Winogradskyella exilis sp. nov., isolated from the starfish *Stellaster equestris*, and emended description of the genus *Winogradskyella*

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A pale-yellowish-pigmented strain, 022-2-26^T, was isolated from a starfish, *Stellaster equestris*. Cells of strain 022-2-26^T were Gram-negative short rods that were chemo-organotrophic, alkali-tolerant and mesophilic. The predominant menaquinone was MK-6. The major cellular fatty acids were iso-C_{15:0}, iso-C_{15:1}, C_{15:0}, iso-C_{15:0} 2-OH and iso-C_{17:0} 3-OH (together representing 87 % of the total fatty acids). The DNA G + C content was 30.1 mol%. A 16S rRNA gene sequence of the isolate was determined and phylogenetic analyses revealed that strain 022-2-26^T formed a robust clade (neighbour-joining algorithm with a bootstrap value of 95 % and parsimony and maximum-likelihood algorithms) with type strains of species in the genus *Winogradskyella*. The closest phylogenetic neighbour of strain 022-2-26^T was *Winogradskyella poriferorum* UST030701-295^T (96 % 16S rRNA gene sequence similarity; 59 differences between sequences). On the basis of the phenotypic and chemotaxonomic characteristics and the phylogenetic evidence, it is proposed that strain 022-2-26^T represents a novel species, *Winogradskyella exilis* sp. nov. The type strain is 022-2-26^T (=KMM 6013^T =CIP 109976^T).

The genus *Winogradskyella* was created within the family *Flavobacteriaceae* in 2005 to accommodate three species, *Winogradskyella thalassocola*, *W. epiphytica* and *W. eximia*, marine bacteria respectively isolated from the green alga *Acrosiphonia sonderi* and the brown algae *Chorda filum* and *Laminaria japonica* (Nedashkovskaya *et al.*, 2005). A fourth species was described shortly afterwards: *Winogradskyella poriferorum*, isolated from a sponge (Lau *et al.*, 2005). We report here the polyphasic characterization of a novel bacterium isolated from the starfish *Stellaster equestris*.

The starfish was collected with dragging equipment in October 1998 at a depth of 100 m (30 ‰ salinity, 15 °C) in the South China Sea (26° 28.3' N 122° 29.0' E). The starfish

was pre-rinsed in sterilized seawater and a piece (about 3 g) of tegument tissue was removed aseptically. Strain 022-2-26^T was isolated from the tissue homogenates by plating samples (0.1 ml) on agar plates of marine agar 2216 (MA; Oxoid) and on medium B. Medium B contained (w/v) 0.2 % Bacto peptone (Difco), 0.2 % casein hydrolysate (Merck), 0.2 % Bacto yeast extract (Oxoid), 0.1 % glucose, 0.02 % KH₂PO₄, 0.005 % MgSO₄ · 7H₂O and 1.5 % Bacto agar (Oxoid) in 50 % (v/v) natural seawater and 50 % (v/v) distilled water at pH 7.5–7.8, as described elsewhere (Ivanova *et al.*, 1996).

The phenotypic properties used for the characterization of *Flavobacterium* species were determined by using standard procedures (Smibert & Krieg, 1994) and as described elsewhere (McMeekin *et al.*, 1971; Ivanova *et al.*, 1996, 1998, 2005; Bernardet *et al.*, 2002). Strain 022-2-26^T was cultured at 22–24 °C. To test for spreading growth and gliding motility, strain 022-2-26^T was grown on medium B with the peptone content reduced to 0.02 % (0.2 g l⁻¹). Gliding motility was verified by using phase-contrast

Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 022-2-26^T is FJ595484.

A table of fatty acid compositions and a 16S rRNA gene sequence-based consensus tree are available as supplementary material with the online version of this paper.

microscopy (Eclipse TE-DH, 100 W; Nikon) of hanging-drop preparations. The bathochromic shift test with 20 % (w/v) KOH was performed to detect flexirubin pigments (Fautz & Reichenbach, 1980). The following physiological and biochemical properties of strain 022-2-26^T were examined: oxidation/fermentation of glucose (Hugh & Leifson, 1953), Gram stain, reduction of nitrate and nitrite, liquefaction of gelatin, activity of catalase (with 5 % H₂O₂), oxidase (Kóvacs, 1956), arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase, production of poly- β -hydroxybutyrate, acetoin (Voges-Proskauer test), indole and H₂S, requirement for sodium ions (0, 1, 3, 6, 8, 10, 12, 15 %, w/v, NaCl) in nutrient broth (Oxoid) and hydrolysis of agar, alginate, casein, chitin, DNA, starch and Tween 80. The temperature range for growth was examined on MA at 4, 10, 30, 35, 37 and 42 °C. Other physiological and biochemical tests were performed with the API 20E, API 50 and API ZYM systems (bioMérieux). Susceptibility to antibiotics was tested by the diffusion plate technique, using solid medium B and discs impregnated with the following antibiotics (μ g per disc unless otherwise indicated): ampicillin (10), benzylpenicillin (10 U), carbenicillin (100), cephalexin (30), cephalosin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), lincomycin (15), nalidixic acid (30), neomycin (30), ofloxacin (5), oleandomycin (15), oxacillin (10), polymyxin (300 U), rifampicin (5), streptomycin (30) and tetracycline (30). The physiological and biochemical characteristics are presented in the species description and in Table 1.

For the extraction and characterization of carotenoid pigments and menaquinones, phospholipids and fatty acids, cell biomass of strain 022-2-26^T was harvested from MA (five plates for carotenoid pigment analysis) after incubation at 28 °C for 48 h as described previously (Ivanova *et al.*, 2005). Pigment extraction and characterization was performed as described previously (Ivanova *et al.*, 2005). The isoprenoid quinone composition was characterized by HPLC-MS (model 1200L; Varian) using a reversed-phase Omnisphere 3 C18 column (20 cm $\times$ 2 mm; Varian) at 55 °C and acetonitrile as the mobile phase (flow rate 0.5 ml min⁻¹). Menaquinones were detected at 270 nm. Lipids were extracted according to Bligh & Dyer (1959). Polar lipids were separated and identified as described previously (Ivanova *et al.*, 2005). The lipids were treated with 5 % HCl in methanol at 80 °C for 180 min to produce fatty acid methyl esters (FAMES; Christie, 1982). FAMES were analysed by GC with flame ionization detection (Shimadzu GC-17) with a fused silica capillary column (30 m $\times$ 0.25 mm) coated with Supelcowax 10 at 210 °C. Helium was used as the carrier gas. FAMES were identified by comparing the retention times with those of authentic standards and using equivalent chain-length measurements. To ensure correct identification, FAMES were analysed by GC-MS using a model GCMS-QP5050A (Shimadzu) fitted with an MDN-5S capillary column (30 m $\times$ 0.25 mm). The column temperature was

Table 1. Characteristics that differentiate strain 022-2-26^T from the type strains of the most closely related *Winogradskyella* species

Strains: 1, *Winogradskyella exilis* sp. nov. 022-2-26^T; 2, *W. thalassocola* KMM 3907^T; 3, *W. epiphytica* KMM 3906^T; 4, *W. eximia* KMM 3944^T; 5, *W. poriferorum* UST030701-295^T. Data were obtained in this study or from Lau *et al.* (2005) and Nedashkovskaya *et al.* (2005). All strains were positive for aerobic and facultatively anaerobic metabolism, gliding motility, catalase and alkaline phosphatase activities and hydrolysis of gelatin and Tween 40. All strains were negative for the presence of flexirubin pigments, β -galactosidase activity, hydrolysis of cellulose (CM-cellulose and filter paper) and chitin, production of indole, acetoin and H₂S and utilization of citrate, D-adonitol, L-arabinose, dulcitol, D-galactose, inositol, lactose, melibiose, D-rhamnose, D-sorbitol and D- and L-xylose. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Ranges for growth					
Temperature (°C)	5–35	12–44	4–37	4–33	4–33
NaCl (% w/v)	0–6	1–4	1–8	1–5	1–8
Oxidase	–	+	+	+	+
Urease	w	–	–	–	–
Denitrification	+	–	–	–	–
Degradation of:					
Agar	–	–	+	+	+
Casein	–	–	–	+	–
DNA	–	+	+	–	–
Starch	w	–	–	+	–
Tween 80	–	+	+	–	–
Utilization of:					
Cellobiose	–	–	–	–	+
D-Glucose	–	–	–	+	+
Maltose	–	–	–	+	+
D-Mannitol	–	–	–	+	–
D-Mannose	–	–	–	+	+
Sucrose	–	–	–	+	+
DNA G + C content (mol%)	30.2	32.8	35.2	36.1	34.6

programmed for a hold at 170 °C for 1 min, an increase to 240 °C at 2 °C min⁻¹ and a hold at 240 °C for 20 min. The temperature of the injector and detector were 250 °C. The results of the fatty acid analysis are presented in the species description and Supplementary Table S1 (available in IJSEM Online). Strain 022-2-26^T contained MK-6, which is the characteristic respiratory quinone for the family *Flavobacteriaceae*, and high levels of branched and hydroxy C₁₅ and C₁₇ fatty acids (representing 92.9 % of the total fatty acid content), which are typical fatty acids for the family *Flavobacteriaceae* (Bowman *et al.*, 1998).

Genomic DNA was extracted from strain 022-2-26^T following the method of Marmur (1961) and the G + C content of the DNA was determined by the thermal denaturation method of Marmur & Doty (1962). The G + C content of the DNA was 30.1 $\pm$ 0.2 mol%. The 16S

rRNA gene was amplified and sequenced at the laboratories of the Australian Genome Research Facility, Brisbane, Australia. Briefly, the PCR primers corresponded to *Escherichia coli* positions 5 and 1540. The amplification products were purified using Microcon 100 molecular-mass cut-off membranes (Millipore) and checked for quality and quantity on separation on agarose gel. Cycle sequencing of the amplification products was performed using AmpliTaq ES DNA polymerase and rhodamine dye terminators and the sequencing reaction products were analysed with an ABI Prism 377 DNA Sequencer (Applied Biosystems).

The 16S rRNA gene sequence of strain 022-2-26^T was compared against a database of cultured species (<http://bioinfo.unice.fr/blast>) and type strains (EzTaxon; <http://147.47.212.35:8080>) in order to retrieve the 110 most similar sequences. A multiple sequence alignment was performed using CLUSTAL W version 2 (Larkin *et al.*, 2007) and checked manually with SeaView (Galtier *et al.*, 1996). Domains common to all sequences were used to derive a preliminary phylogenetic tree (not shown) using the most recent version of SeaView (version 4; Gouy *et al.*, 2010) and the BioNJ neighbour-joining algorithm (Gascuel, 1997). This tree showed that strain 022-2-26^T was most closely related to strains in the genus *Winogradskyella*. According to the preliminary tree, the 44 sequences most closely related to strain 022-2-26^T were selected and the alignment was checked again. Positions containing insertions or deletions were excluded. A full phylogenetic analysis was undertaken using the BioNJ, parsimony and maximum-likelihood algorithms, according to Gascuel (1997), PHYLIP version 3.52 and PhyML version 3.0.1 (Guindon & Gascuel, 2003), respectively. For the neighbour-joining analysis, a distance matrix was calculated using Kimura's two-parameter correction and bootstraps were performed using 1000 replications. Phylogenetic trees were drawn using TreeDyn (Chevenet *et al.*, 2006) or SeaView. A consensus phylogenetic tree is shown in Supplementary Fig. S1. Notably, when using similarity values only, the closest neighbour to strain 022-2-26^T was *W. poriferorum* UST030701-295^T, with which the isolate showed a similarity of about 96 % (59 differences between sequences) and formed a robust cluster with all algorithms and a bootstrap value of 82 % (Fig. 1). Strain 022-2-26^T was also

robustly included in the clade formed by the type strains of *Winogradskyella* species (all methods, bootstrap value of 95 %). Thus, the phylogenetic analyses provide strong evidence that strain 022-2-26^T represents a novel species of the genus *Winogradskyella*.

Strain 022-2-26^T can be differentiated easily from the type strains of previously described species of the genus *Winogradskyella*. For example, strain 022-2-26^T does not have a requirement for NaCl for growth and it is negative for oxidase and positive for denitrification. However, the fatty acid profile of strain 022-2-26^T is characteristic of the genus *Winogradskyella*, members of which have levels of iso-C_{15:0} 2-OH and iso-C_{17:0} 3-OH ranging from 4.2 to 10.2 % and distinct proportions of iso-C_{15:0} and iso-C_{15:1} as the major fatty acids. Consequently, we consider that strain 022-2-26^T represents a novel species in the genus *Winogradskyella*, for which the name *Winogradskyella exilis* sp. nov. is proposed.

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

The description is as given by Nedashkovskaya *et al.* (2005) with the following amendments. Some strains are oxidase-negative and able to reduce nitrate. Some strains do not require NaCl for growth.

Description of *Winogradskyella exilis* sp. nov.

Winogradskyella exilis (e.xi'lis. L. fem. adj. *exilis* small, thin, slender).

Rod-shaped cells with slightly irregular sides and pointed ends, about 0.8–1.8 µm long and 0.4–0.9 µm wide. Gram-negative. No endospores or resting stages. Exhibits gliding motility. Does not accumulate poly-β-hydroxybutyrate as an intracellular reserve product or have an arginine dihydrolase system. Aerobic and facultatively anaerobic. Anaerobic growth occurs by fermentation of D-glucose or anaerobic respiration of nitrate. Chemo-organotrophic. Oxidase-negative and catalase-positive. Colonies are light yellow, circular, 1–3 mm in diameter and low convex on solid media with a high content of nutrient components. Produces carotenoid pigments with absorbance peaks at 455 and 480 nm. Does not produce flexirubin pigments.

Fig. 1. Unrooted neighbour-joining phylogenetic tree showing the position of strain 022-2-26^T among its closest phylogenetic neighbours, based on 16S rRNA gene sequences. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in trees constructed with both the maximum-likelihood and parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

Grows optimally at 23 °C; no growth at 4 or 37 °C. Grows at pH 6.0–10.0 (optimum pH 8.0–8.5) and with 0–6 % NaCl. Weakly hydrolyses starch and gelatin. Does not decompose agar or chitin. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and valine arylamidase. With the API 50 system, utilizes only potassium 5-ketogluconate. Susceptible to (µg per disc unless otherwise indicated) ampicillin (10), benzylpenicillin (10 U), carbenicillin (100), cephalixin (30), cephalosin (30), chloramphenicol (30), lincomycin (15), nalidixic acid (30), ofloxacin (5), oleandomycin (15), rifampicin (5) and tetracycline (30), but not to erythromycin (15), gentamicin (10), kanamycin (30), neomycin (30), oxacillin (10), polymyxin (300 U) or streptomycin (30). The main cellular fatty acids are iso-C_{15:0}, iso-C_{15:1}, C_{15:0}, iso-C_{15:0} 2-OH and iso-C_{17:0} 3-OH. Phosphatidylethanolamine is the only phospholipid detected; aminolipids are not detected. The major isoprenoid quinone is MK-6. The DNA G + C content of the type strain is 30.0 ± 0.2 mol%.

The type strain is 022-2-26^T (=KMM 6013^T =CIP 109976^T), isolated from the starfish *Stellaster equestris*.

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