Winogradskyella lutea sp. nov., isolated from seawater, and emended description of the genus *Winogradskyella*

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A novel Gram-negative, orange-pigmented, rod-shaped, strictly aerobic, gliding, oxidase- and catalase-positive bacterial strain, A73^T, was isolated from seawater collected off Jeju, South Korea. 16S rRNA gene sequence similarity between A73^T and type strains of *Winogradskyella* species with validly published names ranged from 94.1 to 96.2 %. The dominant fatty acids of strain A73^T were iso-C_{15:1} G (19.1 %), iso-C_{15:0} (13.3 %), iso-C_{17:0} 3-OH (10.0 %) and iso-C_{15:0} 3-OH (7.2 %). The DNA G+C content of strain A73^T was 36.0 mol% and its major respiratory quinone was MK-6. On the basis of combined data from phenotypic and phylogenetic analyses, strain A73^T represents a novel species of the genus *Winogradskyella*, for which the name *Winogradskyella lutea* sp. nov. is proposed. The type strain is A73^T (=KCTC 23237^T =DSM 22624^T). An emended description of the genus *Winogradskyella* is also provided.

The genus *Winogradskyella* was established by Nedashkovskaya *et al.* (2005). The genus belongs to the family *Flavobacteriaceae* and, at the time of writing, included nine species with validly published names from various marine habitats: *Winogradskyella thalassocola* (the type species), *W. epiphytica* and *W. eximia* were isolated from marine brown algae (Nedashkovskaya *et al.*, 2005), *W. poriferorum* from a marine sponge (Lau *et al.*, 2005), *W. arenosi* from marine sediments (Romanenko *et al.*, 2009), *W. echinorum* from a sea urchin (Nedashkovskaya *et al.*, 2009), *W. rapida* (Pinhassi *et al.*, 2009) and *W. pacifica* (Kim & Nedashkovskaya, 2010) from seawater and *W. exilis* from a starfish (Ivanova *et al.*, 2010). The aim of the present study was to determine the exact taxonomic position of a novel bacterial isolate, strain A73^T, by using a polyphasic characterization that included determination of phenotypic properties and a detailed phylogenetic investigation based on 16S rRNA gene sequences. The results of phylogenetic analysis based on 16S rRNA gene sequences revealed that the novel isolate

belonged to the family *Flavobacteriaceae* and occupied a distinct lineage within the genus *Winogradskyella*.

Strain A73^T was isolated from a seawater sample that was collected off Jeju Island, Republic of Korea. The seawater sample was diluted with sterilized artificial seawater (Yoon *et al.*, 2002), spread onto a plate of marine agar 2216 (MA; Difco) and incubated at 30 °C. The isolate was routinely cultured on MA and stored as suspensions in 10 % glycerol/marine broth 2216 (MB; Difco) at –80 °C. After the optimum growth temperature had been determined, cultures were maintained on MA or in MB at 30 °C. For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony by a boiling method (Englen & Kelley, 2000). The crude extract served as DNA template for PCRs with *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). PCR products were purified using the AccuPrep PCR purification kit (Bioneer). Sequencing of the amplified 16S rRNA gene fragment was performed with an Applied Biosystems automatic sequencer (ABI 3730XL) at Solgent Co. (Daejeon, Korea). The 16S rRNA gene sequence (1341 bp) of strain A73^T was compared to those available in GenBank using the BLASTN program (Altschul *et al.*, 1990) to determine an approximate phylogenetic

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A73^T is FJ919968.

affiliation, and the sequence was then aligned with those of closely related strains by using the CLUSTAL W software (Thompson *et al.*, 1994). Sequence similarities were computed using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. Sequences were assembled using the BioEdit program (Hall, 2007). Phylogenetic trees were constructed with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Felsenstein, 1978) methods using the software package MEGA version 4.0 (Tamura *et al.*, 2007) and with the maximum-likelihood method (Felsenstein, 1981) using PHYLIP version 3.67 (Felsenstein, 2007). Evolutionary-distance matrices were calculated according to the algorithm of the Jukes–Cantor model (Jukes & Cantor, 1969) for the neighbour-joining method. The topologies of the resultant trees were evaluated using bootstrap analysis (Felsenstein, 1985) based on 1000 replicates. Phylogenetic analysis based on the nearly complete 16S rRNA gene sequence revealed that strain A73^T was a member of the genus *Winogradskyella* (Fig. 1). The distinct phylogenetic position of strain A73^T was confirmed by the maximum-likelihood and maximum-parsimony trees (not shown). Its closest relatives were *W. arenosi* R60^T, *W. echinorum* KMM 6211^T and *W. thalassocola* KMM 3907^T, with 96.5, 96.3 and 96.1% 16S rRNA gene sequence similarity, respectively.

The following tests were performed on strain A73^T only. Cell morphology and the presence of flagella were assessed by light and transmission electron microscopy (JEM100; JEOL) using cells grown on MA at 30 °C for 4 days. The following tests were performed on strains A73^T, *W. arenosi* JCM 15527^T, *W. echinorum* KCTC 22026^T and *W. thalassocola* KCTC 12221^T using the same culture conditions and methods. Gliding motility was assessed on 0.1 × MA plates (containing 10% marine broth and 1% agar) (Bowman, 2000) and by the hanging drop method (Bernardet *et al.*, 2002). The Gram reaction was assessed by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth at 5, 10, 15, 20, 25, 30,

35, 40 and 45 °C was measured on MA, while growth at pH 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5 and 11 and tolerance of 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9 and 10% (w/v) NaCl was measured in MB. The pH range for growth was determined by assessing changes in OD₆₀₀ with time in MB. Tolerance of sea salts was determined on trypticase soy agar (TSA; Difco) supplemented with 0–12% (w/v) sea salts (Sigma). Growth with NaCl as the sole salt was studied on TSA supplemented with 0–12% (w/v) NaCl. Anaerobic growth was assessed on MA incubated in a GasPak anaerobic system (BBL) for 15 days at 30 °C. The presence of catalase activity was revealed by the production of oxygen bubbles by cells when mixed with 3% (v/v) H₂O₂ on a glass slide. The presence of oxidase activity was determined by oxidation of 1% (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine solution. DNase activity was examined by using DNase test agar with methyl green (Difco) supplemented with 2% (w/v) sea salts (Sigma). Hydrolysis of casein, starch and Tweens 20, 40 and 80 was tested on MA, using the substrate concentrations given by Cowan & Steel (1965). Hydrolysis of agar and CM-cellulose was investigated as described by Barrow & Feltham (1993). Hydrolysis of aesculin, gelatin and urea and reduction of nitrate were investigated as described by Lányi (1987) using media supplemented with 2% (w/v) sea salts. H₂S production was tested as described previously (Bruns *et al.*, 2001). Other biochemical tests were performed using API 20NE and API 20E kits (bioMérieux) and enzyme activities were tested using an API ZYM kit (bioMérieux). API strips were inoculated with a heavy bacterial suspension (McFarland value of 0.5) in artificial seawater (Yoon *et al.*, 2002) and used according to the manufacturer's instructions, except that tests were incubated at 30 °C for 4 days. Susceptibility to antibiotics was determined using the disc-diffusion method with commercial antibiotic-impregnated discs (BBL Becton Dickinson). After 4 days of incubation at 30 °C on MA, the results were interpreted according to the guidelines of the CLSI (2003). The main physiological, biochemical and morphological characteristics are given in the species description and in Table 1.

The presence of flexirubin-type pigments was investigated as described by Bernardet *et al.* (2002). Cellular fatty acid methyl esters of strain A73^T, *W. arenosi* JCM 15527^T, *W. echinorum* KCTC 22026^T and *W. thalassocola* KCTC 12221^T were prepared from cultures grown on MA at 30 °C for 4 days and identified and quantified using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's protocol. The dominant fatty acids of strain A73^T were iso-C_{15:1} G (19.1%), iso-C_{15:0} (13.3%), iso-C_{17:0} 3-OH (10.0%) and iso-C_{15:0} 3-OH (7.2%). The detailed fatty acid profile of strain A73^T is compared with those of closely related members of the genus *Winogradskyella* in Table 2. DNA was isolated from strain A73^T, *W. arenosi* JCM 15527^T, *W. echinorum* KCTC 22026^T and *W. thalassocola* KCTC 12221^T by the method of Marmur (1961) and DNA G+C content was determined

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships of strain A73^T and related taxa. Bootstrap percentages (based on 1000 replicates) greater than 70% are shown at branching points. Bar, 0.01 changes per nucleotide position.

Table 1. Differential characteristics of strain A73^T and closely related type strains of the genus *Winogradskyella*

Strains: 1, strain A73^T; 2, *W. arenosi* JCM 15527^T; 3, *W. echinorum* KCTC 22026^T; 4, *W. thalassocola* KCTC 12221^T. Data were obtained in this study unless indicated. All strains were positive for the following: respiratory metabolism; gliding motility; oxidase, catalase and alkaline phosphatase activities; requirement for Na⁺ ions for growth; utilization of D-glucose; hydrolysis of gelatin. All strains were negative for the following: nitrate reductase and urease activity; flexirubin pigments; indole and acetoin production; acid production from L-arabinose, inositol, melibiose, L-rhamnose, D-sorbitol and sucrose; utilization of L-arabinose, inositol, D-sorbitol and sucrose. All strains were susceptible to ampicillin, carbenicillin, chloramphenicol and oleandomycin and resistant to gentamicin, kanamycin, neomycin, polymyxin B and streptomycin (determined according to the criteria of the CLSI, 2003).

Characteristic	1	2	3	4
H ₂ S production	–	+	–	–
Growth at/in:				
37 °C	+	–	+	–
44 °C	–	–	+	–
8 % NaCl	–	+	–	+
Hydrolysis of:				
Agar	–	–	–	+
Casein	+	–	+	–
Starch	+	+	–	–
DNA	+	–	+	–
CM-cellulose	+	–	–	–
Tween 20	–	+	–	–
Tween 40	–	+	–	+
Tween 80	+	+	–	–
Acid formation from:				
D-Glucose	+	+	–	+
Maltose	–	+	–	+
D-Mannitol	–	+	–	–
Utilization of:				
Citrate	+	–	–	–
D-Mannose	–	+	+	+
D-Mannitol	–	+	–	–
Susceptibility to:				
Benzylpenicillin	+	–	+	+
Tetracycline	+	+	+	–
DNA G + C content (mol%)	36.0	34.5	33.5 (33.6) ^a	32.0 (34.6) ^b

*Data in parentheses taken from: a, Nedashkovskaya *et al.* (2009); b, Nedashkovskaya *et al.* (2005).

by using the thermal denaturation method (Marmur & Doty, 1962). The DNA G+C contents of strain A73^T, *W. arenosi* JCM 15527^T, *W. echinorum* KCTC 22026^T and *W. thalassocola* KCTC 12221^T were 36.0, 34.5, 33.5 and 32.0 mol%, respectively. Isoprenoid quinones of strain A73^T only were analysed using an HPLC (ZQ 4000; Waters) and a reversed-phase column (300 × 3.9 mm,

Table 2. Cellular fatty acid contents of strain A73^T and closely related type strains of *Winogradskyella*

Strains: 1, strain A73^T; 2, *W. arenosi* JCM 15527^T; 3, *W. echinorum* KCTC 22026^T; 4, *W. thalassocola* KCTC 12221^T. Values are percentages of total fatty acids and were determined in this study. Fatty acids amounting to <1 % of the total fatty acids in all strains are not shown; –, not detected (<1.0 %).

Fatty acid	1	2	3	4
Saturated				
C _{15:0}	6.4	3.0	2.2	2.0
C _{16:0}	1.3	1.3	–	–
Branched saturated				
iso-C _{15:0}	13.3	18.1	15.2	14.5
iso-C _{16:0}	–	1.5	1.1	1.2
anteiso-C _{15:0}	3.4	11.9	12.4	11.4
Unsaturated				
C _{15:1} ω6c	1.5	1.0	1.8	1.2
Branched unsaturated				
iso-C _{15:1} G	19.1	15.0	14.9	14.1
anteiso-C _{15:1} A	1.5	3.0	2.8	3.0
iso-C _{16:1} H	1.6	1.2	1.5	–
iso-C _{17:1} ω9c	1.1	2.7	1.7	2.2
Hydroxy				
C _{15:0} 2-OH	1.6	1.9	2.3	3.4
C _{16:0} 3-OH	1.6	–	1.1	–
C _{17:0} 2-OH	2.3	2.8	2.6	2.9
iso-C _{15:0} 3-OH	7.2	10.1	12.9	14.8
iso-C _{16:0} 3-OH	6.2	6.0	8.5	8.0
iso-C _{17:0} 3-OH	10.0	10.2	9.1	9.2
Summed feature				
Summed feature 3*	5.0	5.3	5.7	5.5

*Summed features are groups of two or three fatty acids that cannot be separated by the MIDI system. Summed feature 3 comprised iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

Nova-Pak C18; Waters) as described by Komagata & Suzuki (1987). The major respiratory quinone of strain A73^T was MK-6.

Based on the results of the 16S rRNA gene-based phylogenetic analysis and the above-mentioned phenotypic features, we conclude that strain A73^T represents a novel species within the genus *Winogradskyella*, for which the name *Winogradskyella lutea* sp. nov. is proposed. As a consequence of newly reported characteristics, the description of the genus *Winogradskyella* Nedashkovskaya *et al.* 2005 is also emended.

Emended description of genus *Winogradskyella* Nedashkovskaya *et al.* 2005

The main characteristics are as given for the genus by Nedashkovskaya *et al.* (2005). In addition, colonies are yellow- or orange-pigmented.

Description of *Winogradskyella lutea* sp. nov.

Winogradskyella lutea (lu'te.a. L. fem. adj. *lutea* orange-coloured).

Cells are 0.2–0.3 µm wide and 2.0–3.5 µm long. On MA, colonies are 1.0–1.5 mm in diameter, convex and circular/slightly irregular and contain non-diffusible orange pigments. Devoid of flagella. Gliding motility is observed. Grows at 10.0–37.0 °C (optimum, 30.0–35.0 °C) and at pH 6.0–11.0 (optimum, pH 7.0–8.0). Sodium ions are required for growth. Grows in MB supplemented with 1–3 % (w/v) NaCl. Aesculin, Tween 80, gelatin, starch, DNA, casein and CM-cellulose are hydrolysed, but Tweens 20 and 40, agar and urea are not. Acetoin, indole and H₂S are not produced. Nitrate is not reduced. Tryptophan deaminase, lysine decarboxylase and ornithine decarboxylase activities are absent. Acid is produced from D-glucose and citrate, but not from L-arabinose, inositol, melibiose, L-rhamnose, D-sorbitol, sucrose or amygdalin. D-Glucose is assimilated, but L-arabinose, D-mannose, D-mannitol, gluconate, adipic acid, malate, maltose, N-acetylglucosamine, capric acid, trisodium citrate and phenylacetic acid are not. In API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase are present and α-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Susceptible to (µg per disc unless indicated) ampicillin (10), benzylpenicillin (10 U), carbenicillin (100), chloramphenicol (100), oleandomycin (15) and tetracycline (30); resistant to gentamicin (10), kanamycin (30), neomycin (30), polymyxin B (30) and streptomycin (10). The dominant fatty acids are iso-C_{15:1} G, iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{15:0} 3-OH. The DNA G + C content of the type strain is 36.0 mol%.

The type strain, A73^T (=KCTC 23237^T =DSM 22624^T), was isolated from seawater collected off Jeju Island, Republic of Korea.

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