Gramella flava sp. nov., a member of the family *Flavobacteriaceae* isolated from seawater

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A novel Gram-negative, yellow-pigmented, aerobic, motile by gliding, rod-shaped marine bacterium (JLT2011^T) was isolated from surface seawater. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain JLT2011^T could be assigned to the genus *Gramella* and was most closely related to *Gramella gaetbulicola*, with 96.2 % similarity. The genomic DNA G+C content was 42.1 %. The predominant fatty acids were $C_{16:0}$, iso-$C_{15:0}$, $C_{18:0}$ and summed feature 3 ($C_{16:1}\omega6c/C_{16:1}\omega7c$). The major menaquinone was MK-6. The major components of the polar lipid profile were phosphatidylglycerol, diphosphatidylglycerol and sphingoglycolipid. On the basis of phenotypic, genotypic and taxonomic data presented, strain JLT2011^T is considered to represent a novel species of the genus *Gramella*, for which the name *Gramella flava* sp. nov. is proposed; the type strain is JLT2011^T (=CGMCC 1.12375^T=LMG 27360^T).

The genus *Gramella*, a member of the family *Flavobacteriaceae*, was first described by Nedashkovskaya *et al.* (2005). At the time of writing, this genus comprised four species with validly published names: *Gramella echinicola* and *Gramella marina* were isolated from sea urchin (Nedashkovskaya *et al.*, 2005, 2010), *Gramella portivictoriae* was isolated from marine sediment (Lau *et al.*, 2005) and *Gramella gaetbulicola* was isolated from foreshore soil (Cho *et al.*, 2011).

During a survey of the biodiversity of the microbial community associated with the south-eastern Pacific, a novel strain, designated JLT2011^T, was isolated from a surface seawater sample from the cruise of China Global Ocean Sampling with a standard dilution-to-extinction culturing method. After primary incubation on marine agar 2216 (MA; BD) at 30 °C for 2 weeks, strain JLT2011^T was purified as single colonies. The strain was maintained routinely on MA and was preserved in marine broth 2216 (MB; BD) supplemented with 15 % (v/v) glycerol at −80 °C.

The Gram-stain reaction was determined as described by Gerhardt *et al.* (1994). Cell morphology was observed using transmission electron microscopy (JEM-1230; JEOL) after negative staining with 1 % (w/v) phosphotungstic acid with cells grown for 2 days at 30 °C. Cell motility was determined by the semi-solid puncture method.

Oxidase and catalase activity, hydrolysis of casein, starch, gelatin and aesculin were determined according to the protocols of Smibert & Krieg (1994). Other biochemical and physiological properties were determined with the API 20E, API 20 NE and API ZYM strips (BioMérieux) and Biolog GN_2 MicroPlate systems according to the manufacturers' instructions.

Growth at various temperatures (4, 10, 20, 25, 30, 35, 40 and 50 °C) was measured on MB medium. Growth at different pH was determined by adjusting the final pH of MB to pH 4–10 (intervals of 1 pH unit) with 1 M HCl or NaOH. Growth at various NaCl concentrations was investigated on MB medium with final NaCl concentrations of 0, 0.5 and 1–13 % (at intervals of 1 %). Susceptibility to antibiotics was tested by using the disc-diffusion plate (Kirby–Bauer) method according to the methods of Fraser & Jorgensen (1997) with discs containing ampicillin (10 μg), carbenicillin (100 μg), chloromycetin (5 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), lincomycin (2 μg), neomycin (30 μg), novobiocin (5 μg), penicillin (10 μg), polymyxin B (300 μg), rifampicin (5 μg), streptomycin (10 μg), tetracycline (30 μg) or vancomycin (30 μg).

Cellular fatty acid analysis was carried out as described by Komagata & Suzuki (1987) with cells grown on MA medium for 2 days at 30 °C. Polar lipids were separated by two-dimensional TLC (Collins *et al.*, 1980; Kates 1986). Isoprenoid quinones were extracted from 100 mg freeze-dried cells using the two-stage method described by Tindall (1990a, b) and analysed using reverse-phase HPLC.

The genomic DNA was isolated according to the method of Marmur (1961) and the nucleotide sequence of the

The GenBank accession number for the 16S rRNA gene sequence of strain JLT2011^T is JX397931. The whole-genome shotgun sequence of strain JLT2011^T has been deposited under the accession number PRJNA175612.

Six supplementary figures are available with the online version of this paper.

Table 1. Differential characteristics of strain JLT2011^T and type strains of related species of the genus *Gramella*

All data were obtained from this study. Strains: 1, JLT2011^T; 2, *G. echinicola* KMM 6050^T; 3, *G. portivictoriae* UST040801-001^T; 4, *G. marina* KMM 6048^T; 5, *G. gaetbulicola* RA5-111^T. All strains were positive for catalase and oxidase activities; gliding motility; hydrolysis of aesculin; activity of alkaline phosphatase, esterase lipase (C8), leucine arylamidase and β-galactosidase. All taxa were negative for nitrate reduction; hydrolysis of gelatin; H_2S and indole production; citrate utilization; acid production from mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose and amygdalin; and activity of cystine arylamidase, trypsin, β-glucuronidase and β-fucosidase. +, Positive; −, negative.

Characteristic	1	2	3	4	5
Optimum growth temperature (°C)	30	25	35	25–35	25–35
Hydrolysis of:					
DNA	+	−	−	−	−
Starch	+	−	−	−	−
Casein	−	−	−	−	+
Urea	−	+	+	−	+
Acid from glucose	−	−	−	+	−
Acetoin production	+	−	−	−	−
Utilization of:					
Maltose	−	+	+	−	+
L-arabinose	−	−	−	+	−
Activity of					
Esterase (C4)	+	−	−	+	−
Lipase (C14)	+	−	−	−	−
Valine arylamidase	+	−	+	+	+
α-Chymotrypsin	−	+	−	−	−
α-Galactosidase	+	−	−	−	−
α-Glucosidase	+	−	+	+	+
N-acetylglucosaminidase	+	+	+	−	−
α-Mannosidase	+	−	−	−	−

JLT2011^T genome was obtained using massively parallel pyrosequencing technology (454 GS FLX; Roche). This whole-genome shotgun sequence has been deposited at DDBJ/EMBL/GenBank under the accession number PRJNA175612). The DNA G+C content of strain JLT2011^T was 42.1 mol%, which was slightly higher than the range reported previously for the genus *Gramella* (39.6–40.0 mol%).

Phylogenetic analysis based on 16S rRNA gene sequences was performed as described by Kim *et al.* (1998). The 16S rRNA gene sequence of strain JLT2011^T was aligned together with corresponding sequences of representative species of the genus *Gramella* and those available from the GenBank database by using EzTaxon-e (Kim *et al.*, 2012) to determine approximate phylogenetic affiliation. Phylogenetic analysis was performed using BioEdit (Hall, 1999) and phylogenetic trees were reconstructed by using the neighbour-joining and maximum-parsimony methods within the MEGA software (Kumar *et al.*, 2004).

Table 2. Cellular fatty acid profiles of strain JLT2011^T and type strains of species of the genus *Gramella*

All data were obtained from this study. Fatty acid compositions were analysed in this study with cells grown on marine agar 2216 for 2 days. Strains: 1, JLT2011^T; 2, *G. echinicola* KMM 6050^T; 3, *G. portivictoriae* UST040801-001^T; 4, *G. marina* KMM 6048^T; 5, *G. gaetbulicola* RA5-111^T. −, Not detected. Summed features are groups of two or three fatty acids that cannot be separated by the MIDI system.

Fatty acid	1	2	3	4	5
Straight-chain:					
$C_{10:0}$	−	−	2.0	−	−
$C_{12:0}$	5.7	−	22.4	1.9	−
$C_{14:0}$	5.9	−	11.4	6.0	−
$C_{16:0}$	15.2	19.6	17.6	13.6	5.4
$C_{18:0}$	11.6	22.1	9.1	8.8	3.5
$C_{20:0}$	−	−	−	−	7.2
Branched saturated acids:					
iso-$C_{15:0}$	13.3	8.7	7.7	7.9	28.4
anteiso-$C_{15:0}$	3.0	3.2	3.2	6.1	4.3
iso-$C_{16:0}$	1.5	10.2	1.5	5.5	5.5
anteiso-$C_{17:0}$	1.3	−	3.2	−	−
Unsaturated acids:					
$C_{14:1}\omega5c$	2.0	−	−	−	−
$C_{17:1}\omega6c$	1.1	3.7	−	−	−
$C_{18:1}\omega7c$	1.9	−	1.0	6.2	−
$C_{18:1}\omega9c$	3.2	−	2.2	−	−
Branched unsaturated acids:					
$C_{15:0}$ 2-OH	1.1	−	−	−	−
$C_{15:0}$ 3-OH	3.1	−	−	−	−
iso-$C_{15:0}$ 3-OH	2.4	−	−	−	−
iso-$C_{16:0}$ 3-OH	1.8	−	1.6	−	−
iso-$C_{17:0}$ 3-OH	9.9	7.7	5.0	4.9	11.4
$C_{17:0}$ 2-OH	1.7	−	1.5	−	−
iso-$C_{17:1}\omega9c$	1.9	12.1	1.6	8.7	16.4
anteiso-$C_{17:1}\omega9c$	−	−	−	4.3	−
Summed feature 3*	12.5	12.7	6.1	19.7	18.0
Summed feature 4*	−	−	−	6.5	−
Summed feature 6*	−	−	1.1	−	−

*Summed feature 3: $C_{16:1}\omega7c/C_{16:1}\omega6c$; summed feature 4: iso-$C_{17:0}$I/ anteiso- $C_{17:0}$B; summed feature 6: $C_{19:1}\omega11c/C_{19:1}\omega9c$.

Strain JLT2011^T formed yellow, circular, convex and opaque colonies on marine agar at 30 °C for 48 h. Cells were Gram-negative, aerobic, slowly gliding, non-spore-forming and rod-shaped. Growth occurred at 10–35 °C (optimum 30 °C); at pH 4–9 (optimum 5–8) and in 0.5–10 % (w/v) NaCl (optimum 2 % NaCl). Strain JLT2011^T was susceptible to vancomycin, carbenicillin, ampicillin, tetracycline, rifampicin, chloromycetin, erythromycin, penicillin, novobiocin and lincomycin. Other phenotypic characteristics are given in the species description and Table 1.

The major menaquinone was MK-6. As Table 2 shows, the dominant fatty acids of JLT2011^T were $C_{16:0}$ (15.2 %),

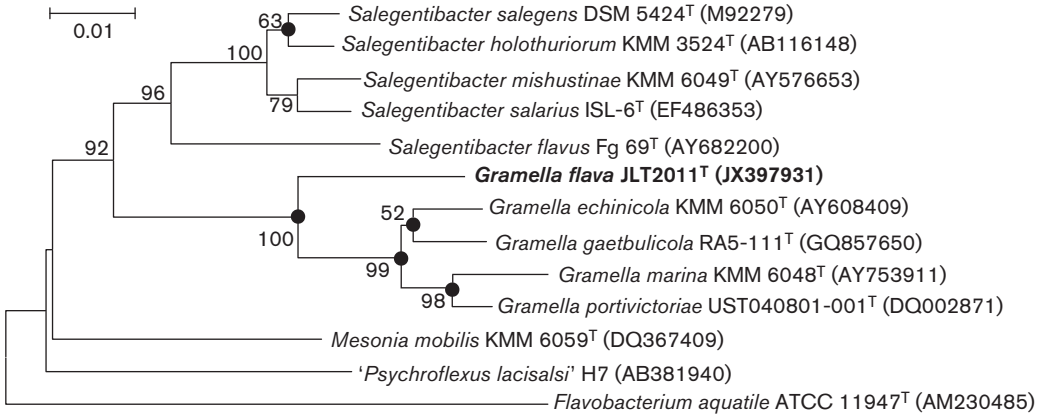

Fig. 1. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain JLT2011^T and members of representative species of the genus *Gramella* and related genera. Bootstrap percentages analyses were based on 1000 replications and only values >50 % are shown. *Flavobacterium aquatile* ATCC 11947^T was used to root the tree. Filled circles indicate that the corresponding nodes were recovered in the trees generated with the neighbour-joining and maximum-parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

iso-$C_{15:0}$ (13.3 %), summed feature 3 consisting of $C_{16:1}\omega6c$/$C_{16:1}\omega7c$ (12.5 %) and $C_{18:0}$ (11.6 %). The polar lipid profile of strain JLT2011^T consisted of phosphatidylglycerol, diphosphatidylglycerol, sphingoglycolipid, one unknown glycolipid and two unknown polar lipids Fig. S2. Strain JLT2011^T was identified as representing a member of the genus *Gramella* based on 16S rRNA gene phylogenetic analysis (Fig. 1). Based on the phylogenetic tree, strain JLT2011^T was closely related to species of the genus *Gramella*: *G. gaetbulicola* (96.2 % similarity), *G. portivictoriae* (95.7 %), *G. marina* (95.0 %) and *G. gaetbulicola* (94.7 %), respectively.

On the basis of phylogenetic analysis and phenotypic characteristics, strain JLT2011^T is considered to represent a novel species of the genus *Gramella*, for which the name *Gramella flava* sp. nov. is proposed.

Description of *Gramella flava* sp. nov.

Gramella flava (fla′va. L. fem. adj. *flava* yellow, the colour of its colonies or pigment).

Cells are Gram-negative, aerobic, motile by gliding, do not form spores and are rod-shaped (0.6–0.8 × 3.0–6.0 μm) (Fig. S1). Colonies are yellow–orange, 2.0–4.0 mm in diameter circular and convex on marine agar. Growth occurs at 10–35 °C (optimum 30 °C), at pH 4–9 (optimum 5–8) and in 0.5–10 % (w/v) NaCl (optimum 2–8 %). Positive for hydrolysis of aesculin, DNA, starch and Tween 40 and Voges–Proskauer test (acetoin production). Negative for gelatin, urea, chitin and casein hydrolysis. Citrate is not utilized. Nitrate is not reduced and indole and H_2S are not produced. Acid is not produced from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin or arabinose in the API 20E tests. According to API ZYM tests, alkaline and acid phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, α- and β-galactosidases, α- and β-glucosidases, *N*-acetyl-β-glucosaminidase and α-mannosidase are positive. In tests with Biolog GN_2 microplates, the following carbon substrates are utilized: α-cyclodextrin, glycogen, α-D-glucose, α-lactose, D-mannose, melibiose, raffinose, sucrose, monomethylsuccinate, trehalose, α-ketoglutaric acid, succinic acid, L-alanine, L-alanyl glycine, 2,3-butanediol, glucose 1-phosphate and glucose 6-phosphate. Sensitive to vancomycin, carbenicillin, ampicillin, tetracycline, rifampicin, chloromycetin, erythromycin, penicillin, novobiocin and lincomycin, but resistant to kanamycin, gentamicin, polymyxin B, neomycin and streptomycin. The major fatty acids are $C_{16:0}$, iso-$C_{15:0}$, summed feature 3 ($C_{16:1}\omega6c$/$C_{16:1}\omega7c$) and $C_{18:0}$. The predominant menaquinone is MK-6. The major polar lipids are phosphatidylglycerol, diphosphatidylglycerol and sphingoglycolipid.

The type strain, JLT2011^T (=CGMCC 1.12375^T=LMG 27360^T), was isolated from surface seawater in the south-eastern Pacific Ocean. The DNA G+C content of the type strain is 42.1 mol%.

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References

Cho, S. H., Chae, S. H., Cho, M., Kim, T. U., Choi, S., Han, J. H., Kim, Y. T., Joung, Y., Joh, K. & other authors (2011). *Gramella gaetbulicola* sp. nov., a member of the family *Flavobacteriaceae* isolated from foreshore soil. *Int J Syst Evol Microbiol* **61**, 2654–2658.

Collins, M. D., Goodfellow, M. & Minnikin, D. E. (1980). Fatty acid, isoprenoid quinone and polar lipid composition in the classification of *Curtobacterium* and related taxa. *J Gen Microbiol* **118**, 29–37.

Fraser, S. L. & Jorgensen, J. H. (1997). Reappraisal of the antimicrobial susceptibilities of *Chryseobacterium* and *Flavobacterium* species and methods for reliable susceptibility testing. *Antimicrob Agents Chemother* **41**, 2738–2741.

Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.

Kates, M. (1986). *Techniques of Lipidology: Isolation, Analysis and Identification of Lipids (Laboratory Techniques in Biochemistry and Molecular Biology)*, vol 3, part 2, 2nd edn, Amsterdam: Elsevier

Kim, S. B., Falconer, C., Williams, E. & Goodfellow, M. (1998). *Streptomyces thermocarboxydovorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov., two moderately thermophilic carboxydotrophic species from soil. *Int J Syst Bacteriol* **48**, 59–68.

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Park, S. C., Jeon, Y. S., Lee, J. H. & other authors (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Lau, S. C. K., Tsoi, M. M. Y., Li, X., Plakhotnikova, I., Dobretsov, S., Wong, P. K. & Qian, P. Y. (2005). *Gramella portivictoriae* sp. nov., a novel member of the family *Flavobacteriaceae* isolated from marine sediment. *Int J Syst Evol Microbiol* **55**, 2497–2500.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Nedashkovskaya, O. I., Kim, S. B., Lysenko, A. M., Frolova, G. M., Mikhailov, V. V., Bae, K. S., Lee, D. H. & Kim, I. S. (2005). *Gramella echinicola* gen. nov., sp. nov., a novel halophilic bacterium of the family *Flavobacteriaceae* isolated from the sea urchin *Strongylocentrotus intermedius*. *Int J Syst Evol Microbiol* **55**, 391–394.

Nedashkovskaya, O. I., Kim, S. B. & Bae, K. S. (2010). *Gramella marina* sp. nov., isolated from the sea urchin *Strongylocentrotus intermedius*. *Int J Syst Evol Microbiol* **60**, 2799–2802.

Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Tindall, B. J. (1990a). A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

Tindall, B. J. (1990b). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.