Winogradskyella arenosi sp. nov., a member of the family *Flavobacteriaceae* isolated from marine sediments from the Sea of Japan

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An aerobic, Gram-negative, yellow-pigmented, gliding bacterial strain, designated R60^T, was isolated from a marine sediment sample obtained from the Sea of Japan and was characterized by using a polyphasic approach. 16S rRNA gene sequence analysis showed that strain R60^T belonged to the genus *Winogradskyella*, sharing <97 % sequence similarity with the type strains of recognized *Winogradskyella* species. The main fatty acids of strain R60^T were iso-$C_{15:0}$ 3-OH, iso-$C_{16:0}$ 3-OH, iso-$C_{17:0}$ 3-OH, anteiso-$C_{17:0}$ 3-OH and iso-$C_{15:0}$, consistent with its assignment to the genus *Winogradskyella*. On the basis of phenotypic distinctiveness and phylogenetic divergence, strain R60^T is considered to represent a novel species of the genus *Winogradskyella*, for which the name *Winogradskyella arenosi* sp. nov. is proposed. The type strain is R60^T (=KMM 3968^T =NRIC 0748^T= JCM 15527^T).

The genus *Winogradskyella* was proposed by Nedashkovskaya *et al.* (2005) to accommodate bacteria recovered from brown and green algae from the Peter the Great Bay of the Sea of Japan, with the description of three species: *Winogradskyella thalassocola*, *Winogradskyella epiphytica* and *Winogradskyella eximia*. Subsequently, Lau *et al.* (2005) described *Winogradskyella poriferorum* to accommodate a strain isolated from a sponge collected from the Bahamas. Here, we report the characterization of a Gram-negative, aerobic, yellow-pigmented gliding bacterial strain, designated R60^T, isolated from a marine sediment sample collected from the Peter the Great Bay of the Sea of Japan. Phylogenetic analysis based on 16S rRNA gene sequence data demonstrated that strain R60^T belongs to the genus *Winogradskyella* and may represent a novel species of this genus. Differential phenotypic properties along with phylogenetic distinctiveness supported the proposal that strain R60^T represents a novel species of the genus *Winogradskyella*.

Strain R60^T was recovered from a marine sediment sample collected from Peter the Great Bay of the Sea of Japan, Russia, in June 2002. It grew aerobically on marine agar 2216 (MA) or marine broth 2216 (MB; both from Difco) at 25–28 °C, and the strain was stored at −80 °C in MB supplemented with 30 % (v/v) glycerol.

Gliding motility was observed according to the method described by Bowman (2000) by using oil-immersion phase-contrast microscopy (AX70; Olympus). The presence of flexirubin pigments was investigated as described by Fautz & Reichenbach (1980). The Gram reaction, oxidase and catalase reactions and hydrolysis of casein, gelatin, starch, alginate, cellulose (CM-cellulose and filter paper), chitin and Tweens 20, 40 and 80 were tested according to the standard methods described by Smibert & Krieg (1994). Formation of H_2S from thiosulfate was tested by using a lead acetate paper strip. Acid production from carbohydrates was examined by using the oxidation/fermentation medium of Leifson (1963) for marine bacteria, with carbohydrates tested at a concentration of 1 % (w/v). Growth at different temperatures, pH and salinities was studied as described previously (Romanenko *et al.*, 2004, 2007). Biochemical tests were carried out by using API 20NE, API ID32 GN, API 50 CH and API ZYM test kits (bioMérieux) as described by the manufacturer, and results were read daily over 5 days incubation at 25 °C. For fatty acid analysis, strain R60^T was cultivated on MA at 25 °C for 3 days, and lipids were extracted by using the chloroform–methanol extraction method of Bligh & Dyer (1959). Fatty acid methyl esters were obtained by using

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R60^T is AB438962.

A maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences showing the position of strain R60^T among recognized *Winogradskyella* species and related taxa is available with the online version of this paper.

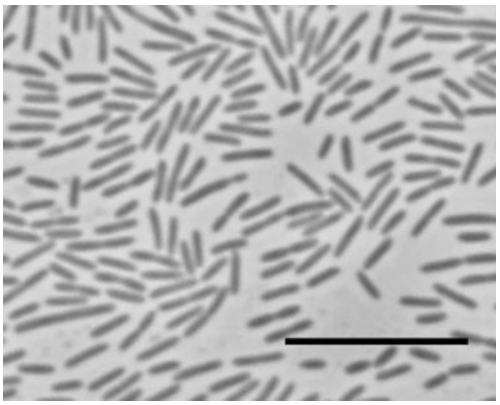

Fig. 1. Photomicrograph showing cells of strain R60^T observed by oil-immersion phase-contrast microscopy. Bar, 10 μm.

Table 1. Differential phenotypic characteristics between strain R60^T and recognized *Winogradskyella* species

Taxa: 1, strain R60^T; 2, *W. poriferorum* (data from Lau *et al.*, 2005); 3, *W. epiphytica*; 4, *W. eximia*; 5, *W. thalassocola* (data for taxa 3–5 from Nedashkovskaya *et al.*, 2005). All are positive for gliding motility, oxidase, catalase and alkaline phosphatase activities, and gelatin and Tween 40 hydrolysis; all are negative for flexirubin pigments, indole production, nitrate reduction, β-galactosidase and urease production, cellulose and chitin hydrolysis, and utilization of L-arabinose, sucrose, D-mannitol, inositol, D-lactose, D-sorbitol and citrate. +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3	4	5
H_2S production	+	−	−	−	−
Growth at/in:					
4 °C	+	−	+	+	+
35 °C	+	+	+	−	−
37 °C	−	+	+	−	−
44 °C	−	+	−	−	−
8 % NaCl	+	−	+	−	+
Acid production from:					
D-Glucose	+	ND	−	+	+
Maltose	+	ND	−	+	+
Sucrose	−	ND	−	+	−
Cellobiose	−	ND	−	−	+
D-Mannitol	+	ND	−	+	−
Hydrolysis of:					
Casein	−	−	−	+	−
DNA	−	+	+	−	−
Tween 20	+	+	+	+	−
Tween 80	+	+	+	−	−
Starch	+	−	−	+	−
Agar	−	−	+	+	+
Utilization of:					
D-Glucose	−	−	−	+	+
Cellobiose	−	−	ND	−	ND
D-Mannose	−	−	−	+	+
Maltose	−	−	ND	+	ND

Table 2. Cellular fatty acid composition (%) of strain R60^T and recognized *Winogradskyella* species

Taxa: 1, strain R60^T; 2, *W. poriferorum* (data from Lau *et al.*, 2005); 3, *W. epiphytica*; 4, *W. eximia*; 5, *W. thalassocola* (data for taxa 3–5 from Nedashkovskaya *et al.*, 2005). Results are percentages of total fatty acids; −, not found.

Fatty acid	1	2	3	4	5
iso-$C_{13:0}$	−	2.6±0.1*	−	−	−
iso-$C_{14:0}$	−	1.2±0.1	4.5	1.4	2.6
iso-$C_{14:0}$ 3-OH	−	0.5±0.1	1.6	−	0.9
iso-$C_{14:1}$	−	2.0±0.3	1.4	−	−
$C_{15:0}$	1.7	−	1.2	6.7	7.9
anteiso-$C_{15:0}$	5.6	−	15.9	7.0	4.9
iso-$C_{15:0}$	9.3	12.6±1.1	6.7	25.6	8.7
anteiso-$C_{15:0}$ 3-OH	3.4	−	−	−	−
iso-$C_{15:0}$ 3-OH	17.0	9.8±1.0	2.9	2.6	11.9
$C_{15:0}$ 2-OH	−	3.1±0.5	3.3	1.0	1.8
$C_{15:0}$ 3-OH	0.9	2.4±0.7	−	−	2.5
$C_{15:1}\omega6$	1.1	−	−	−	6.5
anteiso-$C_{15:1}$	0.9	1.5±0.2	6.3	1.4	1.6
iso-$C_{15:1}$	5.2	20.9±0.6	8.1	10.4	11.4
anteiso-$C_{15:0}$ 2-OH	4.9	−	−	−	−
iso-$C_{15:0}$ 2-OH	3.6	−	−	−	−
iso-$C_{15:0}$ 2-OH/ $C_{16:1}\omega7$	−	9.8±0.8	5.1	6.1	4.2
$C_{16:0}$	1.0	−	−	−	−
$C_{16:0}$ 10-methyl	−	−	−	6.3	−
$C_{16:0}$ 3-OH	−	1.3±0.1	−	−	1.0
iso-$C_{16:0}$	0.6	0.5±0.2	3.7	5.7	0.8
iso-$C_{16:0}$ 3-OH	13.8	11.4±0.1	17.1	32	18.1
anteiso-$C_{16:0}$ 3-OH	1.2	−	−	−	−
iso-$C_{16:1}$	−	0.8±0.3	3.5	4.7	2.7
$C_{17:0}$ 2-OH	−	0.3	5.2	1.0	0.8
$C_{17:0}$ cyclo	−	−	−	2.4	−
iso-$C_{17:0}$ 3-OH	17.1	10.2±0.7	7.3	6.7	5.4
anteiso-$C_{17:0}$ 3-OH	8.5	−	−	−	−
anteiso-$C_{17:1}$	−	−	−	2.3	−
iso-$C_{17:1}\omega9$	−	−	1.1	−	0.6
$C_{17:1}\omega6$	1.0	0.2	1.9	−	0.9
$C_{18:1}$	2.1	−	−	−	−
Unknown	1.0	3.9±0.6	3.7	5.6	4.8

*Mean±SD.

acid methanolysis as described by Rowe *et al.* (2000). The resultant fatty acid methyl esters were extracted by hexane and were analysed by using a GLC-MS Hewlett Packard model 6890 gas chromatograph equipped with an HP 5 MS 5 % phenyl methyl siloxane capillary column (30 m × 250 μm × 0.25 μm), connected to a Hewlett Packard model 5973 mass spectrometer. The 16S rRNA gene sequence of strain R60^T (1430 nt) was determined as described by Shida *et al.* (1997). The sequence obtained was compared with 16S rRNA gene sequences retrieved from GenBank databases by using the FASTA program (Pearson & Lipman, 1988). Phylogenetic analysis of 16S

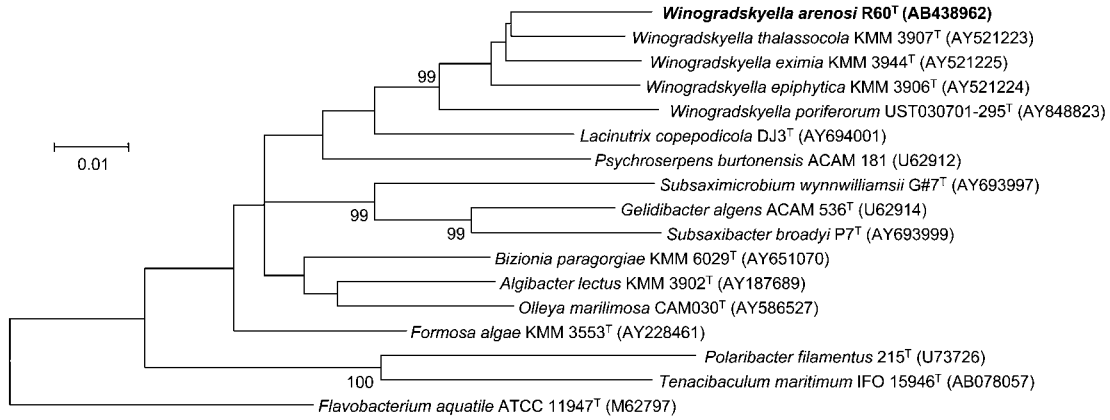

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences available from GenBank (accession numbers in parentheses), showing the position of strain R60^T (=KMM 3968^T) among the type strains of recognized *Winogradskyella* species and related taxa. Phylogenetic analysis was performed by using the software package MEGA4 (Tamura *et al.*, 2007) after multiple alignment of the data by CLUSTAL X (version 1.83; Thompson *et al.*, 1997). Bootstrap values based on 1000 replications are given as percentages at branch points. Bar, 0.01 substitutions per nucleotide position.

rRNA gene sequences was performed by using the software package MEGA4 (Tamura *et al.*, 2007) after multiple alignment of the data by CLUSTAL X (version 1.83; Thompson *et al.*, 1997). Phylogenetic trees were constructed by using the neighbour-joining and maximum-parsimony methods and distances were calculated according to the Kimura two-parameter model. The robustness of the trees was estimated by bootstrap analysis of 1000 replicates.

Phenotypically, strain R60^T matched the description of the genus *Winogradskyella*, being Gram-negative, aerobic, oxidase- and catalase-positive, motile by means of gliding, yellow-pigmented and halophilic. The morphological, physiological and biochemical properties of strain R60^T are shown in Fig. 1, Table 1 and in the species description below. The fatty acid profile of strain R60^T was characterized by the abundance of branched 3-hydroxy components, including iso-$C_{15:0}$ 3-OH, iso-$C_{16:0}$ 3-OH, iso-$C_{17:0}$ 3-OH and anteiso-$C_{17:0}$ 3-OH (47.9 % of the total) as the predominant fatty acids (Table 2). The presence of iso-$C_{15:0}$ (9.3 %), iso-$C_{15:0}$ 3-OH (17.0 %), iso-$C_{16:0}$ 3-OH (13.8 %), iso-$C_{17:0}$ 3-OH (17.1 %) and iso-$C_{15:1}$ (5.2 %) is in accordance with data reported previously for recognized *Winogradskyella* species (Lau *et al.*, 2005; Nedashkovskaya *et al.*, 2005), although some quantitative and qualitative differences were observed (Table 2). Unlike recognized *Winogradskyella* species, strain R60^T contained a significant amount of anteiso-$C_{17:0}$ 3-OH (8.5 %), smaller amounts of anteiso-$C_{15:0}$ 2-OH (4.9 %), anteiso-$C_{15:0}$ 3-OH (3.4 %), $C_{18:1}$ (2.1 %) and anteiso-$C_{16:0}$ 3-OH (1.2 %) and did not contain iso-$C_{14:0}$, iso-$C_{16:1}$ or $C_{15:0}$ 2-OH.

Phylogenetic analysis based on 16S rRNA gene sequencing showed that strain R60^T was affiliated to the genus *Winogradskyella*, and formed an independent lineage in the neighbour-joining phylogenetic tree (Fig. 2). The same relationship was also evident in the 16S rRNA gene sequence dendrogram generated with the maximum-parsimony algorithm (Supplementary Fig. S1, available in IJSEM Online). Levels of 16S rRNA gene sequence similarity between strain R60^T and the type strains of recognized *Winogradskyella* species (94.6–96.6 %) were below the 97.0 % threshold recommended for the discrimination of different bacterial species (Stackebrandt & Goebel, 1994). Based on data from phylogenetic analysis, strain R60^T could not be assigned to any recognized species and thus represents a novel species of the genus *Winogradskyella*. The phylogenetic distinctiveness of strain R60^T was supported by phenotypic differences in its temperature and salinity ranges for growth, substrate utilization pattern and metabolic properties (Table 1). Based on the combination of phenotypic and biochemical characteristics, as well as phylogenetic position, strain R60^T is considered to represent a novel species of the genus *Winogradskyella*, for which the name *Winogradskyella arenosi* sp. nov. is proposed.

Description of *Winogradskyella arenosi* sp. nov.

Winogradskyella arenosi (a.re.no′si. L. gen. n. *arenosi* of a sandy place, dwelling in marine sand).

An aerobic, Gram-negative, oxidase- and catalase-positive, yellow-pigmented, gliding, rod-shaped bacterium (0.2–0.3 μm in diameter and 1.7–2.5 μm in length) (Fig. 1). Cells occur singly or in pairs. On MA, yellow-pigmented, shiny, smooth, slimy colonies, 3–5 mm in diameter, are formed. Flexirubin and diffusible pigments are not produced. Sodium ions are required for growth. Growth occurs in the presence of 0.5–9.0 % NaCl. Growth

temperature range is 4–35 °C, with optimum growth at 25–28 °C. No growth occurs at 37 °C. Casein, chitin, cellulose (CM-cellulose and filter paper), agar and DNA are not hydrolysed. Positive for starch hydrolysis. Gelatin is hydrolysed slowly over 3–5 days in both routine and API 20NE tests. Tweens 20, 40 and 80 are hydrolysed. H_2S production is positive. Acid is produced from D-glucose, maltose, mannose and D-mannitol, but not from sucrose, D-cellobiose, arabinose, rhamnose, galactose or xylose. In API 20NE tests, positive for hydrolysis of aesculin, but negative for reduction of nitrate, production of indole, production of acid from glucose and assimilation of arginine dihydrolase, urease, β-galactosidase, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenylacetate. In API 50CH tests, positive for utilization of aesculin, but negative for utilization of glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, D-lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, D-arabitol, gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. In API ZYM tests, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, weakly positive for α-glucosidase, but negative for lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Predominant fatty acids are iso-$C_{15:0}$ 3-OH, iso-$C_{16:0}$ 3-OH, anteiso-$C_{17:0}$ 3-OH, iso-$C_{17:0}$ 3-OH and iso-$C_{15:0}$; smaller amounts of anteiso-$C_{15:0}$, iso-$C_{15:1}$, anteiso-$C_{15:0}$ 2-OH, iso-$C_{15:0}$ 2-OH and anteiso-$C_{15:0}$ 3-OH are present; and iso-$C_{16:0}$, anteiso-$C_{16:0}$ 3-OH, $C_{16:0}$, $C_{15:0}$ 3-OH, $C_{15:1}\omega6$, anteiso-$C_{15:1}$, $C_{15:0}$, $C_{17:1}\omega6$ and $C_{18:1}$ occur as minor components.

The type strain, R60^T (=KMM 3968^T=NRIC 0748^T=JCM 15527^T), was isolated from a marine sediment sample collected from Peter the Great Bay of the Sea of Japan.

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References

Bligh, E. G. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.

Bowman, J. P. (2000). Description of *Cellulophaga algicola* sp. nov., isolated from the surface of Antarctic algae, and reclassification of *Cytophaga uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Cellulophaga uliginosa* comb. nov. *Int J Syst Evol Microbiol* **50**, 1861–1868.

Fautz, E. & Reichenbach, H. (1980). A simple test for flexirubin-type pigments. *FEMS Microbiol Lett* **8**, 87–91.

Lau, S. C. K., Tsoi, M. M. Y., Li, X., Plakhotnikova, I., Dobretsov, S., Lau, K. W. K., Wu, M., Wong, P.-K., Pawlik, J. R. & Qian, P.-Y. (2005). *Winogradskyella poriferorum* sp. nov., a novel member of the family *Flavobacteriaceae* isolated from a sponge in the Bahamas. *Int J Syst Evol Microbiol* **55**, 1589–1592.

Leifson, E. (1963). Determination of carbohydrate metabolism of marine bacteria. *J Bacteriol* **85**, 1183–1184.

Nedashkovskaya, O. I., Kim, S. B., Han, S. K., Snauwaert, C., Vancanneyt, M., Swings, J., Kim, K. O., Lysenko, A. M., Rohde, M. & other authors (2005). *Winogradskyella thalassocola* gen. nov., sp. nov., *Winogradskyella epiphytica* sp. nov. and *Winogradskyella eximia* sp. nov., marine bacteria of the family *Flavobacteriaceae*. *Int J Syst Evol Microbiol* **55**, 49–55.

Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.

Romanenko, L. A., Schumann, P., Rohde, M., Mikhailov, V. V. & Stackebrandt, E. (2004). *Reinekea marinisedimentorum* gen. nov., sp. nov., a novel gammaproteobacterium from marine coastal sediments. *Int J Syst Evol Microbiol* **54**, 669–673.

Romanenko, L. A., Uchino, M., Frolova, G. M. & Mikhailov, V. V. (2007). *Marixanthomonas ophiurae* gen. nov., sp. nov., a novel marine bacterium of the family *Flavobacteriaceae* isolated from a deep-sea brittle star. *Int J Syst Evol Microbiol* **57**, 457–462.

Rowe, N. J., Tunstall, J., Galbraith, L. & Wilkinson, S. G. (2000). Lipid composition of [*Pseudomonas*] *echinoides*: transfer to the genus *Sphingomonas*. *Microbiology* **146**, 3007–3012.

Shida, O., Takagi, H., Kadowaki, K., Nakamura, L. K. & Komagata, K. (1997). Transfer of *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the genus *Paenibacillus* and emended description of the genus *Paenibacillus*. *Int J Syst Bacteriol* **47**, 289–298.

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.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.