# *Winogradskyella thalassocola* gen. nov., sp. nov., *Winogradskyella epiphytica* sp. nov. and *Winogradskyella eximia* sp. nov., marine bacteria of the family *Flavobacteriaceae*

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Three novel heterotrophic, Gram-negative, yellow-pigmented, aerobic, gliding, oxidase- and catalase-positive bacteria were isolated from algae collected in the Gulf of Peter the Great, Sea of Japan. 16S rRNA gene sequence analysis revealed that the strains studied represented members of the family *Flavobacteriaceae* and showed 93·5–93·8 % similarity with their closest relative, *Psychroserpens burtonensis*. The DNA G+C content of the strains was 34–37 mol%. The major respiratory quinone was MK-6. The predominant fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:1}$, iso-$C_{16:0}$-3OH and iso-$C_{17:0}$-3OH. On the basis of their phenotypic, chemotaxonomic, genotypic and phylogenetic characteristics, the newly described bacteria have been assigned to the new genus *Winogradskyella* gen. nov., as *Winogradskyella thalassocola* sp. nov. (type strain, KMM 3907$^T$=KCTC 12221$^T$=LMG 22492$^T$=DSM 15363$^T$), *Winogradskyella epiphytica* sp. nov. (type strain, KMM 3906$^T$=KCTC 12220$^T$=LMG 22491$^T$=CCUG 47091$^T$) and *Winogradskyella eximia* sp. nov. (type strain, KMM 3944$^T$ (=KCTC 12219$^T$=LMG 22474$^T$).

Bacteria of the family *Flavobacteriaceae* are often found attached to the surfaces of a diverse range of marine algae (Chan & McManus, 1969; Bolinches *et al.*, 1988; Hanzawa *et al.*, 1998). The novel marine bacteria *Arenibacter latericius*, *Cellulophaga fucicola*, *Cellulophaga baltica*, *Cellulophaga algicola*, *Formosa algae*, *Mesonia algae*, *Maribacter ulvicola*, *Tenacibaculum amylolyticum*, *Ulvibacter litoralis* and *Zobellia galactanivorans*, associated with different algae, have been isolated and described (Johansen *et al.*, 1999; Bowman, 2000; Barbeyron *et al.*, 2001; Suzuki *et al.*, 2001; Ivanova *et al.*, 2001, 2004; Nedashkovskaya *et al.*, 2003b, 2004a, b). The above-mentioned flavobacteria are commonly characterized by rod-shaped cells. During studies on microbial communities of algae inhabiting the Sea of Japan, we recovered three novel isolates belonging to the family *Flavobacteriaceae* from the algae frond surfaces; these isolates were able to form cellular network-like structures or aggregates that can be considered to serve for an attachment adaptation.

Based on a polyphasic study of the algal isolates, including

Published online ahead of print on 19 July 2004 as DOI 10.1099/ijs.0.63307-0.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Winogradskyella thalassocola* KMM 3907$^T$, *Winogradskyella epiphytica* KMM 3906$^T$ and *Winogradskyella eximia* KMM 3944$^T$ are AY521223, AY521224 and AY521225, respectively.

phylogenetic, genotypic, chemotaxonomic and phenotypic data, we propose a new genus, *Winogradskyella* gen. nov., containing three novel species.

Strains KMM 3906$^T$, KMM 3907$^T$ and KMM 3944$^T$ were isolated from the green alga *Acrosiphonia sonderi*, and the brown algae *Chorda filum* and *Laminaria japonica*, respectively, collected in the Gulf of Peter the Great of the Sea of Japan during June 2000. Strains were cultivated at 28 °C on marine agar 2216 (MA; Difco) and stored at −80 °C in marine broth 2216 (MB; Difco) supplemented with 20 % (v/v) glycerol. On MA, colonies of strains studied were round, 2–4 mm in diameter, yellow-pigmented, shiny, viscous and with entire edges.

The phylogenetic position of the three isolates was determined using previously described procedures for DNA extraction, PCR and 16S rRNA gene sequence analysis (Kim *et al.*, 1998). The sequence data were aligned with those of representative members of selected genera of the family *Flavobacteriaceae* by using PHYDIT version 3.2 (http://plaza.snu.ac.kr/~jchun/phydit/). Phylogenetic trees were inferred by using suitable programs of the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated from the two-parameter model of Kimura (1980), and trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967) and maximum-likelihood (Felsenstein, 1993) algorithms. Bootstrap analysis was performed with 1000 resampled datasets using the SEQBOOT and CONSENSE programs of the PHYLIP package.

Phylogenetic analysis of almost-complete 16S rRNA gene sequences of strains KMM 3906$^T$, KMM 3907$^T$ and KMM 3944$^T$ revealed that they form a distinct lineage within the family *Flavobacteriaceae* (Fig. 1). *Psychroserpens burtonensis* was found to be the nearest neighbour; this relationship was supported by a high bootstrap value and also by the different tree-making algorithms used. However, 16S rRNA gene sequence similarity between the three strains and *P. burtonensis* was only 93·5–93·8 %. 16S rRNA gene sequence similarity values of the three strains to other close relatives, *Gelidibacter algens* and *Formosa algae*, were 90·8–91·3 and 92·7–92·9 %, respectively. The low sequence similarity values of KMM 3906$^T$, KMM 3907$^T$ and KMM 3944$^T$ to other *Cytophaga*-like bacteria described to date (85·6–92·1 %) demonstrate that the bacteria isolated in this study represent a new genus.

The three strains had 16S rRNA gene sequence similarities in the range 96·3–97·1 %.

For DNA–DNA hybridizations and determination of the G+C content, DNA was isolated following the method of Marmur (1961). The G+C content was determined by the thermal denaturation method of Marmur & Doty (1962). DNA–DNA hybridization was performed spectrophotometrically and initial renaturation rates were recorded as described by De Ley *et al.* (1970).

The DNA G+C contents of strains KMM 3906$^T$, KMM 3907$^T$ and KMM 3944$^T$ were 35·2, 34·6 and 36·1 mol%, respectively. DNA–DNA relatedness between the strains was 34–45 %. These values indicated that the strains represent three separate species. Phenotypic data distinguishing the strains are given in Table 1.

Analysis of fatty acid methyl esters was carried out according to the standard protocol of the Microbial Identification System (Microbial ID). Isoprenoid quinones were extracted from lyophilized cells and analysed as described by Akagawa-Matsushita *et al.* (1992). Isoprenoid quinone composition was characterized by HPLC (Shimadzu Instruments) using a reversed-phase type Zorbax ODS column (250 × 4·6 mm) and acetonitrile/2-propanol (65 : 35, v/v) as a mobile phase at a flow rate of 0·5 ml min$^{-1}$. The column was kept at 40 °C. Menaquinones were detected by monitoring at 270 nm and were identified by comparison with known quinones from reference strain *Salegentibacter salegens* DSM 5424$^T$.

Predominant cellular fatty acids of the strains studied were branched-chain unsaturated and straight-chain saturated, namely iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:1}$, iso-$C_{16:0}$-3OH and iso-$C_{17:0}$-3OH (Table 2). The major isoprenoid quinone was MK-6.

Phenotypic characterization was performed using the tests described previously (Nedashkovskaya *et al.*, 2003a, b). Gliding motility was determined as described by Bowman (2000). Scanning electron microscopy was used to examine the bacteria, which were fixed with a solution containing 2 % glutaraldehyde and 3 % formaldehyde in cacodylate buffer (0·1 M cacodylate, 0·09 M sucrose, 0·01 M $CaCl_2$, 0·01 M $MgCl_2$, pH 6·9) for 1 h on ice and washed with cacodylate buffer. After washing several times in TE buffer (20 mM Tris, 1 mM EDTA, pH 7·0), samples were dehydrated through a graded series of acetone (10, 30, 50, 70, 90, 100 %) on ice, each step for 15 min, followed by critical-point drying with liquid $CO_2$. Samples were sputter-coated with an approximately 10 nm thick gold film before examination in a Zeiss field-emission scanning electron microscope (DSM982 Gemini) at an acceleration voltage of 5 kV using the Everhart Thornley secondary electron (SE) detector and the Inlens-SE detector at a 50 : 50 ratio.

The three bacteria described in this study were Gram-negative, chemo-organotrophic with respiratory-type metabolism, non-motile single flexible rods, 0·4–0·6 μm in diameter and 1·0–1·3 μm in length. All three strains formed unique network-like structures or aggregates (Fig. 2A–C). Growth of strain KMM 3944$^T$ was observed at 1–5 % NaCl; strains KMM 3906$^T$ and KMM 3907$^T$ grew in media containing 1–8 % NaCl. Optimal growth was observed at 1·5–2 % NaCl. The maximum growth temperature for strain KMM 3906$^T$ was 37 °C, and that for strains KMM 3907$^T$ and KMM 3944$^T$ was 33 °C. Strain KMM 3906$^T$ was able to oxidize carbohydrates, but the other two strains were not.

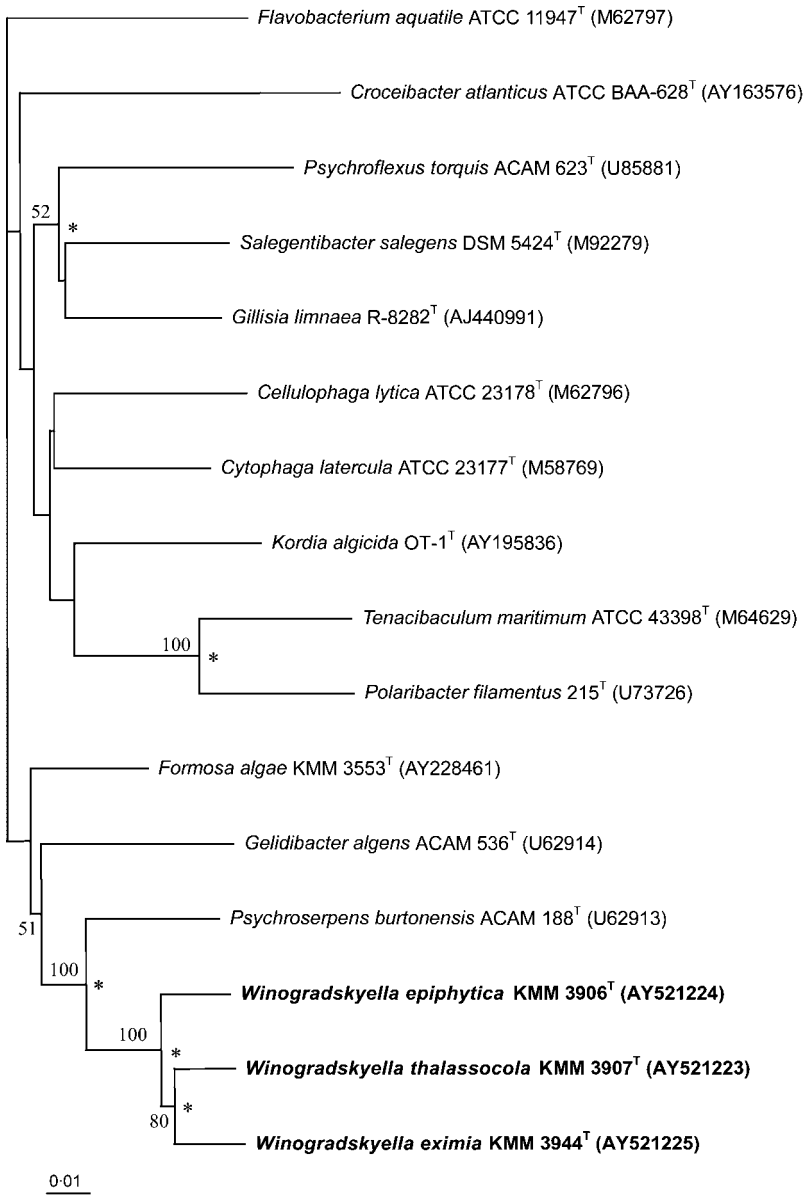


**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequence analysis of strains KMM 3906$^T$, KMM 3907$^T$ and KMM 3944$^T$ and representative members of the family *Flavobacteriaceae*. Asterisks indicate branches that were also recovered using the maximum-likelihood algorithm. The numbers at nodes indicate percentage bootstrap values. Bar, 0·01 substitution per nucleotide position.

Other physiological characteristics of the three strains are given in the species description and in Table 1.

The three isolates occupy a distinct phylogenetic branch and share many common phenotypic traits with other members of the family *Flavobacteriaceae*. Differential features of strains studied and their close relatives are given in Table 3. The algal isolates can be distinguished from their closest relative *P. burtonensis* by the presence of gliding motility, oxidase activity and hydrolysis of agar and casein. The ability to produce oxidase and agarase distinguishes the strains studied and members of the genus *Gelidibacter*. The requirement of $Na^+$ ions for growth and casein hydrolysis separate the strains studied from their close neighbour *Formosa algae*.

Polyphasic data on the strains studied, including their separate phylogenetic branching, morphological characteristics, fatty acid composition, phenotypic features and low levels of DNA–DNA relatedness, indicate that they can not be assigned to any of the taxa currently included in the family *Flavobacteriaceae*. Consequently, we propose that strains KMM 3907$^T$, KMM 3906$^T$ and KMM 3944$^T$ be placed in a new genus, *Winogradskyella* gen. nov., as *Winogradskyella thalassocola* sp. nov., *Winogradskyella epiphytica* sp. nov. and *Winogradskyella eximia* sp. nov., respectively.

## Description of *Winogradskyella* gen. nov.

*Winogradskyella* [Wi.no.grad′sky.el.la. N.L. fem. n. *Winogradskyella* named after Sergey Winogradsky (1856–1953), a Russian microbiologist who made a considerable contribution to the taxonomy of bacteria of the phylum *Cytophaga–Flavobacterium–Bacteroides*].

**Table 1.** Phenotypic properties of the *Winogradskyella* gen. nov. species

Taxa: 1, *W. thalassocola* KMM 3907$^T$; 2, *W. epiphytica* KMM 3906$^T$; 3, *W. eximia* KMM 3944$^T$. All were positive for: respiratory metabolism; gliding motility; oxidase, catalase and alkaline phosphatase activities; requirement for $Na^+$ ions for growth; growth at 33 °C and in 1–5 % NaCl; hydrolysis of agar, gelatin and Tween 40; susceptibility to carbenicillin and lincomycin. All were negative for: flexirubin pigments; nitrate reduction; urease and β-galactosidase activities; hydrolysis of cellulose (carboxymethylcellulose, filter paper) and chitin; acid formation from L-arabinose, D-galactose, D-lactose, D-melibiose, L-rhamnose, DL-xylose, adonitol, dulcitol, inositol, sorbitol and citrate; utilization of L-arabinose, D-lactose, D-sucrose, inositol, mannitol, sorbitol, malonate and citrate; indole, acetoin and $H_2S$ production; susceptibility to benzylpenicillin, gentamicin, kanamycin, neomycin, polymyxin B and streptomycin.

| Characteristic | 1 | 2 | 3 |
|---|---|---|---|
| Degradation of: | | | |
| Casein | − | − | + |
| Starch | − | − | + |
| Tween 20 | − | + | + |
| Tween 80 | − | + | − |
| DNA | − | + | − |
| Growth at/in: | | | |
| 37 °C | − | + | − |
| 8 % NaCl | + | + | − |
| Acid formation from: | | | |
| D-Glucose | + | − | + |
| D-Maltose | + | − | + |
| D-Cellobiose | + | − | − |
| D-Sucrose | − | − | + |
| Mannitol | − | − | + |
| Utilization of: | | | |
| D-Glucose | + | − | + |
| D-Mannose | + | − | + |
| Susceptibility to: | | | |
| Ampicillin | − | + | − |
| Oleandomycin | + | + | − |
| Tetracycline | − | + | + |
| DNA G+C content (mol%) | 34·6 | 35·2 | 36·1 |

Rod-shaped cells, motile by gliding. The cells can form network-like structures. Gram-negative. Do not form endospores. Strictly aerobic. Produce non-diffusible yellow pigments. No flexirubins are formed. Chemo-organotrophic. Cytochrome oxidase-, catalase- and alkaline phosphatase-positive. Can hydrolyse gelatin, starch and DNA. The main cellular fatty acids are straight-chain saturated, branched-chain saturated and unsaturated fatty acids iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{15:1}$, iso-$C_{16:0}$-3OH and iso-$C_{17:0}$-3OH. On the basis of 16S rRNA gene sequence analysis, the genus *Winogradskyella* is a member of the family *Flavobacteriaceae*, phylum '*Bacteroidetes*'.

The type species is *Winogradskyella thalassocola*.

**Table 2.** Whole-cell fatty acid profiles (percentage composition) of the *Winogradskyella* species

Taxa: 1, *W. thalassocola* KMM 3907$^T$; 2, *W. epiphytica* KMM 3906$^T$; 3, *W. eximia* KMM 3944$^T$.

| Fatty acid | 1 | 2 | 3 |
|---|---|---|---|
| iso-$C_{14:0}$ | 2·6 | 4·5 | 1·4 |
| iso-$C_{14:1}$ | | 1·4 | |
| iso-$C_{15:0}$ | **8·7** | **6·7** | **25·6** |
| anteiso-$C_{15:0}$ | **4·9** | **15·9** | **7·0** |
| iso-$C_{15:1}$ | **11·4** | **8·1** | **10·4** |
| anteiso-$C_{15:1}$ | 1·6 | 6·3 | 1·4 |
| $C_{15:0}$ | 7·9 | 1·2 | 6·7 |
| $C15_{:1}\omega6c$ | 6·5 | | |
| iso-$C_{16:0}$ | 0·8 | 3·7 | 5·7 |
| iso-$C_{16:1}$ | 2·7 | 3·5 | 4·7 |
| $C_{16:0}$ 10 methyl | | | 6·3 |
| $C_{16:1}\omega7$, iso-$_{C15:0}$-2OH | 4·2 | 5·1 | 6·1 |
| iso-$C_{17:1}\omega9c$ | 0·6 | 1·1 | |
| anteiso-$C_{17:1}$ | | | 2·3 |
| $C_{17:0}$ cyclo | | | 2·4 |
| $C_{17:1}\omega6c$ | 0·9 | 1·9 | |
| iso-$C_{14:0}$-3OH | 0·9 | 1·6 | |
| $C_{15:0}$-2OH | 1·8 | 3·3 | 1·0 |
| iso-$C_{15:0}$-3OH | 11·9 | 2·9 | 2·6 |
| $C_{15:0}$-3OH | 2·5 | | |
| iso-$C_{16:0}$-3OH | **18·1** | **17·1** | **3·2** |
| $C_{16:0}$-3OH | 1·0 | | |
| iso-$C_{17:0}$-3OH | **5·4** | **7·3** | **6·7** |
| $C_{17:0}$-2OH | 0·8 | 5·2 | 1·0 |
| Unknown | 4·8 | 3·7 | 5·6 |

## Description of *Winogradskyella thalassocola* sp. nov.

*Winogradskyella thalassocola* (tha.las.so.co'la. Gr. n. *thalassa* the sea; L. suffix *-cola* dweller; N.L. n. *thalassocola* a sea-dweller).

Main characteristics are as given for the genus. In addition, cells are 0·5–0·7 μm in width and 4–7·3 μm in length. On MA, colonies are 2–4 mm in diameter, circular, shiny with entire edges, yellow-pigmented and viscous. Growth occurs at 4–33 °C. Optimal temperature for growth is 21–23 °C. Growth occurs in 1–8 % NaCl. Decomposes gelatin and Tween 40. Does not hydrolyse starch, DNA, Tween 20, Tween 80, urea, cellulose (carboxymethylcellulose and filter paper) or chitin. Forms acid from D-glucose, D-maltose and D-cellobiose, but not from L-arabinose, D-galactose, D-lactose, D-melibiose, L-rhamnose, D-sucrose, DL-xylose, citrate, adonitol, dulcitol, inositol or mannitol. Utilizes D-glucose and D-mannose, but not L-arabinose, D-lactose, D-sucrose, mannitol, inositol, sorbitol, malonate or citrate. β-Galactosidase activity is negative. Nitrate is not reduced. $H_2S$, indole and acetoin (Voges–Proskauer reaction) production are negative. Susceptible to

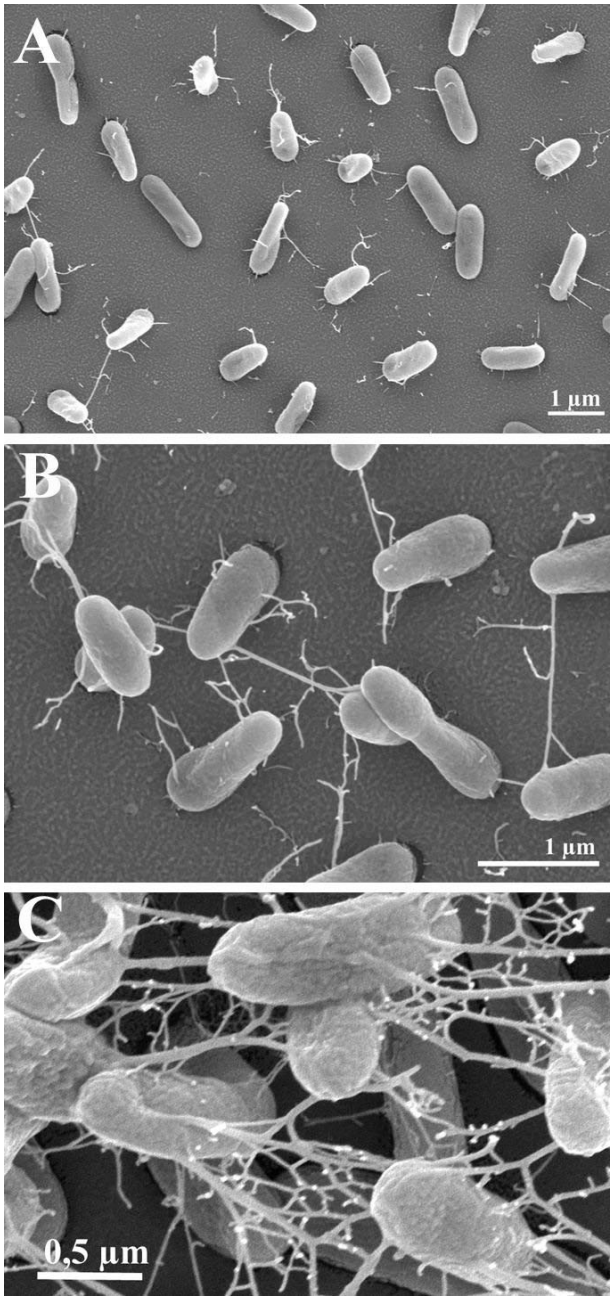


**Fig. 2.** Scanning electron micrographs of cells of strain KMM 3907$^T$ (A, B, C) showing the rod-shaped morphology and network-like structures.

carbenicillin, lincomycin and oleandomycin, but resistant to ampicillin, benzylpenicillin, gentamicin, kanamycin, neomycin, polymyxin B, streptomycin and tetracycline. The DNA G+C content is 34·6 mol%.

The type strain, KMM 3907$^T$ (=KCTC 12221$^T$=LMG 22492$^T$=DSM 15363$^T$), was isolated from the brown alga *Chorda filum*, collected in Troitsa Bay, Gulf of Peter the Great, Sea of Japan.

**Table 3.** Differential characteristics of the genus *Winogradskyella* and allied genera of the family *Flavobacteriaceae*

Genera: 1, *Winogradskyella*; 2, *Psychroserpens*; 3, *Gelidibacter*; 4, *Formosa*. Data from Bowman *et al.* (1997), Macián *et al.* (2002), Ivanova *et al.* (2004) and this study. −, Negative; +, positive; V, variable; ND, not determined.

| Characteristic | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Gliding motility | + | − | + | + |
| Oxidase/catalase | +/+ | −/+ | −/+ | +/+ |
| Requirement for $Na^+$ for growth | + | + | + | − |
| Acid from carbohydrates | V | − | + | + |
| Hydrolysis of: | | | | |
| Agar | + | − | − | − |
| Casein | − | + | V | − |
| Gelatin | V | V | V | + |
| Starch | V | − | + | + |
| DNA | V | − | V | − |
| DNA G+C content (mol%) | 34–37 | 27–29 | 36–40 | 34–35 |

## Description of *Winogradskyella epiphytica* sp. nov.

*Winogradskyella epiphytica* (e.pi.phy′ti.ca. *epiphiticus -a -um* adj. derived from Gr. *epi* on and *phyt-* relating to plants; N.L. *epiphytica* onto plant, pertaining to the original isolation from the surface of the algal fronds).

Main characteristics are as given for the genus. In addition, cells are 0·5–0·7 µm in width and 4–7·3 in length. On MA, colonies are 2–4 mm in diameter, circular, shiny with entire edges, yellow-pigmented and viscous. Growth occurs at 4–37 °C. Optimal temperature for growth is 23–25 °C. Growth occurs in 1–8 % NaCl. Decomposes agar, gelatin, DNA, and Tween 20, Tween 40 and Tween 80. Does not hydrolyse starch, urea, cellulose (carboxymethylcellulose and filter paper) or chitin. Does not form acid from L-arabinose, D-cellobiose, D-galactose, D-glucose, D-lactose, D-maltose, D-melibiose, L-rhamnose, D-sucrose, DL-xylose, citrate, adonitol, dulcitol, inositol or mannitol. Does not utilize L-arabinose, D-glucose, D-lactose, D-mannose, D-sucrose, mannitol, inositol, sorbitol, malonate or citrate. $\beta$-Galactosidase activity is negative. Nitrate is not reduced. $H_2S$, indole and acetoin (Voges–Proskauer reaction) production are negative. Susceptible to ampicillin, carbenicillin, lincomycin, oleandomycin and tetracycline, but resistant to benzylpenicillin, gentamicin, kanamycin, neomycin, polymyxin B and streptomycin. The DNA G+C content is 35·2 mol%.

The type strain, KMM 3906$^T$ (=KCTC 12220$^T$=LMG 22491$^T$=CCUG 47091$^T$), was isolated from the green alga *Acrosiphonia sonderi*, collected in Troitsa Bay, Gulf of Peter the Great, Sea of Japan.

### Description of *Winogradskyella eximia* sp. nov.

*Winogradskyella eximia* (e.xi′mi.a. L. fem. adj. *eximia* excellent).

Main characteristics are as given for the genus. In addition, cells are 0·5–0·7 μm in width and 4–7·3 in length. On MA, colonies are 2–4 mm in diameter, circular, shiny with entire edges, yellow-pigmented and viscous. Growth occurs at 4–33 °C. Optimal temperature for growth is 21–23 °C. Growth occurs in 1–5 % NaCl. Decomposes casein, gelatin, starch, Tween 20 and Tween 40. Does not hydrolyse DNA, urea, Tween 80, cellulose (carboxymethylcellulose and filter paper) or chitin. Forms acid from D-glucose, D-maltose, D-sucrose and mannitol, but not from L-arabinose, D-cellobiose, D-galactose, D-lactose, D-melibiose, L-rhamnose, DL-xylose, citrate, adonitol, dulcitol or inositol. Utilizes D-glucose and D-mannose, but not L-arabinose, D-lactose, D-sucrose, mannitol, inositol, sorbitol, malonate or citrate. β-Galactosidase activity is negative. Nitrate is not reduced. $H_2S$ is produced but indole and acetoin (Voges–Proskauer reaction) are not. Susceptible to lincomycin, but resistant to ampicillin, benzylpenicillin, carbenicillin, gentamicin, kanamycin, oleandomycin, neomycin, polymyxin B, streptomycin and tetracycline. The DNA G+C content is 36·1 mol%.

The type strain, KMM 3944$^T$ (=KCTC 12219$^T$=LMG 22474$^T$), was isolated from the brown alga *Laminaria japonica*, collected in the Gulf of Peter the Great, Sea of Japan.

## Acknowledgements

This research was supported by grants from the Ministry for Industry, Science and Technologies of the Russian Federation no. 2-2.16, and from the Presidium of the Russian Academy of Sciences 'Molecular and Cell Biology' and Russian Foundation for Basic Research no. 02-04-49517. K. S. Bae, S. K. Han and S. B. Kim are also grateful for the support from the Korea Research Council of Fundamental Science & Technology (grant no. KBM1000212).

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