Winogradskyella pulchriflava sp. nov., isolated from marine sediment

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A taxonomic study was conducted on strain EM106T, isolated from a sediment sample of the East Sea, Republic of Korea. Comparative 16S rRNA gene sequence analyses showed that strain EM106T belongs to the family Flavobacteriaceae and is most closely related to Winogradskyella echinorum KMM 6211T and Winogradskyella ulvae KMM 6390T (97.8 and 97.3 % 16S rRNA gene sequence similarities, respectively). The G+C content of the genomic DNA of strain EM106T was 33.3 mol%, and the major respiratory quinone was menaquinone-6. The polar lipids of EM106T were phosphatidylethanolamine, two unidentified aminolipids and two unidentified lipids. DNA–DNA relatedness data indicated that strain EM106T represented a distinct species, separate from W. echinorum KMM 6211T and W. ulvae KMM 6390T. Strain EM106T possessed iso-C15:0, iso-C15:1 G and iso-C16:0 3-OH as the major cellular fatty acids. The isolate was Gram-staining-negative, strictly aerobic, short rod-shaped and motile by gliding. The strain grew at 10–35 °C (optimum, 25 °C), pH 6.5–9.0 (optimum, 7.5), and with 0.5–5 % (w/v) NaCl (optimum, 0.5–1 % NaCl). The overall physiological features of strain EM106T were very similar to those of W. echinorum KMM 6211T but only strain EM106T had nitrate reductase activity. On the basis of phenotypic and phylogenetic analyses, strain EM106T is proposed to represent a novel species, Winogradskyella pulchriflava. The type strain is EM106T (=KCTC 23858T =NCAIM B 02481T).

The genus Winogradskyella, within the family Flavobacteriaceae, was first described by Nedashkovskaya et al. (2005) to accommodate Gram-staining-negative, heterotrophic, strictly aerobic, rod-shaped bacteria (without production of flexirubin-type pigments) isolated from the sea urchin Strongylocentrotus intermedius. At the time of writing, the genus comprises 15 recognized species: Winogradskyella aquimarins, Winogradskyella arenosi, Winogradskyella damptonensis, Winogradskyella echinorum, Winogradskyella epiphytica, Winogradskyella exilis, Winogradskyella eximia, Winogradskyella litorisediminis, Winogradskyella lutea, Winogradskyella pacifica, Winogradskyella poriferorum, Winogradskyella psychrotolerans, Winogradskyella rapida, Winogradskyella thalassocola and Winogradskyella ulvae (Begum et al., 2013; Ivanova et al., 2010; Kim & Nedashkovskaya, 2010; Kang et al., 2013; Lau et al., 2005; Lee et al., 2012, 2013; Nedashkovskaya et al., 2005, 2009, 2012; Pinhassi et al., 2009; Romanenko et al., 2009; Yoon et al., 2011). The genus Winogradskyella is composed of marine strains isolated from sediments, seaweed, marine sponges and seawater. Here, we provide a polyphasic taxonomic characterization of a Winogradskyella-like bacterial strain, EM106T, which was isolated from marine sediment. The isolated strain, EM106T, is proposed to represent a novel species of the genus Winogradskyella.

A novel bacterial strain, EM106T, was isolated from marine sediment of the East Sea, Republic of Korea (38° 20’ N 128° 35’ E). A sediment sample was placed in a sterile conical tube and transported to the laboratory on the same day. The sediment sample (10 g) was suspended in filter-sterilized (0.22 μm pore size; Millipore) natural seawater (100 ml) and was vortexed for homogenization. One millilitre of the suspension was serially diluted with filter-sterilized (0.22 μm pore size; Millipore) natural seawater containing 0.1 % yeast extract (Difco). After incubation at 25 °C under aerobic conditions for 2 weeks, an aliquot of the last dilution showing turbidity was spread onto modified artificial seawater medium (AM; per litre distilled water: 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl2 .6H2O, 15 g agar, 0.1 g yeast extract) (Levring, 1946) and was incubated at 25 °C for 2 weeks. Among the various colonies which were present, a yellow-coloured
single colony was purified by transferring onto marine agar 2216 (MA; Difco) and subjecting it to an additional incubation at 25 °C for 3 days. The isolated strain was stored as a glycerol suspension (25%, w/v) at −70 °C.

Bacterial genomic DNA was extracted using a commercial genomic DNA extraction kit (Cosmogenetech). The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primers 27F (5′- AGAGTTTGATCMTGGCTCAG-3′; *Escherichia coli* positions 8–27) and 1492R (5′-TACGYYTACCTGTAGGACTTT-3′; *E. coli* positions 1492–1510) (Lane, 1991) and the purified PCR product was sequenced by Cosmogenetech. The 16S rRNA gene sequences were assembled using SeqMan software (DNASTAR). The 16S rRNA gene sequence of strain EM106T determined in this study was 1431 bp in length. The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Sequence alignments were performed using SILVA (http://www.arb-silva.de/aligner), considering the secondary structure of the rRNA gene (Pruesse et al., 2007). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were reconstructed based on the maximum-likelihood (Felsenstein, 1981), neighbour-joining (Saitou & Nei, 1987) and minimum-evolution (Kidd & Sgaramella-Zonta, 1971) methods by using the MEGA5 program (Tamura et al., 2011). The tree topologies were not significantly different from each other. In the analysis of phylogeny, strain EM106T was determined to belong to the family *Flavobacteriaceae*. On the basis of pairwise comparisons of the 16S rRNA gene sequences using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net) (Kim et al., 2012), the highest degrees of sequence similarity were found to be with *W. echinorum* KMM 6211T (97.8% 16S rRNA gene sequence similarity) and *W. ulvae* KMM 6390T (97.3% similarity). The range of 16S rRNA gene sequence similarity of with other species of genus *Winogradskyella* was 93.6–96.3%. Strain EM106T clearly belonged to the genus *Winogradskyella*, as shown by the bootstrap value in the phylogenetic tree (Fig. 1) of 16S rRNA gene sequences. The selected reference strains, *W. echinorum* KMM 6211T (=KCTC 22026T) and *W. ulvae* KMM 6390T (=KCTC 23626T), for comparison with the isolated strain were obtained from an established culture collection, Korean Collection for type Cultures (KCTC, Republic of Korea). Unless otherwise stated, all reference strains were grown on MA 2216 under their optimal culture conditions (Nedashkovskaya et al., 2009, 2012).

The Gram stain reaction was determined by using a Gram stain kit (Difco) according to the manufacturer’s instructions. Morphology of the Gram-stained cells was examined by light microscopy (Eclipse 80i; Nikon). Transmission electron microscopy (Tecnai G2 Sprite; FEI; as installed at the Korean Basic Science Institute) was performed after negative staining with 1% (w/v) phosphotungstic acid. Catalase activity was determined by bubble production in 3% (v/v) hydrogen peroxide solution and oxidase activity was determined using 1% (w/v) tetramethyl-p-phenylenediamine. Cell motility was examined by the semisolid agar method (Tittsler & Sandholzer, 1936) and gliding motility was observed by direct phase-contrast microscopic examination with a Nikon Eclipse 80i microscope according to the method described by Bowman (2000). Detection of flexirubin pigments was tested by flooding the plates with 20% (w/v) potassium hydroxide according to Fautz & Reichenbach (1980). For pigment tests, cells were extracted with acetone/methanol (7:2, v/v) and scanned between 300 and 600 nm with a UV spectrophotometer (Optizen 3220UV bio; Mecasys) as described Nedashkovskaya et al. (2006). Cells of strain EM106T were Gram-staining-negative, flexirubin-negative, oxidase- and catalase-positive and strictly aerobic. Strain EM106T produced carotenoid pigments with maximum absorption at 447 and 449 nm. Colonies were golden-yellow, circular with entire edges and had a diameter of 1–2 mm when grown on MA 2216 at 25 °C for 3 days. Cells of variable sizes were observed (0.3–0.4 × 0.8–1.2 μm) (Fig. S1, available in IJSEM Online), which were motile by gliding without discernible flagella. Carbon source utilization and enzyme activities were determined with API 20NE, API ZYM, and Biolog (GEN III MicroPlate) galleries according to the instructions of the manufacturers (bioMerieux and Biolog). Bacterial suspensions were made in sterile, chilled AM without yeast extract. After inoculation, the galleries were incubated at 25 °C for 3 days and reactions were read. Hydrolysis of starch and Tween 80 were determined as described by Cowan & Steel (1965) with modified AM. DNA hydrolysis was observed by using DNase test agar with methyl green (Difco). Cellulose hydrolysis was tested on MA 2216 overlaid with 0.5% CM-cellulose (Bowman, 2000). The production of H2S was tested on peptone iron agar (Difco). DNase test agar and peptone iron agar media were supplemented with 1% NaCl (final concentration). For antibiotic susceptibility tests, discs containing the following antibiotics were used: ampicillin (10 μg), chloramphenicol (25 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin G (10 μg), streptomycin (10 μg) and tetracycline (30 μg).

Growth at different temperatures (0, 5, 10, 15, 20, 25, 30, 35, and 40 °C) was assessed after 3 days of incubation on MA 2216. Strain EM106T was able to grow at 10–35 °C, but not at 5 or above 35 °C (optimum, 25 °C). Growth at different NaCl concentrations (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 10 and 12 %, w/v) was measured using AM without NaCl. Strain EM106T required sodium ions for growth and grew in 0.5–5% NaCl (optimum, 0.5–1%). The response to pH (pH 5.0–10.0 at intervals of 0.5 pH units) was determined in marine broth 2216 at 25 °C for 3 days. Three different buffers were used (final concentration, 10 mM): acetate buffer was used for pH 5.0–5.5 (Green, 1933); phosphate buffer was used for pH 6.0–8.0 (Green, 1933); Tris buffer was used for pH 8.5–10.0 [Tris/HC1, pH 8.5 (Biosesang, Korea), adjusted with 1 M NaOH]. Strain EM106T grew at...
pH 6.5–9.0 but not at pH below 6.5 or above 9.0 (optimum, pH 7.5). The physiological characteristics of strain EM106T are summarized in the species description and selective characteristics are compared with those of related type strains in Table 1. The overall physiological features of strain EM106T were very similar to those of *W. echinorum* KMM 6211T, but differ in nitrate reduction.

Cellular fatty acids of the isolated strain and reference organisms (*W. echinorum* KCTC 22026T and *W. ulvae* KCTC 23626T) were analysed after cultivation on MA 2216 at 25 °C at pH 7.5 for 3 days. The cells of all strains were harvested at the late exponential phase of growth. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the MIDI system (Microbial ID). The fatty acids were analysed by GC (6890N; Agilent Technologies) and were identified using the Microbial Identification software package (version 6.1). The major cellular fatty acids of strain EM106T were iso-C15 : 0 (24.3 %), iso-C15 : 1G (17.5 %) and iso-C16 : 03-OH (12.8 %) (Table 2). The fatty acid profile of strain EM106T was similar to those of *W. echinorum* KCTC 22026T and *W. ulvae* KCTC 23626T.

Polar lipids of strain EM106T were extracted and analysed by two-dimensional ascending TLC as described by Minnikin *et al.* (1984). Alpha-naphthol solution was used as a glycolipid stain solution as described by Torreblanca *et al.* (1986). The major polar lipid represented was phosphatidylethanolamine. Some unknown aminolipids and unidentified lipids were also detected as minor components (see Fig. S2). Quinones were extracted with a chloroform/methanol mixture (2 : 1, v/v), evaporated in a vacuum and re-extracted three times with *n*-hexane/water (1 : 1, v/v). The crude quinone extract in *n*-hexane was then concentrated and applied to a Sep-Pak Plus silica column (Waters). Quinone components were separated and identified by reverse-phase HPLC and photodiode array detection, with internal and external standard quinones, as described by Hiraishi *et al.* (1996). Similar to *W. ulvae* KMM 6390T, strain EM106T also had menaquinone 6 (MK-6) as the sole respiratory quinone (Nedashkovskaya *et al.*, 2012). MK-6 as a sole quinone is widely observed in all other members of the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006).

Chromosomal DNA extracted for 16S rRNA gene amplification was used for determination of G+C content. RNA in the DNA solution was removed by incubation with a mixture of RNase A and T1 (each 20 units ml⁻¹) at 30 °C for 1 h. The G+C content of the chromosomal DNA was determined according to Gonzalez & Saiz-Jimenez (2002).

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**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain EM106T, representatives of the genus *Winogradskyella* and related genera. *Zobellia amurskyensis* KMM 3526T served as the outgroup. Bootstrap values ≥70 % (based on 1000 replicates) from maximum-likelihood, neighbour-joining and minimum-evolution methods, respectively, are indicated at branch points; values <70 % are indicated by a dash. GenBank accession numbers are shown in parentheses. Bar, 0.02 substitutions per nucleotide position.
The G+C content of chromosomal DNA of strain EM106\(^\top\) was 33.3 mol%, which is quite similar to those of reference species (Table 1).

DNA–DNA hybridization experiments were carried out with EM106\(^\top\), \textit{W. echinorum} KCTC 22026\(^\top\) and \textit{W. ulvae} KCTC 23626\(^\top\) using the method described by Ezaki \textit{et al.} (1989). The genomic DNA of strain EM106\(^\top\) and reference micro-organisms (\textit{W. echinorum} KCTC 22026\(^\top\) and \textit{W. ulvae} KCTC 23626\(^\top\)) was extracted using a genomic DNA extraction kit (Cosmogenetech) and was used for hybridization templates and probes. Probe DNA was biotinylated with photobiotin and hybridized with single-stranded unlabelled chromosomal DNA fragments of reference or test micro-organisms. Means from three independent determinations of DNA–DNA hybridization levels were determined. The hybridization levels of strain EM106\(^\top\) with \textit{W. echinorum} KCTC 22026\(^\top\) and \textit{W. ulvae} KCTC 23626\(^\top\) were 26.1 ± 0.5 and 8.5 ± 1.8 %, respectively (Table S1).

The present data demonstrated that the novel isolate, designated EM106\(^\top\), is closely related to members of the genus \textit{Winogradskyella}. The phylogenetic trees generated by neighbour-joining, minimum-evolution and maximum-parsimony algorithms showed that strain EM106\(^\top\) formed a robust cluster with members of the genus \textit{Winogradskyella}.
Table 2. Cellular fatty acid compositions of strains EM106<sup>T</sup>, W. echinorum KCTC 22026<sup>T</sup> and W. ulvae KCTC 23626<sup>T</sup>.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;16&lt;/sub&gt;:0</td>
<td>1.95</td>
<td>2.54</td>
<td>2.00</td>
</tr>
<tr>
<td>C&lt;sub&gt;16&lt;/sub&gt;:0 3-OH</td>
<td>0.50</td>
<td>1.19</td>
<td>0.63</td>
</tr>
<tr>
<td>C&lt;sub&gt;17&lt;/sub&gt;:0 2-OH</td>
<td>0.42</td>
<td>1.20</td>
<td>1.86</td>
</tr>
</tbody>
</table>

*Summed features represent groups of fatty acids that could not be separated by GLC with the MIDI system (version 6.1). Summed feature 3 contained C<sub>16</sub>:<i>c</i>17:<i>c</i>6<sub>c</sub> and/or C<sub>16</sub>:<i>c</i>16:<i>c</i>6<sub>c</sub>. Summed feature 9 contained iso-C<sub>17</sub>:1<sub>0</sub>3<sub>c</sub> and/or 10-Methyl C<sub>16</sub>:0.

(1). In current bacterial systematics, two strains belong to separate species when DNA–DNA relatedness between the two strains is below 70% (Wayne et al., 1987). The low level of DNA–DNA relatedness (<30%) with the most closely related species (W. echinorum and W. ulvae) and other differential features compared with other species of the genus Winogradskyella demonstrate clearly that this isolate differs sufficiently to be regarded as a representative of a novel species. Therefore, on the basis of the phenotypic, genotypic and phylogenetic characteristics, strain EM106<sup>T</sup> is considered to represent a novel species of the genus Winogradskyella, for which the name Winogradskyella pulchriflava sp. nov. is proposed.

Description of Winogradskyella pulchriflava sp. nov.

Winogradskyella pulchriflava (pul.chri fla’va. L. adj. pulcher -chra -chrom beautiful; L. adj. flavus -a -um golden-yellow; N.L. fem. adj. pulchriflava beautifully golden-coloured).

Cells are Gram-staining-negative, flexirubin-negative, strictly aerobic, oxidase- and catalase-positive, rod-shaped (0.3–0.4 μm wide and 0.8–1.2 μm long) and are motile by gliding. Carotenoid pigments are produced. Favourable growth occurs aerobically, forming circular colonies with regular edges within 3 days, with diameters of approximately 1.0–2.0 mm. Growth occurs at 10–35 °C (optimum, 25 °C) and at pH 6.5–9.0 (optimum, pH 7.5) and in 0.5–5% (w/v) NaCl (optimum, 0.5–1%). NaCl is required for growth. Reduction of nitrate to nitrite and nitrogen is positive. Indole production, arginine dihydrolase, cellulase and urease are negative. DNA, gelatin, ascusin, Tween 40 and Tween 80 are hydrolysed. D-Glucose and maltose are utilized, but not L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid (API 20NE). Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase enzyme activity tests are positive, but lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activity tests are negative (API ZYM). Utilizes dextrin, maltose, α-D-glucose, D-galactose, glycerol-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, gelatin, L-serine, pectin and D-galacturonic acid, but does not utilize trehalose, cellobiose, gentiobiose, sucrose, turanose, stachyose, raffinose, Z-lactose, melibiose, β-methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, D-mannose, D-fructose, 3-methyl glucose, D-fucose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycerol, D-glucose 6-phosphate, D-fructose 6-phosphate, D-aspartic acid, D-serine, L-histidine, L-pyroglutamic acid, L-galactonic acid lactone, D-glucuronic acid, D-galacturonic acid, glucuronic mide, mucus acid, quinic acid, D-saccharic acid, D-hydroxyphenylacetic acid, methyl pyruvate, D-lactic acid, methyl ester, D-malic acid, L-malic acid, bromosuccinic acid, γ-aminobutyric acid, α-hydroxybutyric acid, α-ketobutyric acid, acetoclastic acid, propionic acid or formic acid (Biolog). Susceptible to ampicillin, kanamycin, gentamicin, penicillin G, streptomycin and tetracycline, but tolerant of chloramphenicol and erythromycin. Menaquinone 6 and phosphatidylethanolamine are detected as the major respiratory quinone and major polar lipid, respectively. The major cellular fatty acids are iso-C<sub>15</sub>:0, iso-C<sub>15</sub>:1<sub>V</sub> and iso-C<sub>16</sub>:0 3-OH.

The type strain, EM106<sup>T</sup> (=KCTC 23858<sup>T</sup>=NCAIM B 02481<sup>T</sup>), was isolated from marine sediment of the East Sea. The DNA G+C content of the type strain is 33.3 mol%.

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References


