Gramella gaetbulicola sp. nov., a member of the family *Flavobacteriaceae* isolated from foreshore soil

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A Gram-reaction-negative, yellow-pigmented, gliding, rod-shaped, aerobic bacterium (RA5-111^T) was isolated from foreshore soil. The taxonomic status of the novel isolate was determined using a polyphasic approach. On the basis of 16S rRNA gene sequence similarities, strain RA5-111^T could be assigned to the genus *Gramella*, with sequence similarities of 97.7, 97.3 and 96.2 % to the type strains of *Gramella echinicola*, *Gramella portivictoriae* and *Gramella marina*, respectively. Chemotaxonomic and phenotypic characteristics also supported the affiliation of strain RA5-111^T with the genus *Gramella*. The genomic DNA G + C content was 39.1 mol%. The isolate contained MK-6 as the predominant menaquinone, iso-C_{15:0}, iso-C_{17:0} 3-OH and a summed feature (iso-C_{15:0} 2-OH and/or C_{16:1ω7c}) as major fatty acids, and phosphatidylethanolamine and unknown phospholipids as the polar lipids. DNA–DNA relatedness, phenotypic, genotypic and chemotaxonomic data clearly indicate that the isolate represents a novel species of the genus *Gramella*, for which the name *Gramella gaetbulicola* sp. nov. is proposed. The type strain is RA5-111^T (=KCTC 23022^T =JCM 16528^T =NBRC 106272^T).

The genus *Gramella*, which belongs to the family *Flavobacteriaceae*, was first described by Nedashkovskaya *et al.* (2005) and, at the time of writing, comprised three species: *Gramella echinicola* (Nedashkovskaya *et al.*, 2005), *Gramella portivictoriae* (Lau *et al.*, 2005) and *Gramella marina* (Nedashkovskaya *et al.*, 2010), all of which were found in marine environments. *G. echinicola* and *G. marina* were isolated from the sea urchin *Strongylocentrotus intermedius* (Nedashkovskaya *et al.*, 2005, 2010), whereas *G. portivictoriae* was isolated from marine sediment (Lau *et al.*, 2005). Eilers *et al.* (2001) reported that species of the genus *Gramella* are commonly found in surface seawater. The whole genome of ‘*Gramella forsetii*’, a representative member of the former study, was determined and the organism had interesting features showing it to be a well-adapted inhabitant of the marine environment (Bauer *et al.*, 2006).

Abbreviation: PE, phosphatidylethanolamine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of RA5-111^T is GQ857650.

In the present study, the taxonomic status of a novel member of the genus *Gramella*, isolated during a study of halophilic bacterial diversity associated with marine environments, was determined using a polyphasic approach.

Strain RA5-111^T was isolated from foreshore soil in the Buan area, Jeonbuk, Republic of Korea. The soil sample was suspended in Ringer’s solution (2.25 g NaCl, 0.105 g KCl, 0.12 g CaCl₂ and 0.05 g NaHCO₃ per litre distilled water), serially diluted and spread on marine agar 2216 (Difco). The strain was maintained using the same medium and also as a suspension in 20 % (w/v) glycerol at –70 °C.

The Gram-stain reaction was determined using a non-staining method as described by Buck (1982). Cell morphology was observed with a light microscope (Olympus) using cells grown for 48 h at 30 °C. Other phenotypic characteristics, including gliding motility, catalase and oxidase activities, and hydrolysis of casein, cellulose, DNA, starch, Tweens 20, 40, 60 and 80, and urea, were ascertained using methods described by Nedashkovskaya *et al.* (2005) and Cho *et al.* (2010). Marine broth (Difco)

was used for determination of growth at 4, 10, 20, 25, 30, 37 and 42 °C and at pH 4–12 (intervals of 1 pH unit) adjusted with 1 M HCl or NaOH. Growth in the presence of 0, 3, 5, 10, 15 and 20 % (w/v) NaCl was investigated using a basal medium consisting of 10 g peptone and 5 g yeast extract per litre distilled water. API 20E, API 20 NE and API ZYM strips (bioMérieux) and GN2 MicroPlates (Biolog) were used to determine other biochemical and physiological properties according to the manufacturers' instructions. The isoprenoid quinone composition was analysed using reversed-phase HPLC as described by Cho *et al.* (2010). For fatty acid methyl ester analysis, strain RA5-111^T and the three reference strains were grown on marine agar at 30 °C for 2 days and fatty

acid methyl esters were extracted and prepared according to the standard protocol of the Sherlock Microbial Identification System (MIDI). Polar lipids were determined using two-dimensional TLC followed by spraying with the appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The genomic DNA G+C content was determined according to Cui *et al.* (2007).

The 16S rRNA gene of RA5-111^T was amplified by PCR from chromosomal DNA and sequenced as described previously (Cho *et al.*, 2008). The sequence was aligned together with corresponding sequences of representative species of the genus *Gramella* using the program PHYDIT version 3.1

Table 1. Phenotypic characteristics differentiating strain RA5-111^T from type strains of related species of the genus *Gramella*

Strains: 1, RA5-111^T; 2, *G. echinicola* KCTC 12278^T; 3, *G. marina* KCTC 12366^T; 4, *G. portivictoriae* KCTC 22434^T. All data were obtained from this study except for data obtained with the Biolog GN2 system for *G. marina* KCTC 12366^T (Nedashkovskaya *et al.*, 2010). All taxa were positive for the following: respiratory metabolism, gliding motility, oxidase and catalase activities, hydrolysis of DNA, gelatin, starch, and Tweens 20, 40, 60 and 80, and presence of acid phosphatase, alkaline phosphatase, α -chymotrypsin, cystine arylamidase, leucine arylamidase, valine arylamidase, esterase (C4), esterase lipase (C8), α - and β -glucosidase, trypsin and naphthol-AS-BI-phosphohydrolase. All taxa were negative for the following: nitrate reduction, hydrogen sulfide and indole production, hydrolysis of casein and cellulose, presence of α -fucosidase, β -glucuronidase and urease activities, and acid production from rhamnose, inositol, mannitol and sorbitol.

Characteristic	1	2	3	4
Range for growth (optimum)				
Temperature (°C)	4–42 (30–37)	4–42 (25)	4–42 (25)	4–42 (25)
pH	4–11 (7)	4–11 (7–8)	5–11 (7)	5–11 (7)
NaCl (%)	3–10 (3–5)	0–15 (0–3)	0–15 (3–5)	0–5 (0–3)
Activity of:				
Lipase (C14)	–	+	+	+
α -Galactosidase	+	–	+	+
β -Galactosidase	+	+	–	–
N-Acetyl- β -glucosaminidase	+	+	–	–
α -Mannosidase	–	–	+	–
Utilization of (Biolog GN2):				
α -Cyclodextrin, dextrin, D-galactose, raffinose, pyruvic acid methyl ester, succinic acid monomethyl ester, D-gluconic acid, succinic acid, glycyl-L-glutamic acid, L-leucine, L-threonine, uridine, thymidine, α -D-glucose 1-phosphate	+	–	–	–
Glycogen, cellobiose, D-psicose, trehalose, turanose, acetic acid, α -ketobutyric acid, inosine	+	+	–	–
N-Acetyl-D-galactosamine, inositol, lactulose, L-rhamnose, malonic acid, quinic acid, D-saccharic acid, glucuronamide, L-alaninamide, hydroxy-L-proline, urocanic acid, putrescine, glycerol, DL- α -glycerol phosphate	–	–	–	+
<i>i</i> -Erythritol, methyl β -D-glucoside, xylitol, citric acid, D-galactonic acid lactone, D-glucuronic acid, β -hydroxybutyric acid, itaconic acid, D-alanine, L-asparagine, D-serine, L-serine, DL-carnitine	–	+	–	–
D-Fructose, gentiobiose, α -ketovaleric acid, succinamic acid, D-glucose 6-phosphate	+	–	–	+
D-Mannitol, D-sorbitol, L-aspartic acid	–	+	–	+
Sucrose	+	–	+	–
D-Galacturonic acid, L-alanyl-glycine	–	+	+	–
L-Glutamic acid	+	–	+	+
DNA G+C content (mol%)	39.1	41.1	40.0	41.1

(<http://plaza.snu.ac.kr/~jchun/phydit>). Phylogenetic trees were reconstructed on the basis of the Fitch–Margoliash, maximum-likelihood, maximum-parsimony and neighbour-joining algorithms following previously described procedures (Kim *et al.*, 2003). Levels of DNA–DNA relatedness were determined using photobiotin-labelled DNA probes and microdilution wells as described previously (Ezaki *et al.*, 1989). DNA–DNA relatedness data were obtained from experiments carried out in triplicate and reciprocal hybridization reactions.

Strain RA5-111^T formed yellow–orange, circular, convex colonies with entire margins on marine agar at 30 °C within 48 h. Growth occurred at 4–42 °C (optimum 30–37 °C) and in 3–10 % (w/v) NaCl (optimum 3–5 % NaCl). Cells were Gram-reaction-negative, strictly aerobic, non-spore-forming and rod-shaped with gliding ability. Other phenotypic characteristics are given in the species description and Table 1.

Table 2. Fatty acid contents of strain RA5-111^T and type strains of species of the genus *Gramella*

Strains: 1, RA5-111^T; 2, *G. echinicola* KCTC 12278^T; 3, *G. marina* KCTC 12366^T; 4, *G. portivictoriae* KCTC 22434^T. –, not detected or >1.0 % of total fatty acids.

Fatty acid	1	2	3	4
Straight-chain				
C _{15:0}	—	—	3.9	—
C _{16:0}	5.8	6.9	—	4.4
C _{18:0}	3.7	—	—	—
Branched saturated acids				
iso-C _{14:0}	—	1.8	—	1.5
iso-C _{15:0}	21.4	14.9	17.9	19.7
anteiso-C _{15:0}	4.0	7.8	8.6	9.1
iso-C _{16:0}	1.5	5.3	6.3	4.3
iso-C _{16:1}	—	—	2.7	—
anteiso-C _{16:0}	1.3	—	—	—
iso-C _{17:0}	1.5	—	—	—
anteiso-C _{17:1ω9c}	—	—	2.2	2.7
Unsaturated acids:				
C _{15:1ω6c}	—	1.4	1.1	1.2
C _{17:1ω6c}	—	—	3.6	—
C _{18:1ω9c}	1.7	—	—	—
Branched unsaturated acids:				
iso-C _{15:0} 3-OH	3.2	2.0	1.5	2.0
C _{15:0} 2-OH	1.5	2.7	2.8	3.4
iso-C _{16:0} 3-OH	2.6	8.1	3.9	4.5
iso-C _{17:0} 3-OH	22.5	20.0	12.4	15.9
C _{17:0} 2-OH	3.8	—	5.1	6.4
iso-C _{17:1ω9c}	5.7	2.9	5.5	4.0
Summed feature*	16.7	20.5	11.6	13.0

*Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system, here, comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH.

MK-6 was found to be the predominant isoprenoid quinone. The strain contained iso-C_{15:0} (21.4 %), iso-C_{17:0} 3-OH (22.5 %) and a summed feature consisting of iso-C_{15:0} 2-OH and/or C_{16:1ω7c} (16.7 %) as the predominant fatty acids. Table 2 shows the detailed fatty acid profile of strain RA5-111^T and related species. The presence of anteiso-C_{16:0} (1.3 %), iso-C_{17:0} (1.5 %), C_{18:0} (3.7 %) and C_{18:1ω9c} (1.7 %) was characteristic for strain RA5-111^T. The major polar lipid was phosphatidylethanolamine (PE); unknown aminophospholipids and other unknown polar lipids were also present. The presence of PE as the major lipid is in line with data reported for members of related genera (Nedashkovskaya *et al.*, 2004, 2006; Lim *et al.*, 2008; Kang & Lee, 2010). The DNA G+C content of RA5-111^T was 39.1 mol%, which was close to the range reported for the genus *Gramella* (40.0–41.1 mol%).

Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain RA5-111^T is a member of the family *Flavobacteriaceae*, forming a coherent cluster with species of the genus *Gramella* (Fig. 1). 16S rRNA sequence similarities between RA5-111^T and the type strains of *G. echinicola*, *G. portivictoriae* and *G. marina* were 97.7 % (31 bp differences out of 1356), 97.3 % (36 bp differences) and 96.2 % (51 bp differences), respectively. The mean DNA–DNA relatedness values between RA5-111^T and the type strains of *G. echinicola* and *G. portivictoriae* were 48 % and 47 %, respectively, thus confirming that the isolate belongs to a novel taxon in the genus *Gramella*.

The combined phenotypic, chemotaxonomic and phylogenetic evidence clearly indicates that strain RA5-111^T

Fig. 1. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing the relationship between strain RA5-111^T and members of representative species of the genus *Gramella*. Asterisks indicate branches that were also recovered using the Fitch–Margoliash, maximum-likelihood and maximum-parsimony algorithms. Levels of bootstrap support >50 %, based on neighbour-joining analysis of 1000 resampled datasets using Jukes–Cantor distances, are given at branch points. Bar, 0.01 substitutions per nucleotide position.

represents a novel species of the genus *Gramella*, for which the name *Gramella gaetbulicola* sp. nov. is proposed.

Description of *Gramella gaetbulicola* sp. nov.

Gramella gaetbulicola (ga.et.bu.li'co.la. N.L. n. *gaetbulum* *gaetbul*, the Korean name for a tidal flat; L. suff. *-cola* from L. masc. or fem. n. *incola* a dweller, inhabitant; N.L. masc. n. *gaetbulicola* a dweller of a tidal flat).

Cells are Gram-reaction-negative, strictly aerobic, non-spore-forming, motile by gliding and rod-shaped ($1.2\text{--}2.7 \times 0.6\text{--}0.7\ \mu\text{m}$). Colonies are yellow–orange, circular and convex with entire margins on marine agar. Growth occurs at $4\text{--}42\ ^\circ\text{C}$ (optimum $30\text{--}37\ ^\circ\text{C}$), at pH 4–11 (optimum pH 7) and in 3–10 % (w/v) NaCl within 7 days (optimum 3–5 %). Catalase- and oxidase-positive. Acetoin is produced (positive Voges–Proskauer test). Citrate is not utilized. Nitrate and nitrite are not reduced and indole and hydrogen sulfide are not produced. Hydrolyses DNA, starch and Tweens 20, 40, 60, and 80, but not casein or cellulose. Acid is not produced from amygdalin, arabinose, glucose, inositol, mannitol, melibiose, rhamnose, sorbitol or sucrose. As determined by API ZYM and API 20 E strips, alkaline and acid phosphatase, esterase (C4), esterase lipase (C8), α - and β -galactosidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, tryptophan deaminase, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase and gelatinase activities are present, but lipase (C14), β -glucuronidase, α -mannosidase, α -fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and urease activities are absent. Using Biolog GN2 MicroPlates, the following substrates are utilized: α -cyclo-dextrin, dextrin, glycogen, cellobiose, D-fructose, D-galactose, gentiobiose, α -D-glucose, maltose, D-psicose, raffinose, sucrose, trehalose, turanose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, D-gluconic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, succinic acid, succinamic acid, L-alanine, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-leucine, L-proline, L-threonine, inosine, uridine, thymidine, α -D-glucose 1-phosphate and D-glucose 6-phosphate. The predominant menaquinone is MK-6. The major fatty acids are iso-C_{15:0}, iso-C_{17:0} 3-OH and a summed feature (iso-C_{15:0} 2-OH and/or C_{16:1} ω 7c). PE, unknown aminophospholipids and other unknown polar lipids are present.

The type strain, RA5-111^T (=KCTC 23022^T =JCM 16528^T =NBRC 106272^T), was isolated from the foreshore soil in the Buan area, Jeonbuk, Republic of Korea. The DNA G+C content of the type strain is 39.1 mol%.

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