Winogradskyella flava sp. nov., isolated from the brown alga, Sargassum fulvulum

Ji Hee Lee,1 Joo Won Kang,2 Soon Bum Shin1 and Chi Nam Seong1,2,*

Abstract
An aerobic, gliding and yellow-pigmented bacterium, designated strain SFD31T, was isolated from brown alga collected from the South Sea, Republic of Korea. Cells were Gram-stain-negative, and catalase- and oxidase-positive. The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed that strain SFD31T forms an independent lineage within the genus Winogradskyella. Strain SFD31T was related distantly to Winogradskyella echinorum KMM 6211T (97.9 %, 16S rRNA gene sequence similarity), Winogradskyella litoriviva KMM 6491T (97.4 %), Winogradskyella pulchrlflava EM106T (97.2 %) and Winogradskyella eckloniae EC29T (96.9 %). The major fatty acids of strain SFD31T were iso-C15:0, iso-C15:1 G, summed feature 3 (C16:1ω7c and/or C16:1ω6c) and unknown 13:0. The only isoprenoid quinone of the isolate was menaquinone 6. The major polar lipids were phosphatidylethanolamine, four unidentified aminolipids and two unidentified lipids. The DNA G+C content of strain SFD31T was 36.0 mol%. Phenotypic characteristics distinguished strain SFD31T from the related species of the genus Winogradskyella. On the basis of the evidence presented in this study, a novel species, Winogradskyella flava sp. nov., is proposed for strain SFD31T (=KCTC 52348T=JCM 31798T).

The genus Winogradskyella belonging to the family Flavobacteriaceae of the phylum Bacteroidetes was first described by Nedashkovskaya et al. [1] with Winogradskyella thalassocola as the type species to accommodate Gram-stain-negative, heterotrophic, strictly aerobic, motile by gliding and rod-shaped bacteria. The genus description has been subsequently emended by Ivanova et al. [2], Yoon et al. [3], Nedashkovskaya et al. [4] and Begum et al. [5]. At the time of writing, the genus Winogradskyella consists of 23 species with validly published names. Members of the genus Winogradskyella have been isolated from various marine environments, such as seawater, seaweeds, marine animals and marine sediment [1–19]. The present study reports on the taxonomic characterization of a Winogradskyella-like bacterial strain, SFD31T, which was isolated from brown alga (Sargassum fulvulum).

Strain SFD31T was isolated from the brown alga, Sargassum fulvulum, collected from a natural seaweed bed in the South Sea (33° 29‘ 52.4” N, 126° 54‘ 59.2” E), Republic of Korea. The seaweed sample was wiped and immersed into saline. The immersed sample was rotated for 30 min at 4 °C. Saline supernatant was inoculated onto marine agar 2216 (MA; Becton Dickinson) and incubated for 5 days at 25 °C. The isolate was routinely cultured on MA and preserved at −80 °C as a suspension in marine 2216 broth (MB; Becton Dickinson) containing 20 % glycerol (v/v). Reference strains Winogradskyella echinorum KCTC 22026T, Winogradskyella litoriviva KCTC 23972T, Winogradskyella pulchrlflava KCTC 23858T and Winogradskyella eckloniae KCTC 32172T were purchased from the Korean Collection for Type Cultures (KCTC).

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously [20]. Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved by using the EzTaxon-e server (www.ezbiocloud.net); [21]) and the BLAST search program on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The novel sequence and related sequences were aligned by using CLUSTAL_W [22] and the alignment was refined by using BioEdit version 7.2.0 [23]. Phylogenetic analysis was performed by using the software package MEGA version 6.06 [24]. Phylogenetic trees were inferred using the neighbour-joining [25], maximum-likelihood [26] and maximum-parsimony [27] algorithms. The distance matrix of the neighbour-joining method was generated...
The 16S rRNA gene sequence of strain SFD31\textsuperscript{T} was continuous stretches of 1445 nt. The closest relatives of strain SFD31\textsuperscript{T} were \textit{W. echinorum} KMM 6211\textsuperscript{T} (97.9\%, 16S rRNA gene sequence similarity), \textit{W. litoriviva} KMM 6491\textsuperscript{T} (97.4\%), \textit{W. pulchriflava} EM106\textsuperscript{T} (97.2\%) and \textit{W. eckloniae} EC29\textsuperscript{T} (96.9\%). 16S rRNA gene sequence similarity of strain SFD31\textsuperscript{T} with other members of the genus \textit{Winogradskyella} was less than 96.5\%. The neighbour-joining tree (Fig. 1) showed that strain SFD31\textsuperscript{T} was closely related to members of the genus \textit{Winogradskyella} and formed distinct branches with the clade comprising \textit{W. echinorum} and \textit{W. pulchriflava}. The trees based on maximum-likelihood and maximum-parsimony methods showed essentially similar topology (Fig. S1, available in the online Supplementary Material). Strain SFD31\textsuperscript{T} shared a low DNA–DNA relatedness value of 28.9, 22.0, 19.0 and 17.4\% with \textit{W. echinorum} KCTC 22026\textsuperscript{T}, \textit{W. litoriviva} KCTC 23972\textsuperscript{T}, \textit{W. pulchriflava} KCTC 23858\textsuperscript{T} and \textit{W. eckloniae} KCTC 32172\textsuperscript{T}, respectively. The values are below the threshold (70\%) for determining bacterial species [32]; the finding strongly suggested that the isolate should belong to a separate species in the genus \textit{Winogradskyella}.

Growth on various standard bacteriological media was tested by using nutrient agar (NA; Becton Dickinson), Reasoner’s 2A (R2A; Becton Dickinson) agar, plate-count agar (PCA; Becton Dickinson), tryptic soy agar (TSA; Becton Dickinson) and Zobell’s agar ([33]; 5g Bacto peptone, 1g yeast extract, 0.1g ferric citrate and 15g Bacto agar in 1 litre distilled water). Cells grown on MA at 25 °C for 2–3 days were used for the physiological and biochemical tests. The Gram-reaction test was performed by using the bioMérieux Gram stain kit and the Ryu non-staining KOH method [34]. Cell morphology was observed by phase-contrast (IC50; Leika) and transmission electron (CM-20; Philips) microscopy using cells grown at 25 °C for 3 days on MA. Motility was examined by observing the cells grown in wet mounts using phase-contrast microscopy (IC50; Leika). Flagellation was determined with a transmission electron microscope (CM-20; Philips) using cells cultured for 48 h in MB. Growth at various NaCl concentrations (0–10.0\%, w/v, in increments of 1.0\%) was investigated in MA except that no NaCl was used. The growth experiment at pH 4–11 (increments of 1 pH units) was performed using MB containing 100 mM acetate buffer, 100 mM NaH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}PO\textsubscript{4} buffer and 100 mM NaHCO\textsubscript{3}/Na\textsubscript{2}CO\textsubscript{3} buffer, at pH 4–5, 6–8 and 9–11, respectively. The optimal temperature and temperature range for growth was tested on MA at 4 and 10–45 °C (at 5 °C intervals). Anaerobic growth was tested on MA in a jar containing an AnaeroPack-Anaero packet (Mitsubishi Gas Chemical), which works as an oxygen absorber and a CO\textsubscript{2} generator, for up to 10 days. Catalase and oxidase activities were tested in 3 \%/v/v hydrogen peroxide and oxidase reagent (bioMérieux), respectively. Acid production from sugars was tested as described by Yamaguchi and Yokoe [35]. Simmon’s citrate test was carried out in Simmons’ citrate agar (Sigma). Indole production was determined with Kovac’s indole reagent on SIM agar (Becton Dickinson). H\textsubscript{2}S production was determined on Kligler iron agar (Becton Dickinson) according to Smibert and Krieg [36]. Degradation of the following macromolecules was tested using MA as the basal medium and incubation at 25 °C for 10 days: carboxymethyl cellulose (CMC; 1\%, w/v), casein (5\% skimmed milk), chitin (1\% colloidal chitin, w/v), hypoxanthine (1\%, w/v), starch (0.2\%), Tween 20 (1\%), Tween 80 (1\%) and xanthine (1\% w/v). Degradation was revealed by formation of clear zones around the colonies either directly [37] or after flooding with adequate-staining solutions [36]. Decomposition of xylan (1\%, w/v) was tested using MA as the basal medium [38]. DNase activity was determined with DNase test agar (Becton Dickinson). The presence of flexirubin-type pigments was tested as described by Bernardet et al. [39]. Cell extracts for carotenoid analysis were prepared with methanol [40] and the absorption spectrum (200–800 nm) was recorded using a UV/VIS spectrophotometer (Ultrospec 2100pro; Biochrom). Some physiological characteristics and enzyme activities were determined using API 20NE and API ZYM kits (bioMérieux) prepared according to the manufacturer’s instructions. Antibiotic resistance was determined with disc diffusion method [41] using commercial antibiotic-impregnated discs (Becton Dickinson). After 5 days of incubation at 25 °C on MA, the results were interpreted according to the guidelines set by the Clinical and Laboratory Standards Institute [42].

Cells were Gram-stain-negative, motile by gliding, rod-shaped, approximately 0.3–0.4 µm in diameter and 0.8–1.0 µm in length (Fig. S2). Colonies were circular, convex, smooth, 0.5–1.0 mm in diameter and yellow coloured on MA after 5 days. Strain SFD31\textsuperscript{T} was sensitive to the following antibiotics (µg per disc, unless otherwise indicated): ampicillin (10), chloramphenicol (30), erythromycin (15) and vancomycin (30), but resistant to amikacin (30), gentamicin (10), kanamycin (30), nalidixic acid (30), penicillin (10 IU) and streptomycin (10). The detailed results of physiological and biochemical analyses are given in Table 1 and the species description.

For cellular fatty acid analysis, strain SFD31\textsuperscript{T} and the reference strains were grown on MA and harvested at late exponential growth phase, i.e. after 2 days at 25 °C. Extraction of fatty acid methyl esters and separation by gas
Fig. 1. Neighbour-joining phylogenetic tree based on 1294 nt in unambiguously aligned 16S rRNA gene sequences, showing relationships between strain SFD31T and members of the genus Winogradskyella. The percentage numbers at the nodes are the levels of bootstrap support (>70%) based on neighbour-joining analyses of 1000 resampled data sets. The sequence of Capnocytophaga ochracea ATCC 27872T (U41350) was used as an outgroup. Closed circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood algorithms. Bar, 0.01 nucleotide substitution per position.

The fatty acid profiles of strain SFD31 were determined by the FAME method of the Microbial Identification System (MIDI) version 6.1 and the TSBA6 database. For analyses of polar lipids and isoprenoid quinone, cells grown in MB for 3 days were harvested and freeze-dried. Polar lipids were extracted, separated by two-dimensional thin-layer chromatography (TLC) and identified by spraying the plates with appropriate detection reagents such as ethanolic molybdate and 4 % NaCl. 

The fatty acid profiles of strain SFD31 were described in Table 2. The predominant fatty acids (>10.0 % of total fatty acids) are iso-C<sub>15:1</sub> G (26.6 %), iso-C<sub>15:0</sub> (20.0 %), unknown 13.565 (12.0 %) and summed feature 3 (C<sub>16:1ω7c</sub> and/or C<sub>16:1ω6c</sub>) (10.6 %). The fatty acid compositions of the isolate and the reference strains were similar, although there were differences in the proportions and the presence/absence of some fatty acids. The only isoprenoid quinone was menaquinone 6 (MK-6). The polar lipid profile of strain SFD31<sup>T</sup> contained phosphatidylethanolamine, four unidentified aminolipids and two unidentified lipids (Fig. S3).

The phylogenetic distinctness of the novel isolate SFD31<sup>T</sup> was supported by both physiological difference in the NaCl tolerance and starch hydrolysis, and chemotaxonomic differences in the composition of fatty acids iso-C<sub>15:1</sub> G and summed feature 3. Based on the combination of phenotypic, biochemical characteristics and DNA–DNA relatedness, as well as the phylogenetic position, strain SFD31<sup>T</sup> represents a novel species of the genus Winogradskyella, for which the name Winogradskyella flava sp. nov., is proposed.

**DESCRIPTION OF WINOGRADSKYELLA FLAVA SP. NOV.**

*Winogradskyella flava* (fla‘va. L. fem. adj. *flava* yellow, pertaining to the yellow colour of the colonies).

Cells are Gram-stain-negative, aerobic, non-spore-forming, gliding, rod-shaped and approximately 0.4–0.6 μm in diameter and 0.8–1.4 μm in length (Fig. S2). Colonies are circular, convex, smooth, 0.5–1.0 mm in diameter and yellow...
Table 2. Cellular fatty acid composition (%) of strain SFD31<sup>T</sup> and related type strains of Winogradskyella species

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>Straight-chain saturated</td>
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<tr>
<td>C&lt;sub&gt;15&lt;/sub&gt;:0</td>
<td>4.2</td>
<td>4.7</td>
<td>6.1</td>
<td>3.7</td>
<td>9.1</td>
</tr>
<tr>
<td>C&lt;sub&gt;16&lt;/sub&gt;:0</td>
<td>TR</td>
<td>1.2</td>
<td>1.3</td>
<td>2.7</td>
<td>1.5</td>
</tr>
<tr>
<td>C&lt;sub&gt;15&lt;/sub&gt;:0 2-OH</td>
<td>TR</td>
<td>–</td>
<td>1.3</td>
<td>–</td>
<td>TR</td>
</tr>
<tr>
<td>Branched saturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-C&lt;sub&gt;14&lt;/sub&gt;:0</td>
<td>2.6</td>
<td>2.2</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>iso-C&lt;sub&gt;15&lt;/sub&gt;:0</td>
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<td>22.4</td>
<td>26.4</td>
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<td>TR</td>
<td>TR</td>
<td>5.1</td>
<td>–</td>
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<tr>
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<td>1.2</td>
<td>1.3</td>
<td>3.1</td>
<td>TR</td>
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<tr>
<td>iso-C&lt;sub&gt;17&lt;/sub&gt;:0 3-OH</td>
<td>4.2</td>
<td>2.3</td>
<td>1.6</td>
<td>2.7</td>
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<td>12.3</td>
<td>TR</td>
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<td>1.3</td>
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<tr>
<td>iso-C&lt;sub&gt;14&lt;/sub&gt;:1 E</td>
<td>–</td>
<td>2.8</td>
<td>TR</td>
<td>–</td>
<td>2.1</td>
</tr>
<tr>
<td>iso-C&lt;sub&gt;15&lt;/sub&gt;:1 G</td>
<td>26.6</td>
<td>20.4</td>
<td>21.1</td>
<td>17.9</td>
<td>21.4</td>
</tr>
<tr>
<td>iso-C&lt;sub&gt;16&lt;/sub&gt;:1 H</td>
<td>–</td>
<td>–</td>
<td>TR</td>
<td>1.9</td>
<td>–</td>
</tr>
<tr>
<td>C&lt;sub&gt;15&lt;/sub&gt;:1ω6c</td>
<td>–</td>
<td>–</td>
<td>1.6</td>
<td>–</td>
<td>4.1</td>
</tr>
<tr>
<td>Summed feature&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
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<td>3</td>
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<td>5.1</td>
<td>4.4</td>
<td>3.4</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>1.4</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>Unknown 11.543</td>
<td>5.1</td>
<td>15.7</td>
<td>5.4</td>
<td>8.5</td>
<td>6.7</td>
</tr>
<tr>
<td>Unknown 13.565</td>
<td>12.0</td>
<td>8.7</td>
<td>5.4</td>
<td>12.8</td>
<td>9.7</td>
</tr>
</tbody>
</table>

<sup>*</sup>Summed features are groups of two or three fatty acids that cannot be separated by gas-liquid chromatography with the MIDI system. Summed feature 3 contained C<sub>16</sub>:1ω7c and/or C<sub>16</sub>:1ω6c. Summed feature 9 contained iso-C<sub>17</sub>:1ω9c and/or 10-methyl C<sub>16</sub>:0.

coloured on MA after 5 days. Growth occurs on MA but not on MacConkey agar, NA, PCA, R2A, TSA and Zobell agar. Growth occurs at 4–35 °C (optimum, 20–30 °C), at pH 7–9 (optimum, pH 8) and at 2.0–3.0 % (w/v) NaCl (optimum, 2 % NaCl). Catalase and oxidase activities are positive. Flexirubin-type pigments are absent. Methanol extracts show the typical spectrum of carotenoid pigment with the maximum absorption at 451 nm. Nitrate is not reduced to nitrite. Indole and H<sub>2</sub>S are not produced. Aesculin, gelatin and Tween 80 are hydrolysed, but arginine, casein, chitin, CMC, DNA, hypoxanthine, starch, Tween 20, urea, xanthine and xylan are not. Does not assimilate adipic acid, arabinose, capric acid, glucose, malic acid, maltose, mannotol, mannose, phenylacetic acid, potassium gluconate, trisodium citrate and N-acetyl-glucosamine. Tests for acid production from fructose, galactose, glucose, lactose, maltose, mannotol, mannose, rhamnose, sucrose, trehalose and xylose are negative. In the API ZYM gallery, acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase (C4), esterase lipase (C8), β-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities are present, but cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, N-acetyl-β-glucosaminidase, α-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase and trypsin activities are absent. The predominant fatty acids (>10.0 % of total fatty acids) are iso-C<sub>15</sub>:0, iso-C<sub>15</sub>:1 G, summed feature 3 (C<sub>16</sub>:1ω7c and/or C<sub>16</sub>:1ω6c) and unknown 13.565. The only respiratory quinone detected is menaquinone 6 (MK-6). The major polar lipid is phosphatidylethanolamine; four unidentified aminolipids and two unidentified lipids were also detected. The DNA G+C content is 36.0 mol%.

The type strain is SFD31<sup>T</sup> (=KCTC 52348<sup>T</sup>=JCM 31798<sup>T</sup>), isolated from the brown alga, Sargassum fulvellum, collected from the South Sea, Republic of Korea.

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Conflicts of interest
The authors declare that there are no conflicts of interest.
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