Zobellia amurskyensis sp. nov., *Zobellia laminariae* sp. nov. and *Zobellia russellii* sp. nov., novel marine bacteria of the family *Flavobacteriaceae*

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The taxonomic position of four newly isolated marine, heterotrophic, gliding, Gram-negative, aerobic, pigmented, agarolytic bacteria was established. 16S rRNA gene sequence analysis indicated affiliation of the isolates to the genus *Zobellia* in the family *Flavobacteriaceae*. DNA–DNA hybridization experiments revealed that the strains studied represent three distinct and novel species, for which the names *Zobellia amurskyensis* sp. nov., *Zobellia laminariae* sp. nov. and *Zobellia russellii* sp. nov. are proposed, with KMM 3526^T (=LMG 22069^T=CCUG 47080^T), KMM 3676^T (=LMG 22070^T=CCUG 47083^T) and KMM 3677^T (=LMG 22071^T=CCUG 47084^T), respectively, as the type strains.

The genus *Zobellia* was created by Barbeyron *et al.* (2001) and contains Gram-negative, aerobic, gliding, agarolytic bacteria that produce flexirubin-type pigments. To date, two species of the genus *Zobellia* have validly published names, *Zobellia galactanivorans* and *Zobellia uliginosa*, and both originate from marine environments. The latter species was isolated from beach sand in California and was originally classified as *Flavobacterium uliginosum* (ZoBell & Upham, 1944). Later, it was transferred to the genus *Cytophaga* as *Cytophaga uliginosa* (Reichenbach, 1989), reclassified in the genus *Cellulophaga* as *Cellulophaga uliginosa* (Bowman, 2000) and finally assigned to the new genus *Zobellia* on the basis of a higher DNA G+C content (mol%), its maximum growth temperature, the presence of flexirubin pigments and its unique phylogenetic position (Barbeyron *et al.*, 2001). *Z. galactanivorans*, a marine species isolated from a red alga, was chosen as the type species.

In the course of a study on 450 strains isolated from sea water and bottom sediment samples, the sea urchin *Strongulocentrotus intermedius* and red (*Polysiphonia japonica*), green (*Acrosiphonia sonderi* and *Ulva fenestrata*) and brown (*Chorda filum* and *Laminaria japonica*) algae, the taxonomic position of four isolates was unclear and was further investigated in the present study. We report the results of phenotypic, physiological and genomic analyses on the latter strains, which led to the description of three novel species of the genus *Zobellia*, for which the names *Zobellia amurskyensis* sp. nov., *Zobellia laminariae* sp. nov. and *Zobellia russellii* sp. nov. are proposed.

The isolates investigated in this study were obtained during sampling in the Gulf of Peter the Great, Sea of Japan, Pacific Ocean, in June 2000. Strain KMM 3526^T was isolated from a sea-water sample collected in Amursky Bay. Strains KMM 3676^T, KMM 3926 and KMM 3677^T were recovered from the brown alga *L. japonica* (KMM 3676^T, KMM 3926) and the green alga *A. sonderi* (KMM 3677^T), collected in Troitsa Bay. For the isolation, 0·1 ml sea water or algal tissue homogenates was transferred to marine agar 2216 (Difco).

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Abbreviation: CM-cellulose, carboxymethylcellulose.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Zobellia amurskyensis* KMM 3526^T, *Zobellia laminariae* KMM 3676^T and *Zobellia russellii* KMM 3677^T are AB121974, AB121975 and AB121976, respectively.

After primary isolation and purification, strains were cultivated at 28 °C on the same medium and stored at −80 °C in marine broth (Difco) supplemented with 20 % (v/v) glycerol.

The almost complete 16S rRNA gene sequences of isolates KMM 3526^T, KMM 3676^T and KMM 3677^T were determined by PCR amplification and direct sequencing (Hiraishi, 1992). The conditions and reagents used for PCR amplification and sequencing of 16S rRNA gene were as described previously (Suzuki *et al.*, 2001). The sequences were aligned on the secondary-structure model, maintained by the SSU rRNA database (Van de Peer *et al.*, 2000), using the profile-alignment program of the CLUSTAL W software (Thompson *et al.*, 1994). Evolutionary distances were then computed with the DNADIST program in the PHYLIP 3.572 package (Felsenstein, 1995) with the two-parameter model (Kimura, 1980); a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987). To evaluate the phylogenetic trees, a bootstrap analysis with 1000 sample replications was performed with the SEQBOOT and CONSENSE programs in the PHYLIP 3.572 package.

The 16S rRNA gene-based analysis revealed that KMM 3526^T, KMM 3676^T and KMM 3677^T formed a coherent cluster within the genus *Zobellia* of the family *Flavobacteriaceae* (Fig. 1). The level of 16S rRNA gene sequence similarity between the KMM strains and *Z. galactanivorans* and *Z. uliginosa* ranged from 97·4 to 98·3 %. The 16S rRNA gene sequence similarities between strains KMM 3526^T, KMM 3676^T and KMM 3677^T ranged from 98·2 to 99·3 %.

Genomic DNA was prepared from cells cultivated on marine agar (Difco) for 24–48 h at 25 °C and extracted by following the DNA-extraction protocol of Pitcher *et al.* (1989), as modified by Leisner *et al.* (2002). DNA–DNA hybridizations were performed using the microplate method as described by Ezaki *et al.* (1989). Hybridizations were performed at 37 °C in a hybridization mixture containing 50 % formamide (2× SSC, 5× Denhardt's solution, 2·5 % dextran sulphate, 50 % formamide, 100 μg denaturated salmon sperm DNA ml^{-1} and 1250 ng biotinylated probe DNA ml^{-1}). The DNA G+C content was determined using two methods: (i) the HPLC method of Mesbah *et al.* (1989) on DNA extracted as indicated above and (ii) the thermal denaturation method of Marmur & Doty (1962) on DNA extracted according to the protocol of Marmur (1961).

The G+C contents of the DNA of strains KMM 3526^T, KMM 3676^T and KMM 3677^T were 37·1, 36·1 and 38·6 mol%, respectively, when determined by the thermal denaturation method. Slightly higher values were observed when determined by HPLC: 37·7 mol% for KMM 3526^T, 36·3 mol% for KMM 3676^T and 38·8 mol% for KMM 3677^T. DNA–DNA relatedness between strains KMM 3526^T, KMM 3676^T and KMM 3677^T and the type strains *Z. galactanivorans* Dsij^T and *Z. uliginosa* CIP 104808^T ranged from 8 to 29 %. The DNA–DNA binding value for strains KMM 3676^T and KMM 3926 was 93 %.

To obtain whole-cell fatty acid profiles, the strains studied were grown at 28 °C for 24 h on marine agar 2216 (Difco). Analysis of fatty acid methyl esters was carried out according to the standard protocol of the Microbial Identification System (Microbial ID).

The dominant cellular fatty acids of the novel isolates and the type strains of *Z. galactanivorans* and *Z. uliginosa* were the straight-chain and branched-chain saturated and unsaturated fatty acids 15:0, iso-15:0, iso-15:0 3-OH,

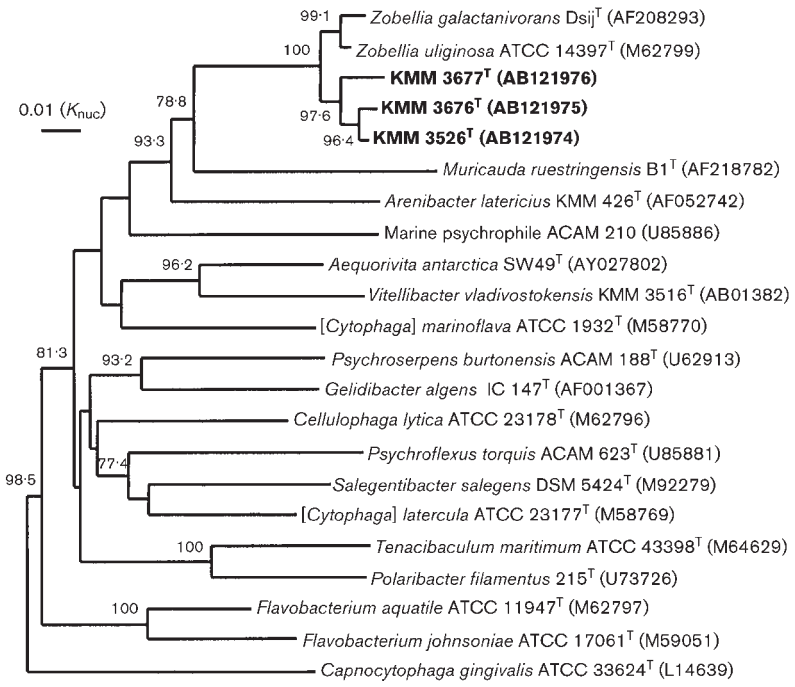

Fig. 1. Phylogenetic relationship between strains KMM 3526^T, KMM 3676^T and KMM 3677^T and marine species of the family *Flavobacteriaceae*, based on 16S rRNA gene sequence comparisons. The phylogenetic tree was generated by using the neighbour-joining method (Saitou & Nei, 1987). The 16S rRNA gene sequence of *Capnocytophaga gingivalis* was used as the outgroup. The number shown next to each node indicates the percentage bootstrap value of 1000 replicates (only values of 70 % or higher were cited). The scale bar indicates a genetic distance of 0·01 (K_{nuc}).

iso-15 : 1 and iso-17 : 0 3-OH (Table 1). No significant differences were found between the species.

Isoprenoid quinones were extracted and analysed by using the method of Nakagawa & Yamasato (1993). The major lipoquinone of the novel isolates, *Z. galactanivorans* Dsij^T and *Z. uliginosa* CIP 104808^T was MK-6.

Gram-staining, hydrolysis of agar, starch, casein, elastin, gelatin, cellulose [filter paper and carboxymethylcellulose (CM-cellulose)], chitin, DNA, urea and alginic acids, flexirubin production, growth at different pH values, production of acid from carbohydrates, hydrolysis of Tweens 20, 40 and 80, nitrate reduction, production of hydrogen sulphide and indole and β-galactosidase, oxidase, catalase and alkaline phosphatase activities were tested according to Gerhardt *et al.* (1994). Oxidative versus fermentative utilization of glucose was determined using Hugh & Leifson medium modified for marine bacteria (Lemos *et al.*, 1985). Susceptibility to antibiotics was tested as described earlier (Nedashkovskaya *et al.*, 2003). To examine carbon-source utilization, a medium containing 0·2 g $NaNO_3$, 0·2 g NH_4Cl, 0·05 g yeast extract (Difco) and 0·4 % (w/v) carbon source in 1000 ml artificial sea water was used. The carbon sources tested were L-arabinose, D-glucose, D-lactose, D-mannose, D-sucrose, inositol, sorbitol, mannitol, fumarate, citrate and malonate. Spreading growth was observed with cultivation on medium B containing (l^{-1}) 1 g Bactopeptone (Difco), 1 g yeast extract (Difco), 15 g agar and half-strength natural sea water under high moisture conditions. Gliding motility was determined as described by Bowman (2000).

The physiological, morphological and biochemical characteristics of the strains studied are listed in the species descriptions and in Table 2. The presence of oxidase, catalase, β-galactosidase, agarase and alkaline phosphatase activities, the absence of urease activity, flexirubin-type pigment production, the reduction of nitrate to nitrite and the oxidation of carbohydrates, the absence of crystalline and amorphous cellulose hydrolysis and the respiratory quinone and fatty acid compositions of strains KMM 3526^T, KMM 3676^T and KMM 3677^T are consistent with the characteristics of *Z. galactanivorans* Dsij^T and *Z. uliginosa* CIP 104808^T (Table 1). However, the isolates differed clearly from *Z. galactanivorans* and *Z. uliginosa* by their inability to grow at 42 °C and to hydrolyse casein, their ability to oxidize L-rhamnose and the lower G+C content of their DNA (Table 2). Strain KMM 3526^T is distinguished from strain KMM 3677^T by the inability to grow with 8 % NaCl or at 37 °C or to hydrolyse Tween 40, by the absence of acid production from L-arabinose, D-cellobiose, DL-xylose and mannitol, by susceptibility to streptomycin and by resistance to tetracycline (Table 2). It is possible to differentiate strains KMM 3526^T and KMM 3676^T by the hydrolysis of starch, alginate, DNA, Tweens 20, 40 and 80, by the

Table 1. Whole-cell fatty acid composition of the *Zobellia* species studied

Those fatty acids for which the mean amount for all taxa was less than 1 % are not given. Tr, Trace amount (less than 1 %); ND, not detected.

Fatty acid	KMM 3526^T	KMM 3676^T	KMM 3677^T	*Z. galactanivorans* Dsij^T	*Z. uliginosa* CIP 104808^T
Straight-chain fatty acids:					
$C_{14:0}$	1·0	Tr	Tr	Tr	ND
$C_{15:0}$	14·4	12·5	11·0	7·5	10·2
$C_{15:0}$ 3-OH	Tr	ND	ND	ND	ND
$C_{15:1}\omega6c$	3·2	2·7	1·7	1·1	1·4
$C_{16:0}$	1·0	Tr	2·4	2·2	2·6
$C_{16:0}$ 3-OH	2·4	2·6	4·9	3·0	2·9
$C_{17:1}\omega6c$	1·2	1·0	ND	ND	ND
$C_{18:1}\omega6c$	0·7	1·1	ND	ND	ND
Branched fatty acids:					
iso-$C_{15:0}$	22·5	16·8	20·1	21·1	21·9
iso-$C_{15:0}$ 3-OH	4·6	6·1	5·9	8·3	6·7
anteiso-$C_{15:0}$	1·0	1·0	ND	1·8	1·4
iso-$C_{15:1}$	10·4	12·3	14·9	8·8	12·0
iso-$C_{17:0}$ 3-OH	15·1	22·4	19·7	23·7	25·9
iso-$C_{17:1}\omega9$	3·8	3·1	2·4	5·1	3·6
Summed feature 3*	15·5	14·9	14·3	14·5	9·9
ECL 13·565†	ND	1·4	1·8	2·4	1·4

*Summed feature 3 consisted of one or more of the following fatty acids, which could not be separated by the Microbial Identification System: $C_{16:1}\omega7c$, $C_{16:1}\omega7t$ and iso-$C_{15:0}$ 2-OH.

†ECL, Equivalent chain-length. The identity of the fatty acid is not known.

Table 2. Phenotypic characteristics of the *Zobellia* species

All strains were positive for the following: respiratory type of metabolism; movement by gliding; a requirement for NaCl for growth; flexirubin production; oxidase, catalase, β-galactosidase and alkaline phosphatase activities; growth at 4 °C (minimum temperature for growth of *Z. galactanivorans* Dsij^T is 10 °C; Barbeyron *et al.*, 2001) and with 6 % NaCl; hydrolysis of agar and gelatin; acid formation from L-fucose; nitrate reduction; utilization of L-arabinose, D-glucose, D-lactose, D-mannose, D-sucrose and mannitol; susceptibility to carbenicillin, lincomycin and oleandomycin. All strains were negative for the following: hydrolysis of D-cellulose (CM-cellulose and filter paper), chitin and urea; production of H_2S, indole and acetoin; oxidation of D-galactose, D-lactose, D-melibiose, L-sorbose, *N*-acetylglucosamine, citrate, adonitol, dulcitol, glycerol and inositol; utilization of inositol, sorbitol, malonate and citrate; susceptibility to gentamicin, kanamycin, neomycin and polymixin B.

Characteristic	*Z. amurskyensis* KMM 3526^T	*Z. laminariae* KMM 3676^T, KMM 3926	*Z. russellii* KMM 3677^T	*Z. galactanivorans* Dsij^T	*Z. uliginosa* CIP 104808^T
Growth at:					
8 % NaCl	−	−	+	+	−
10 % NaCl	−	−	+	−	−
32 °C	+	−	+	+	+
37 °C	−	−	+	+	+
42 °C	−	−	−	+	+
Hydrolysis of:					
Casein	−	−	−	+	+
Starch	+	−	+	+	+
Alginate	+	−	+	+	+
DNA	+	−	+	−	+
Tween 20	+	−	+	+	−
Tween 40	−	+	+	−	−
Tween 80	+	−	+	−	+
Acid from:					
L-Arabinose	−	+	+	+	−
D-Cellobiose	−	+	+	+	−
D-Glucose	+	+	+	−	−
D-Maltose	+	+	+	+	−
D-Raffinose	−	+	−	−	−
L-Rhamnose	+	+	+	−	−
D-Sucrose	+	+	+	−	+
L-Xylose	−	−	+	−	−
Mannitol	−	+	+	−	−
Susceptibility to:					
Ampicillin	−	−	−	−	+
Benzylpenicillin	−	−	−	−	+
Streptomycin	+	−	−	−	−
Tetracycline	−	−	+	−	−
DNA G+C content (mol%) determined by:					
Thermal denaturation	37·1	36·1*–36·7	38·6	43·4	42·9
HPLC	37·7	36·3	38·8	43·0	42·9

*Determined for the type strain.

oxidation of L-arabinose, D-cellobiose, D-raffinose and mannitol, and by susceptibility to streptomycin. KMM 3677^T is distinguished from KMM 3676^T by the ability to grow with 10 % NaCl and at 37 °C, the ability to oxidize DL-xylose and the ability to hydrolyse starch, alginate, DNA, Tween 20 and Tween 80.

Phenotypic findings in combination with the differences in the phylogenetic positions, based on 16S rRNA gene sequence analysis, and the DNA–DNA relatedness between the strains studied and the existing *Zobellia* species, support the inclusion of strains KMM 3526^T, KMM 3676^T and KMM 3677^T in the genus *Zobellia* as three distinct species, for

which the names *Zobellia amurskyensis* sp. nov., *Zobellia laminariae* sp. nov. and *Zobellia russelii* sp. nov are proposed.

Description of *Zobellia amurskyensis* sp. nov.

Zobellia amurskyensis (a.mur.sky.en′sis. N.L. fem. adj. *amurskyensis* of Amursky Bay, in which the type strain was isolated).

Cells range from 0·4 to 0·5 μm in width and from 1·2 to 1·4 μm in length. On marine agar, colonies are 2–4 mm in diameter, circular, shiny with entire edges, pigmented dark orange and sunken in the agar. Growth occurs at 4–32 °C, with the optimum at 23–25 °C, and at 1–6 % NaCl, with the optimum at 2 % NaCl. Decomposes agar, gelatin, starch, alginate, DNA, Tween 20 and Tween 80. Does not hydrolyse casein, cellulose (CM-cellulose and filter paper), chitin or Tween 40. Forms acid from D-glucose, L-fucose, D-maltose, L-rhamnose and D-sucrose, but not from L-arabinose, D-cellobiose, D-galactose, D-lactose, D-melibiose, L-sorbose, L-raffinose, DL-xylose, *N*-acetylglucosamine, citrate, adonitol, dulcitol, glycerol, inositol or mannitol. Utilizes L-arabinose, D-lactose, D-mannose and mannitol, but not inositol, sorbitol, malonate or citrate. Nitrate is reduced. H_2S, indole and acetoin (Voges–Proskauer reaction) are not produced. Susceptible to carbenicillin, lincomycin, oleandomycin and streptomycin, but resistant to ampicillin, benzylpenicillin, gentamicin, kanamycin, neomycin, polymyxin B and tetracycline. The predominant fatty acids are 15 : 0 (14·4 %), i15 : 0 (22·5 %), i15 : 0 3-OH (4·6 %), i15 : 1 (10·4 %) and i17 : 0 3-OH (15·1 %). The major lipoquinone is MK-6. The G + C content of the DNA is 37·1 mol%.

The type strain is KMM 3526^T (= LMG 22069^T = CCUG 47080^T). Isolated from sea water.

Description of *Zobellia laminariae* sp. nov.

Zobellia laminariae (la.mi.na′ri.ae. N.L. gen. n. *laminariae* of *Laminaria*, the generic name of the brown alga *Laminaria japonica*, from which the bacteria were isolated).

Cells range from 0·4 to 0·5 μm in width and from 1·2 to 1·4 μm in length. On marine agar, colonies are 2–4 mm in diameter, circular, shiny with entire edges, pigmented dark red and sunken in the agar. Growth occurs at 4–30 °C, with the optimum at 21–23 °C, and at salt concentrations from 1·5 to 6 % NaCl, with an optimum at 2 %. Decomposes agar, gelatin and Tween 40. Does not hydrolyse casein, starch, alginate, DNA, Tween 20, Tween 80, cellulose (CM-cellulose and filter paper) or chitin. Forms acid from L-arabinose, D-cellobiose, D-glucose, L-fucose, D-maltose, D-raffinose, L-rhamnose, D-sucrose and mannitol, but not from D-galactose, D-lactose, D-melibiose, L-sorbose, DL-xylose, *N*-acetylglucosamine, citrate, adonitol, dulcitol, glycerol or inositol. Utilizes D-lactose and D-mannose, but not inositol, sorbitol, malonate or citrate. Nitrate is reduced. H_2S, indole and acetoin (Voges–Proskauer reaction) are not produced. Susceptible to carbenicillin, lincomycin and oleandomycin, but resistant to ampicillin, benzylpenicillin, gentamicin, kanamycin, neomycin, polymyxin B, streptomycin and tetracycline. The predominant fatty acids are 15 : 0 (12·5 %), i15 : 0 (16·8 %), i15 : 0 3-OH (6·1 %), i15 : 1 (12·3 %) and i17 : 0 3-OH (22·4 %). The major lipoquinone is MK-6. The G + C content of the DNA is 36–37 mol%.

The type strain is KMM 3676^T (= LMG 22070^T = CCUG 47083^T). Isolated from the brown alga *Laminaria japonica*.

Description of *Zobellia russellii* sp. nov.

Zobellia russellii (rus′sel.li.i. N.L. gen. n. *russellii* of H. L. Russell, the American scientist, for his contribution to the development of marine microbiology).

Cells range from 0·4–0·5 μm in width and from 1·2 to 1·4 μm in length. On marine agar, colonies are 2–4 mm in diameter, circular, shiny with entire edges, pigmented dark orange and sunken in the agar. Growth occurs at 4–38 °C, with the optimum at 25–28 °C, and at salt concentrations between 1 and 10 % NaCl, with the optimum at 2–3 %. Decomposes agar, gelatin, starch, alginate, DNA, Tween 20, Tween 40 and Tween 80. Does not hydrolyse casein, cellulose (CM-cellulose and filter paper) or chitin. Forms acid from L-arabinose, D-cellobiose, D-glucose, L-fucose, D-maltose, L-rhamnose, D-sucrose, DL-xylose and mannitol, but not from D-galactose, D-lactose, D-melibiose, L-sorbose, D-raffinose, *N*-acetylglucosamine, citrate, adonitol, dulcitol, glycerol or inositol. Utilizes D-lactose and D-mannose, but not inositol, sorbitol, malonate or citrate. Nitrate is reduced. H_2S, indole and acetoin (Voges–Proskauer reaction) are not produced. Susceptible to carbenicillin, lincomycin, oleandomycin and tetracycline, but resistant to ampicillin, benzylpenicillin, gentamicin, kanamycin, neomycin, polymyxin B and streptomycin. The predominant fatty acids are 15 : 0 (11·0 %), i15 : 0 (20·1 %), i15 : 0 3-OH (5·9 %), i15 : 1 (14·9 %) and i17 : 0 3-OH (19·7 %). The major lipoquinone is MK-6. The G + C content of the DNA is 38·6 mol%.

The type strain is KMM 3677^T (= LMG 22071^T = CCUG 47084^T). Isolated from the green alga *Acrosiphonia sonderi*.

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