Gramella echinicola gen. nov., sp. nov., a novel halophilic bacterium of the family *Flavobacteriaceae* isolated from the sea urchin *Strongylocentrotus intermedius*

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A novel marine bacterium, strain KMM 6050^T, was isolated from the sea urchin *Strongylocentrotus intermedius*, which inhabits the Sea of Japan. The strain studied was strictly aerobic, heterotrophic, yellow–orange-pigmented, motile by gliding, Gram-negative and oxidase-, catalase-, β -galactosidase- and alkaline phosphatase-positive. The results of 16S rRNA gene sequence analysis showed that strain KMM 6050^T occupies a distinct lineage within the family *Flavobacteriaceae* and is most closely related to the species *Mesonía algae* and *Salegentibacter salegens* (sequence similarity of 92.5–92.6%). The DNA G + C content of KMM 6050^T was 39.6 mol%. The major respiratory quinone was MK-6. The predominant fatty acids were i15:0, a15:0, 15:0, i16:1, i16:0, i16:0 3-OH and i17:0 3-OH. On the basis of phenotypic, chemotaxonomic, genotypic and phylogenetic characteristics, the novel bacterium has been assigned to the genus *Gramella* gen. nov., as *Gramella echinicola* sp. nov. The type strain is KMM 6050^T (=KCTC 12278^T=NBRC 100593^T=LMG 22585^T).

Flavobacteria are often found in different marine habitats, including sea water, sediments or algae (Hanzawa *et al.*, 1998; Glöckner *et al.*, 1999; Patel *et al.*, 2003). Also, the representatives of this group are considered to be an important part of marine-animal microbial populations, as indicated by data from culture-independent molecular analyses (Cooney *et al.*, 2002; López-García *et al.*, 2002; Webster *et al.*, 2004). For example, Alain *et al.* (2002) examined the microbial assemblage associated with the hydrothermal vent polychaete *Paralvinella palmiformis* of the north-eastern Pacific by using 16S rRNA gene sequence-based phylogenetic analysis; they reported that members of the *Cytophaga*–*Flavobacterium* group were identified frequently in 16S rRNA gene clone libraries.

In the course of a study on the microbial population of the sea urchin *Strongylocentrotus intermedius* (a common member of the fauna of the Sea of Japan), a novel heterotrophic, aerobic, yellow–orange-pigmented, gliding, Gram-negative bacterium was isolated. 16S rRNA gene sequence-based phylogenetic analysis of strain KMM 6050^T revealed it to be a potentially distinct and novel member of the family *Flavobacteriaceae*. The nearest neighbours of the strain studied were *Mesonía algae* and *Salegentibacter salegens* (92.6 and 92.5% sequence similarity, respectively). On the basis of the results of 16S rRNA gene sequence phylogenetic analysis and the chemotaxonomic and phenotypic features of strain KMM 6050^T, obtained and presented in this work, we propose the description of a new genus of the family *Flavobacteriaceae*, *Gramella* gen. nov., and novel species, *Gramella echinicola* sp. nov.

Strain KMM 6050^T was isolated from the sea urchin *Strongylocentrotus intermedius*, which was collected at a

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Gramella echinicola* strain KMM 6050^T is AY608409.

depth of 3 m in Troitsa Bay, Gulf of Peter the Great, Sea of Japan, Pacific Ocean, in September 2002. For strain isolation, 0.1 ml homogenates of sea-urchin tissues were transferred onto plates of marine agar 2216 (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.

DNA extraction, PCRs and sequencing of the 16S rRNA gene followed procedures described previously (Kim *et al.*, 1998). The sequences obtained were 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 from the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated from the models of Kimura (1980) and the 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 16S rRNA gene sequence analysis indicated that strain KMM 6050^T is a member of the family *Flavobacteriaceae* and forms a cluster with species of the genera *Psychroflexus*, *Salegentibacter* and *Mesonina* (Fig. 1). The closest relatives of this strain are *M. algae* KMM 3909^T and *Salegentibacter salegens* DSM 5424^T, there being 16S rRNA gene sequence similarities of 92.6 and 92.5 %, respectively, without significant bootstrap support.

DNA was isolated according to 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 6050^T was 39.6 mol%.

Analysis of fatty acid methyl esters was carried out according to the standard protocol of the Microbial Identification System (Microbial ID). The main cellular fatty acids of KMM 6050^T were i15:0, a15:0, 15:0, i16:1, i16:0, i16:0 3-OH, i17:0 3-OH and summed feature 3, comprising 16:1ω7 or i15:0 2-OH fatty acids (Table 1). Isoprenoid

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of strain KMM 6050^T and related members of the family *Flavobacteriaceae*. The asterisks indicate branches that were also recovered with the maximum-likelihood algorithm, while the numbers at the nodes are the levels of bootstrap support (%) from the 1000 resamplings in the dataset. Bar, 0.01 substitution per nucleotide position.

Table 1. Cellular fatty acid compositions (percentage content) of *Gramella echinicola* KMM 6050^T and its closest relatives

Species: 1, *G. echinicola* KMM 6050^T; 2, *M. algae* KMM 3909^T; 3, *Salegentibacter salegens* DSM 5424^T. Data are from Nedashkovskaya *et al.* (2003a) and this study.

Fatty acid	1	2	3
i14:0	1.4	0.9	
i15:1	1.2	7.9	17.7
a15:1		0.4	3.5
i15:0	14.4	21.2	8.5
a15:0	7.6	4.1	8.5
15:0	7.1	4.8	7.6
15:1ω6c	1.9	1.8	5.7
i16:1	5.8	4.2	2.6
i16:0	13.1	6.3	3.1
i15:0 3-OH	1.3	3.2	2.5
15:0 3-OH			3.9
15:0 2-OH	2.0	1.4	2.4
i17:1ω9c	3.5	5.1	2.0
a17:1ω9c	2.0	1.9	
17:1ω6c	3.6	2.4	4.1
i16:0 3-OH	5.9	6.0	5.9
i17:0 3-OH	6.7	14.6	5.9
17:0 2-OH	2.6	1.8	4.7
Summed feature 3*	11.4	5.1	6.1
Unknown	4.6	3.3	2.2

*Summed feature 3 consists of 16:1ω7 and/or i15:0 2-OH fatty acids that could not be separated by the Microbial Identification System.

quinones were extracted from lyophilized cells and analysed as described previously (Nedashkovskaya *et al.*, 2003a). The major respiratory quinone was MK-6.

A phenotypic analysis was performed by using previously described methods (Nedashkovskaya *et al.*, 2003a, b). Gliding motility was determined as described by Bowman (2000).

The physiological, biochemical and morphological characteristics of strain KMM 6050^T are listed in the species description and are shown in Table 2. The results of the phenotypic examination demonstrated that the strain studied and *M. algae* had many traits in common. However, strain KMM 6050^T differs sufficiently from its closest relative by having the ability to move by gliding, by producing acid from carbohydrates, by growing at 37 °C, by hydrolysing starch and DNA, by producing hydrogen sulfide and by having a higher G+C content (Table 2). The sea-urchin isolate can be clearly differentiated from the other nearest neighbour, *Salegentibacter salegens*, by the presence of gliding motility and by the ability to degrade casein (Table 2). Significant differences in the cellular fatty acid composition of the strain studied and type strains of the genera *Mesonina* and *Salegentibacter* support the creation of a new genus for strain KMM 6050^T (Table 1). The low sequence similarities between the strain tested and other flavobacteria described to date (89.3–91.3 %) demonstrate clearly that the bacterium isolated in this study represents a new genus.

It is, therefore, evident from the phylogenetic distinctness in combination with significant differences in the phenotypic findings and cellular fatty acid composition that the strain studied should be differentiated from the nearest

neighbours *M. algae* and *Salegentibacter salegens*. Thus, the results of the polyphasic analysis presented in this work clearly demonstrate that the strain studied cannot be assigned to any of the currently described taxa of the family *Flavobacteriaceae*, and support the placement of strain KMM 6050^T in a new genus, *Gramella* gen. nov., as *Gramella echinicola* sp. nov.

Description of *Gramella* gen. nov.

Gramella [Gra.mel'la. L. dim. ending -ella; N.L. fem. n. *Gramella* of Gram. Named in honour of the famous Danish pharmacologist and pathologist Hans Christian Gram (1853–1938), who proposed the differentiating staining of bacteria by gentian violet].

Rod-shaped cells, motile by gliding. Gram-negative. Do not form endospores. Strictly aerobic. Produce non-diffusible carotenoid pigments. Chemo-organotrophic. Cytochrome oxidase-, catalase- and alkaline phosphatase-positive. The major respiratory quinone is MK-6. The main cellular fatty acids are straight-chain unsaturated and branched-chain unsaturated fatty acids i15:0, a15:0, 15:0, i16:1, i16:0, i16:0 3-OH and i17:0 3-OH and summed feature 3, comprising fatty acids 15:0 iso 2-OH and/or 16:1 ω 7 or both. As determined by 16S rRNA gene sequence analysis, the genus *Gramella* is a member of the family *Flavobacteriaceae*.

The type species is *Gramella echinicola*.

Description of *Gramella echinicola* sp. nov.

Gramella echinicola (e.chi.ni.co'la. L. n. *echinus* a hedgehog, a sea urchin; L. suffix -cola dweller; N.L. n. *echinicola* a sea-urchin-dweller).

The main characteristics are the same as those given for the genus. In addition, cells are 0.5–0.7 μ m in width and 2.1–2.7 μ m in length. On marine agar, colonies are 2–4 mm in diameter, circular, shiny with entire edges and yellow–orange in colour. Growth is observed at 4–37 °C. Optimal temperature for growth is 23–25 °C. Growth occurs in 1–15 % (w/v) NaCl. β -Galactosidase-positive. Casein, gelatin, starch, DNA, Tween 40 and Tween 80 are decomposed. Agar, urea, Tween 20, cellulose (CM-cellulose and filter paper) and chitin are not degraded. Acid is formed from D-galactose, D-glucose, D-maltose, D-sucrose and L-raffinose, but not from L-arabinose, D-cellobiose, L-fucose, D-lactose, D-melibiose, L-rhamnose, DL-xylose, citrate, adonitol, dulcitol, glycerol, inositol or mannitol. L-Arabinose, D-glucose and D-sucrose are utilized, but D-lactose, D-mannose, mannitol, inositol, sorbitol, malonate and citrate are not utilized. Nitrate is not reduced. H₂S, indole and acetoin (Voges–Proskauer reaction) are not produced. The G+C content of the DNA is 39.6 mol%.

The type strain is KMM 6050^T (=KCTC 12278^T=NBRC 100593^T=LMG 22585^T). Isolated from the sea urchin *Strongylocentrotus intermedius*, collected in Troitsa Bay, Gulf of Peter the Great, Sea of Japan.

Table 2. Differential characteristics of *Gramella* gen. nov. and related genera of the family *Flavobacteriaceae*

Genera: 1, *Gramella*; 2, *Mesonina*; 3, *Salegentibacter*; 4, *Psychroflexus*. Data are from Dobson *et al.* (1993), McCammon & Bowman (2000), Bowman *et al.* (1998), Nedashkovskaya *et al.* (2003a, 2004) and this study. –, Negative; +, positive; v, variable.

Characteristic	1	2	3	4
Gliding motility	+	–	–	v
NaCl requirement for growth	+	+	v	v
Acid formation from carbohydrates	+	–	+	+
Growth at 37 °C	+	–	v	–
Growth in 15 % NaCl	+	+	v	v
Hydrolysis of:				
Casein	+	+	–	–
Starch	+	–	+	+
DNA	+	–	+	+
Nitrate reduction	–	–	+	–
H ₂ S production	–	+	+	–
DNA G+C content (mol%)	39.6	32–34	37–38	32–36

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