Winogradskyella damuponensis sp. nov., isolated from seawater

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A novel bacterium, designated F081-2¹, isolated from seawater from Damupo beach in Pohang, Korea, was investigated using a polyphasic taxonomic approach. Cells were yellow-pigmented, strictly aerobic, motile by gliding, Gram-negative-staining and rod-shaped. The temperature, pH and NaCl ranges for growth were 4–35 °C, pH 5.5–9.5 and 1.0–5.0 %, respectively. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain F081-2¹ belonged to a distinct lineage in the genus Winogradskyella of the family Flavobacteriaceae, sharing 93.7–98.1 % similarity with recognized members of the genus. Low levels of DNA–DNA relatedness values were found between strain F081-2¹ and Winogradskyella eximia KCTC 12219¹ (61.1 %), Winogradskyella thalassocola KCTC 12221¹ (47.0 %), Winogradskyella echinorum KCTC 22026¹ (39.3 %), Winogradskyella rapida CCUG 56098¹ (34.3 %) and Winogradskyella arenosi JCM 17633¹ (33.4 %). The major cellular fatty acids were iso-C₁₅:0 (25.3 %), iso-C₁₅:₁ G (14.6 %), iso-C₁₇:₀ 3-OH (9.3 %), anteiso-C₁₅:₀ (7.8 %) and iso-C₁₅:₀ 3-OH (7.6 %). The polar lipid profile was composed of phosphatidylethanolamine, one unidentified aminolipid, one unidentified phospholipid, one unidentified aminophospholipid and six unidentified lipids. The major respiratory quinone was menaquinone-6 and the DNA G+C content of the strain was 32.3 mol%. On the basis of phenotypic, phylogenetic and genotypic data, strain F081-2¹ represents a novel species within the genus Winogradskyella, for which the name Winogradskyella damuponensis sp. nov. is proposed. The type strain is F081-2¹ (=KCTC 23552¹ =JCM 17633¹).

The genus Winogradskyella, belonging to the family Flavobacteriaceae, was first proposed with three species by Nedashkovskaya et al. (2005) through the description of Winogradskyella thalassocola, Winogradskyella epiphytica and Winogradskyella eximia. At the time of writing, the genus Winogradskyella comprised ten recognized species isolated from various marine habitats: Winogradskyella epiphytica, Winogradskyella eximia and Winogradskyella thalassocola were isolated from marine algae (Nedashkovskaya et al., 2005); Winogradskyella poriferorum, Winogradskyella echinorum and Winogradskyella exilis were isolated from marine animals (Lau et al., 2005; Nedashkovskaya et al., 2009; Ivanova et al., 2010); Winogradskyella arenosi was isolated from marine sediments (Romanenko et al., 2009); and Winogradskyella rapida, Winogradskyella pacifica and Winogradskyella lutea were isolated from seawater samples (Pinhasi et al., 2009; Kim & Nedashkovskaya, 2010; Yoon et al., 2011). In this study, we report the taxonomic characterization of a bacterial strain similar to members of the genus Winogradskyella, F081-2¹, which was isolated from seawater at Damupo beach in Pohang, Korea.

Strain F081-2¹ was isolated from a seawater sample collected from Damupo beach in Pohang, Korea, by using a standard serial dilution plating method and incubation on marine agar 2216 (MA; Difco) at 25 °C for 7 days. Subcultivation was routinely performed on MA at 25 °C for 3 days under aerobic conditions and the strain was preserved at −80 °C in marine broth (MB, Difco) supplemented with 20 % v/v glycerol. This strain was deposited in the Korean Collection for type Cultures (=KCTC 23552¹) and the Japan Collection of Microorganisms (=JCM 17633¹). For comparative analyses of phenotypic characterization, fatty acid analysis and DNA–DNA hybridization, W. arenosi R60¹ (=JCM 15527¹), W. echinorum KMM 6211¹ (=KCTC 22026¹), W. eximia KMM 3944¹ (=KCTC 12219¹), W. rapida SCB36¹ (=CCUG 56098¹) and W. thalassocola KMM 3907¹ (=KCTC 12221¹) were used as reference strains. Bacterial genomic DNA was extracted and purified by using an Exgene genomic extraction kit (GeneAll...
Biotecnology), and the nearly complete 16S rRNA gene sequence was amplified using bacterial universal primers, 27F and 1492R (Weisburg et al., 1991). The PCR product was purified by using an Expi DNA purification kit (GeneAll Biotechnology) and was ligated into the T-Blunt vector (SolGent) according to the manufacturer’s instructions. The ligation product was transformed into competent Escherichia coli DH5α cells. The cloned 16S rRNA gene sequencing was performed with an Applied Biosystems automated sequencer (ABI 3730XL) at Macrogen. The resulting 16S rRNA gene sequences were compiled using SeqMan software (DNASTAR) and compared with available 16S rRNA gene sequences in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/blast.cgi). The 16S rRNA gene sequences of recognized species of the genus Winogradskyella and sequence similarity values were obtained from the EzTaxon service (http://www.eztaxon.org/; Chun et al., 2007). Multiple alignments were performed using the CLUSTAL_X program (Thompson et al., 1997) and gaps at the 5′ and 3′ ends and ambiguous bases were removed from the alignment using BioEdit program (Hall, 2007). Phylogenetic trees based on comparison of 1308 bases were constructed by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA 5 software (Kumar et al., 2008), with bootstrap values based on 1000 replications (Felsenstein, 1985). Evolutionary distance matrices were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1983).

The nearly complete 16S rRNA gene sequence of strain F081-2T was determined and deposited in the GenBank database under the accession number HQ336488. The 16S rRNA gene sequence similarities using the pairwise alignment obtained from the EzTaxon database version 2.1 (Chun et al., 2007) showed that strain F081-2T was closely related to the genus Winogradskyella in the family Flavobacteriaceae. Comparisons with sequences from taxa with validly published names showed that strain F081-2T shared the highest similarity (98.1%) with W. eximia KMM3944T, followed by W. thalassocolla KMM 3907T (97.8%), W. rapida SCB36T (97.6%), W. echinorum KMM 6211T (97.2%) and W. arenosi R60T (97.1%) and lower sequence similarities (<97.0%) with all other species in the genus Winogradskyella. In the phylogenetic trees based on the neighbour-joining method, strain F081-2T fell within the cluster of the genus Winogradskyella and joined W. eximia KMM 3944T with a bootstrap value of 76% (Fig. 1). This relationship was also maintained in the trees based on the maximum-parsimony and maximum-likelihood algorithms (data not shown).

DNA–DNA hybridization was carried out by the membrane filter hybridization method of Tourouva & Antonov (1988). Probe labelling was conducted by using the non-radioactive DIG High Prime system and hybridized DNA was visualized using the DIG Luminescent Detection kit according to the manufacturer’s instructions (Roche Diagnostics). DNA–DNA relatedness was quantified using a densitometer (Bio-Rad). Strain F081-2T showed relatively low DNA–DNA relatedness to W. eximia KMM 3944T (61.1%), W. thalassocolla KMM 3907T (47.0%), W. echinorum KMM 6211T (39.3%), W. rapida SCB36T (34.3%) and W. arenosi R60T (33.4%). These DNA–DNA relatedness values were lower than 70%, a threshold used to delineate a species (Wayne et al., 1987).

Cells of strain F081-2T grown aerobically on MA at 25 °C for 3 days were used for the examination of colony morphology, size and colour. The cell morphology and size were examined by light microscopy (FXD-35, Nikon) and transmission electron microscopy (JEM-1010; JEOL) using cells from exponentially growing cultures (Jeon et al., 2004). Gliding motility of strain F081-2T was investigated using phase-contrast light microscopy by the hanging drop method described by Bernardet et al. (2002).

The following tests were performed on strain F081-2T and the reference strains. The optimal temperature and temperature range for growth were tested on MA at 4, 10, 15, 20, 25, 30, 35, 37 and 42 °C. NaCl requirement and tolerance were determined in the presence of 0–10% (w/v) NaCl in synthetic Zobell agar medium (ZoBell, 1941; 5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate and 15 g Bacto agar in 1 l distilled water) prepared with modified artificial seawater [0–10% (w/v) NaCl, 5.94 g MgSO₄·7H₂O, 4.53 g MgCl₂·6H₂O, 0.64 g KCl and 1.3 g CaCl₂ per litre]. The pH range for growth was determined in marine broth 2216 (MB; Difco) that was adjusted to various pH values (pH 5.0–10.0 at intervals of 0.5 pH unit) with HCl or NaOH after sterilization. Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer’s instructions. To detect flexirubin-type pigments, the cell mass was subjected to the KOH test as described by Bernardet et al. (2002).

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**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence, showing relationships between strain F081-2T and type strains of species of the genus Winogradskyella. Bootstrap values are shown as percentages of 1000 replicates, when >70%. Lutaonella thermophila CC-MHSW-2T (GenBank accession number, EU287913) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.
Table 1. Phenotypic characteristics of strain F081-2T and type strains of the most closely related members of the genus *Winogradskyella*

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</table>

*DNA G+C content data from Nedashkovskaya et al. (2005); Lau et al. (2005); Nedashkovskaya et al. (2009); Pinhassi et al. (2009); Romanenko et al. (2009); Ivanova et al. (2010); Kim & Nedashkovskaya (2010) and Yoon et al. (2011).
Anaerobic growth was assessed on MA incubated in a GasPak anaerobic system (BBL) for up to 20 days at 25 °C. Catalase and oxidase activities were determined using standard methods (Tindall et al., 2007). The degradation of agar, starch and carboxymethyl cellulose were tested according to the methods of Smibert & Krieg (1994). Casein hydrolysis was determined according to the method of Lánfyi (1987) and hydrolysis of Tweens 20, 40, 60 and 80 was determined according to Baumann & Baumann (1981). DNase activity was determined with DNase test agar (Difco) supplemented seawater (3% sea salts in distilled water). Antibiotic sensitivity was determined with the disc diffusion method using commercial antibiotic discs (Oxoid) with the following antibiotics: ampicillin (10 μg), carbenicillin (100 μg), chloramphenicol (30 μg) erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), lincomycin (15 μg), nalidixic acid (30 μg), neomycin (30 μg), novobiocin (5 μg), oleandomycin (15 μg), penicillin (10 U), polymyxin B (300 μg), rifampicin (5 μg), streptomycin (25 μg) and tetracycline (30 μg). The results were interpreted according to the guidelines set forth by CLSI (2003). Other phenotypic and enzymic characterizations of strain F081-2T and the reference strains were assessed using API 20E and API 20NE kits (bioMérieux), incubated at 25 °C for 3 days, and API ZYM kits, incubated at 25 °C for 12 h. The oxidation of different carbon sources for strain F081-2T was assessed using GN2 MicroPlates (Biolog) according to the manufacturer’s instructions. All commercial kits were inoculated with bacterial suspensions in 3% sea salts. Detailed results of morphological, physiological and biochemical tests are given in the species description and in Table 1.

For cellular fatty acid analysis, cells of strain F081-2T and the reference strains were grown aerobically on MA at 25 °C. Cells were harvested by centrifugation after 3 days of cultivation and subjected to fatty acid analysis. Fatty acid methyl esters were obtained by saponification, methylation and extraction by using standard protocol of the MIDI (Sherlock Microbial Identification System, version 6.1) (Sasser, 1990). Fatty acids were analysed by GC (Agilent 7890A) and identified by using the TSBA6.0 database of the Microbial Identification System. The major respiratory quinone of strain F081-2T was analysed as described previously (Minnikin et al., 1984; Komagata & Suzuki, 1987). Polar lipids were separated using TLC silica gel 60 F254 plates (Merck) according to the procedures described by Minnikin et al. (1984) and individual polar lipids were identified by spraying with the appropriate detection reagents and through co-migration with authentic standards (Sigma) (Komagata & Suzuki, 1987). The G+C content of the genomic DNA of strain F081-2T was determined using the thermal denaturation method (Marmur & Doty, 1962) using an Ultraspec 2100 spectrophotometer (Pharmacia Biotech). DNA from E. coli K-12 was used as a control.

The major cellular fatty acids of strain F081-2T were iso-C15:0 (25.3%), iso-C15:1 G (14.6%), iso-C17:0 3-OH (9.3%), anteiso-C15:0 (7.8%) and iso-C15:0 3-OH (7.6%). The detailed fatty acid compositions of strain F081-2T and the reference strains are compared in Table 2. Although the reference strains grown under the same conditions shared similar overall fatty acid compositions, strain F081-2T differed from the reference strains in the respective proportions of several fatty acids, particularly, iso-C15:0 and anteiso-C15:0. The major respiratory quinone of strain F081-2T was menaquinone-6 (MK-6) in line with all members of the family Flavobacteriaceae (Bernardet & Nakagawa, 2006). The polar lipid profile of strain F081-2T was composed of phosphatidylethanolamine, one unidentified aminolipid, one unidentified phospholipid and six unidentified lipids (see Fig. S1 available at IJSEM online). The polar lipid profile of strain F081-2T was similar to those of W. eximia.

### Table 2. Cellular fatty acid compositions (%) of strain F081-2T and type strains of the most closely related members of the genus Winogradskyella

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<tr>
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<td>23.2</td>
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<td>9.8</td>
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<td>–</td>
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<td>–</td>
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<td>13.9</td>
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<td>iso-C16:0 3-OH</td>
<td>6.7</td>
<td>6.1</td>
<td>7.5</td>
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<td>2.5</td>
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<td>1.8</td>
<td>1.6</td>
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</tbody>
</table>

Summed feature 3 comprised C16:1 106c and/or C16:1 107c; summed feature 9 comprised C10-methyl and/or iso-C17:1 109c.
KCTC 12219^T and \emph{W. thalassocola} KCTC 12221^T, (see Fig. S1, available in IJSEM online). The G+ C content of the DNA determined for strain F081-2^T was 32.3 mol\%, which is within the range of DNA G+ C content of the other type strains of the genus \textit{Winogradskyella} (30.1–36.3 mol\%).

In summary, the mean DNA–DNA relatedness values between strain F081-2^T and recognized species of the genus \textit{Winogradskyella} were lower than 70\%, a threshold for the delineation of bacterial species (Wayne \textit{et al.}, 1987). Strain F081-2^T was also distinguishable from recognized species of the genus \textit{Winogradskyella} through differences in several phenotypic characteristics and in the proportions of some fatty acids, particularly iso-C_{15:0} and anteiso-C_{15:0} (Tables 1 and 2). The phylogenetic distinctiveness, together with the genetic distinctiveness and different phenotypic properties, is sufficient to show that strain F081-2^T is separate from recognized species of the genus \textit{Winogradskyella}. On the basis of the data presented, strain F081-2^T represents a novel species within the genus \textit{Winogradskyella}, for which the name \textit{Winogradskyella damuponensis} sp. nov. is proposed.

**Description of \textit{Winogradskyella damuponensis} sp. nov.**

\textit{Winogradskyella damuponensis} (da.mu.po.nen‘sis. N.L. fem. adj. \textit{damuponensis} of or pertaining to Damupo beach in Pohang, Korea, where the type strain was isolated).

Cells are Gram-negative, motile by gliding and rod-shaped (0.3–0.5 x 0.5–1.5 \(\mu\)m). Colonies on marine agar are yellow, circular, 0.5–3.0 mm in diameter and convex with smooth surfaces. Cells are positive for catalase and oxidase activities. Flexirubin-type pigments are not produced. Growth occurs at 4–35 °C (25–30 °C optimum), pH 6.0–9.5 and 1–5 % NaCl. Anaerobic growth does not occur on marine agar. Nitrate is not reduced. Indole and H_{2}S are not produced. Hydrolyses aesculin, DNA, gelatin, Tween 20, Tween 40 and Tween 80, but not agar, casein, carboxymethyl cellulose, starch or urea. Utilizes D-glucose and D-mannose, positive in tests for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities. Negative in tests for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, urease, lipase (C14), trypsin, x-chymotrypsin, x-galactosidase, \(\beta\)-galactosidase, \(\beta\)-glucuronidase, \(\beta\)-glucosidase, N-acetyl-\(\beta\)-glucosaminidase, x-mannosidase and x-fucosidase activities. In the API 50CH test, acid is produced aerobically from aesculin ferric citrate. In the GN2 Microplate test, the following substrates are oxidized: x-cyclodextrin, dextrin, N-acetyl-d-galactosamine, N-acetyl-D-glucosamine, adonitol, D-arabitol, cellobiose, i-erythritol, d-fructose, L-fucose (weakly), D-galactose, gentiobiase, x-D-glucose, \textit{myo}-inositol, lactose, maltose, D-mannitol (weakly), D-mannose, methyl \(\beta\)-D-glucoside, raffinose, D-sorbitol, sucrose, trehalose, acetic acid, D-galacturonic acid, D-gluconic acid (weakly), D-glucosaminic acid, succinic acid, gluconuramide (weakly), L-alaninamide, L-alanine, L-alaenyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycol L-glutamic acid, L-ornithine, L-phenylalanine, L-proline, L-pyrogulatamic acid (weakly), inosine, uridine, thymidine, phenylethylamine (weakly) and 2,3-butanediol. All other carbon sources on the GN2 Microplate are not oxidized. Susceptible to ampicillin (10 \(\mu\)g), carbenicillin (100 \(\mu\)g), chloramphenicol (30 \(\mu\)g), erythromycin (15 \(\mu\)g), lincomycin (15 \(\mu\)g), novobiocin (5 \(\mu\)g), oleandomycin (15 \(\mu\)g), penicillin G (10 U) and tetracycline (30 \(\mu\)g), but resistant to gentamicin (10 \(\mu\)g), kanamycin (30 \(\mu\)g) nalidixic acid (30 \(\mu\)g), neomycin (30 \(\mu\)g), polymyxin B (300 \(\mu\)g) and streptomycin (10 \(\mu\)g). The major cellular fatty acids are iso-C_{15:0}, iso-C_{15:1} \textit{G}, iso-C_{17:0} 3-OH, anteiso-C_{15:0} and iso-C_{15:0} 3-OH. The polar lipids are phosphatidylethanolamine, one unidentified aminolipid, one unidentified phospholipid, one unidentified aminophospholipid and six unidentified lipids. The major respiratory quinone is menaquinone-6.

The type strain, F081-2^T (=KCTC 23552^T=JCM 17633^T), was isolated from seawater at Damupo beach in Pohang, Korea. The DNA G+ C content of the type strain is 32.3 mol\%.

**Acknowledgements**

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**References**


