; iso-C_{16:0}, 6.3%; iso-C_{17:1}ω9c, 5.5%; and C_{17:0} 2-OH, 5.1%. The fatty acid profile of the novel isolate was similar to those of recognized species of the genus *Gramella* (Lau *et al.*, 2005; Nedashkovskaya *et al.*, 2005), but it differed from them in the proportions of iso-C_{16:0}, iso-C_{15:0} 3-OH

and C_{17:0} 2-OH (Table 1). Also, C_{15:0}, iso-C_{15:1} G, iso-C_{16:1} G and anteiso-C_{17:1}ω9c were detected only in cells of strains KMM 6048^T and *G. echinicola* KMM 6050^T, whereas C_{15:0} 3-OH was found only in cells of *G. portivictoriae* UST040801-001^T; however, these differences may be a result of different cultivation conditions. Isoprenoid quinones were extracted and analysed using a standard procedure (Minnikin *et al.*, 1984). The major respiratory quinone of strain KMM 6048^T was MK-6, which was in line with that of the two recognized species of the genus *Gramella* and all other members of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002; Lau *et al.*, 2005; Nedashkovskaya *et al.*, 2005).

Phenotypic analysis of strain KMM 6048^T was performed by using previously described methods (Nedashkovskaya *et al.*, 2003a, b). In addition, API 20E, API 20NE and API ZYM galleries (bioMérieux) and GN2 MicroPlates (Biolog) were used according to the manufacturers' instructions, except that the galleries were incubated at 28 °C. Susceptibility to antibiotics was tested using the diffusion method with discs containing ampicillin (10 µg), benzylpenicillin (10 U), carbenicillin (100 µg), cefalexin (30 µg), cefazolin (30 µg), chloramphenicol (30 µg), doxycycline (10 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), lincomycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), ofloxacin (5 µg), oleandomycin (15 µg), oxacillin (10 µg), polymyxin (300 U), rifampicin (5 µg), streptomycin

Table 1. Fatty acid profiles of species of the genus *Gramella*

Strains: 1, KMM 6048^T; 2, *G. echinicola* KMM 6050^T; 3, *G. portivictoriae* UST040801-001^T. Data from Lau *et al.* (2005), Nedashkovskaya *et al.* (2005) and this study. All strains were grown on marine agar 2216 for 48 h. Strain KMM 6048^T and *G. echinicola* KMM 6050^T were grown at 25 °C, whereas *G. portivictoriae* UST040801-001^T was grown at 30 °C. Fatty acids amounting to <1% of the total fatty acids in all strains are not shown. ND, Not detected; tr, trace (<1%).

Fatty acid	1	2	3
iso-C _{14:0}	tr	1.4	tr
C _{15:0}	3.9	7.1	ND
C _{15:1} ω6c	1.1	1.9	1.8
anteiso-C _{15:0}	8.6	7.6	6.2
iso-C _{15:0}	17.9	14.4	38.1
iso-C _{15:1} G	2.1	1.2	ND
iso-C _{16:0}	6.3	13.1	1.9
iso-C _{16:1} G	2.7	5.8	ND
C _{15:0} 2-OH	2.8	2.0	1.0
C _{15:0} 3-OH	ND	ND	2.5
iso-C _{15:0} 3-OH	1.5	1.3	6.2
iso-C _{16:0} 3-OH	3.9	5.9	3.4
C _{17:0} 2-OH	5.1	2.6	1.5
C _{17:1} ω6c	3.6	3.6	1.7
anteiso-C _{17:1} ω9c	2.2	2.0	ND
iso-C _{17:1} ω9c	5.5	3.5	5.7
iso-C _{17:0} 3-OH	12.4	6.7	11.2
C _{18:1} ω5c	tr	ND	1.0
Summed feature 3*	11.6	11.4	8.8

*Summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1}ω7c that could not be separated by the Microbial Identification System.

cin (30 µg), tetracycline (30 µg) and vancomycin (30 µg) (Research Center of Pharmacotherapy, Russia). Gliding motility was determined as described by Bowman (2000).

In accordance with the two other recognized species of the genus *Gramella*, strain KMM 6048^T was heterotrophic, strictly aerobic, yellow–orange-pigmented and motile by gliding. It was oxidase-, catalase- and alkaline phosphatase-positive, required NaCl or seawater for growth, hydrolysed aesculin, gelatin and starch, and utilized carbohydrates. However, strain KMM 6048^T differed from the type strains of the two other species of the genus in a number of phenotypic properties (Table 2).

Consequently, phylogenetic distinctiveness and differential phenotypic characteristics warrant description of strain KMM 6048^T as a representative of a novel species in the genus *Gramella*, for which the name *Gramella marina* sp. nov. is proposed.

Description of *Gramella marina* sp. nov.

Gramella marina (ma.ri'na. L. fem. adj. *marina* of the sea, marine, a bacterium isolated from a marine environment).

Table 2. Phenotypic characteristics that differentiate strain KMM 6048^T from other species of the genus *Gramella*

Strains: 1, KMM 6048^T; 2, *G. echinicola* KMM 6050^T; 3, *G. portivictoriae* UST040801-001^T. All strains were positive for the following tests: respiratory metabolism; gliding motility; oxidase and catalase activities; requirement for Na⁺ ions for growth; growth with 1–6% NaCl and at 4–36 °C; hydrolysis of aesculin, gelatin, starch, Tween 40 and Tween 80; utilization of arabinose, galactose, glucose and sucrose; acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, leucine arylamidase, valine arylamidase, esterase (C4), esterase lipase (C8), lipase (C14), α-galactosidase, α-glucosidase, β-glucosidase, trypsin and naphthol-AS-BI-phosphohydrolase activities; susceptibility to ampicillin, benzylpenicillin, chloramphenicol and tetracycline; and resistance to kanamycin. All strains were negative for the following tests: production of flexirubin-type pigments; nitrate reduction; α-fucosidase and urease activities; H₂S and indole production; hydrolysis of agar, cellulose (CM-cellulose and filter paper) and chitin; acid production from fucose, lactose, rhamnose, sorbose, cellobiose, xylose, glycerol, adonitol, inositol, mannitol, sorbitol and citrate; and utilization of lactose, inositol and malonate. Data are from Lau *et al.* (2005), Nedashkovskaya *et al.* (2005) and this study. ND, Not determined.

Characteristic	1	2	3
Acetoin production	+	–	+
Growth with 15% NaCl	+	+	–
Hydrolysis of:			
Casein	+	+	–
DNA	–	+	–
Tween 20	+	–	+
Acid production from:			
Arabinose, melibiose	+	–	–
Galactose, glucose, maltose, sucrose, trehalose, raffinose	+	+	–
N-Acetylglucosamine	–	+	–
Utilization of:			
Acetic acid, L-asparagine, glycyl-L-aspartic acid, L-threonine	–	+	–
D-Galacturonic acid	+	–	–
γ-Hydroxybutyric acid	–	–	+
α-Ketoglutaric acid, L-alanine, L-alanyl-glycine, L-glutamic acid, L-proline	+	+	–
Melibiose	+	–	+
Mannitol, sorbitol	–	–	+
N-Acetylglucosamine	+	+	–
Citrate	+	–	–
Enzyme activities:			
β-Galactosidase	+	+	–
β-Glucuronidase, N-acetylglucosaminidase	–	+	–
α-Mannosidase	+	–	–
Susceptibility to:			
Streptomycin	+	–	–
Vancomycin	+	–	ND
DNA G + C content (mol%)	40.0	39.6	39.9

The main characteristics are the same as those given for the genus. In addition, cells are 0.4–0.6 µm in width and 1.7–2.5 µm in length. On marine agar, colonies are 2–3 mm in

diameter, circular, shiny with entire edges and yellow–orange-pigmented. Growth is observed at 4–37 °C (optimum, 28–30 °C) and with 1–15 % (w/v) NaCl (optimum, 2–5 % NaCl). Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities are absent. Acid is produced from amygdalin, but not from cellobiose. D-Mannose, adipate, gluconate, malate and phenylacetate are utilized, but caprate is not. The substrates utilized in the GN2 MicroPlate are listed in Table 1. Susceptible to ampicillin, benzylpenicillin, carbenicillin, cefalexin, cefazolin, chloramphenicol, doxycycline, erythromycin, lincomycin, nalidixic acid, ofloxacin, oleandomycin, streptomycin, tetracycline, rifampicin and vancomycin, but resistant to gentamicin, kanamycin, neomycin, oxacillin and polymyxin. Other biochemical and physiological properties are shown in Table 2. The predominant fatty acids (i.e. >5 % of the total fatty acids) are iso-C_{15:0}, iso-C_{17:0} 3-OH, anteiso-C_{15:0}, iso-C_{16:0}, iso-C_{17:1}ω9c and summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH and C_{17:0} 2-OH).

The type strain is KMM 6048^T (=KCTC 12366^T=LMG 25418^T), isolated from the sea urchin *Strongylocentrotus intermedius* collected in Troitsa Bay, Gulf of Peter the Great, Sea of Japan (also known as the East Sea). The DNA G + C content of the type strain is 40.0 mol%.

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