Formosa algae gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*

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Four light-yellow-pigmented, Gram-negative, short-rod-shaped, non-motile isolates were obtained from enrichment culture during degradation of the thallus of the brown alga *Fucus evanescens*. The isolates studied were chemo-organotrophic, alkalitolerant and mesophilic. Polar lipids were analysed and phosphatidylethanolamine was the only phospholipid identified. The predominant cellular fatty acids were 15 : 0, i15 : 0, ai15 : 0, i15 : 1 and 15 : 1(n-6). The DNA G+C contents of the four strains were 34·0–34·4 mol%. The level of DNA relatedness of the four isolates was conspecific (88–98 %), indicating that they belong to the same species. The 16S rDNA sequence of strain KMM 3553^T was determined. Phylogenetic analysis revealed that KMM 3553^T formed a distinct phyletic line in the phylum *Bacteroidetes*, class *Flavobacteria* in the family *Flavobacteriaceae* and that, phylogenetically, this strain could be placed almost equidistant from the genera *Gelidibacter* and *Psychroserpens* (16S rRNA gene sequence similarities of 94 %). On the basis of significant differences in phenotypic and chemotaxonomic characteristics, it is suggested that the isolates represent a novel species in a new genus; the name *Formosa algae* gen. nov., sp. nov. is proposed. The type strain is KMM 3553^T (=CIP 107684^T).

INTRODUCTION

According to the phylogenetic arrangement of taxa presented in the second edition of *Bergey's Manual of Systematic Bacteriology*, the family *Flavobacteriaceae* Reichenbach 1992 emend. Bernardet *et al.* 1996, 2002 is placed in the class *Flavobacteria*, one of three classes of the phylum *Bacteroidetes* (Bernardet *et al.*, 1996, 2002; Garrity & Holt, 2001). Currently, the family *Flavobacteriaceae* comprises more than 20 genera (*Flavobacterium*, *Aequorivita*, *Arenibacter*, *Bergeyella*, *Capnocytophaga*, *Cellulophaga*, *Chryseobacterium*, *Coenonia*, *Croceibacter*, *Empedobacter*, *Gelidibacter*, *Muricauda*, *Myroides*, *Ornithobacterium*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Riemerella*, *Salegentibacter*, *Tenacibaculum*, *Vitellibacter*, *Weeksella* and *Zobellia*) of ubiquitous bacteria from an array of environments that often overlap phenotypically with members of other phyla (Gherna & Woese, 1992; Holmes, 1993; Segers *et al.*, 1993; Bernardet *et al.*, 1996; Nakagawa *et al.*, 1997). It is believed that members of the *Flavobacteria* play an important role in the degradation of complex polysaccharides and other biomacromolecules (Bernardet *et al.*, 2002).

Polyphasic characterization of four bacterial isolates representing one of the numerically dominant groups of the brown alga-degrading enrichment community was carried out. On the basis of the results obtained and presented in this paper, it is proposed that these organisms represent a novel species in a new genus, *Formosa algae* gen. nov., sp. nov., in the family *Flavobacteriaceae*.

Abbreviations: AFM, atomic force microscopy; PE, phosphatidylethanolamine.

The GenBank accession number for the 16S rRNA gene sequence of *Formosa algae* KMM 3553^T is AY228461.

METHODS

Bacterial cultures, sampling and isolation procedure. Brown alga (*Fucus evanescens*) samples were collected by scuba divers in

mid-summer (July 1999) in Kraternaya Bay, Kuril Islands, Pacific Ocean, during the 23rd scientific expedition of the R/V *Akademician Oparin*. The set-up of the enrichment experiments and bacterial isolation were carried out as described elsewhere (Ivanova *et al.*, 2002a, b) with the only modification being addition of protein inhibitor for endo-(1→3)-β-D-glucanases (Yermakova *et al.*, 2002) to the enrichment culture (E. Ivanova, unpublished results). Cultures were maintained on marine agar 2216 (Difco) plates and medium B [0·2 % (w/v) Bacto peptone (Difco), 0·2 % (w/v) casein hydrolysate (Merck), 0·2 % (w/v) Bacto yeast extract (Difco), 0·1 % (w/v) glucose, 0·02 % (w/v) KH_2PO_4, 0·005 % (w/v) $MgSO_4.7H_2O$, 1·5 % (w/v) Bacto agar (Difco), 50 % (v/v) natural sea water and 50 % (v/v) distilled water at pH 7·5–7·8] and preserved in marine broth supplemented with 30 % glycerol at −80 °C.

Phenotypic characterization. Unless otherwise indicated, the phenotypic properties used for characterization of *Flavobacterium*-related species were tested following established procedures (McMeekin *et al.*, 1971; Smibert & Krieg, 1994; Ivanova *et al.*, 1996; Bernardet *et al.*, 2002). To test for spreading growth and gliding motility, strains were grown on medium B with a reduced peptone content (0·2 g l^{-1}). Gliding motility was verified using phase-contrast microscopy (Nikon) of hanging drop preparations. The bathochromic shift test with 20 % (w/v) KOH was performed to detect flexirubin pigmentation (Fautz & Reichenbach, 1980).

Starch, casein and gelatin hydrolysis was tested by the methods of Smibert & Krieg (1994). Degradation of macromolecules was tested using medium B. Chitin (1 %, w/v), elastin (0·1 %, w/v) and alginate (sodium salt; 0·1 %, w/v) hydrolysis was determined by development of clear zones around colonies. Cellulose hydrolysis was tested both by using cellulose overlay plates (1 % carboxymethylcellulose) and by examining strips of filter paper in liquid cell cultures for dissolution (Smibert & Krieg, 1994). Oxidation of 95 carbon sources was tested using Biolog GN microplates (Rüger & Krambeck, 1994; Ivanova *et al.*, 1998).

Growth at different temperatures (4–45 °C), NaCl concentrations (0–15 %) and pH (4·5–12·0, adjusted using HCl and NaOH) was measured by OD_{660} after 24 h incubation in marine broth. Cultures were incubated on a rotary shaker at 160 r.p.m. for 24 h at 28 °C (4–45 °C for temperature experiments).

Susceptibility to antibiotics was tested by the disc-diffusion plate method using medium B agar and discs impregnated with following antibiotics: kanamycin (30 μg), ampicillin (10 μg), benzylpenicillin (10 μg), streptomycin (30 μg), gentamicin (10 μg), lincomycin (15 μg), neomycin (30 μg), polymyxin B (25 μg) and tetracycline (30 μg).

Atomic force microscopic (AFM) imaging. AFM characterization was carried out on a TopoMetrix Explorer (ThermoMicroscopes) in non-contact mode using either a 2 μm liquid scanner (0·8 μm *z* range) or a 100 μm liquid scanner (10 μm *z* range). Silicon cantilevers with a spring constant of 42 N m^{-1} and resonant frequency of 320 kHz were used and all imaging was performed in ethanol. All samples were prepared on freshly cleaved mica.

Pigment characterization. The strains were grown on medium B for 2 days at 28 °C. Pigments were extracted using methanol. Absorption spectra were determined between 250 and 700 nm using a Specord M40 spectrophotometer.

Polar lipids. For lipid analyses followed by fatty acid methyl ester generation, cells were grown as described earlier (Svetashev *et al.*, 1995). Lipids were extracted according to Bligh & Dyer (1953). Two-dimensional micro-TLC of polar lipids was carried out using the method of Svetashev & Vaskovsky (1972), with chloroform/methanol/benzene/28 % NH_4OH (65 : 30 : 10 : 6, by vol.) for the first dimension and chloroform/methanol/benzene/acetone/acetic acid/water (70 : 30 : 10 : 5 : 4 : 1, by vol.) for the second dimension (Vaskovsky & Terekhova, 1979). Non-specific detection of lipids on the TLC was performed with a 10 % solution of H_2SO_4 in methanol at 180 °C. Specific reagents used for phospholipids were as described by Vaskovsky *et al.* (1975); 2 % (w/v) ninhydrin in acetone was used for amino-containing lipids. The phosphorus content in phospholipid spots was determined using micromethods described by Vaskovsky *et al.* (1975).

Fatty acid methyl ester analysis. Analyses of fatty acid methyl esters were carried out on a Shimadzu GC-14A GC with an FID using both a non-polar SPB-5 fused-silica column (30 m × 0·25 mm i.d.) at 210 °C and a polar Supelcowax-10 fused-silica column (30 m × 0·25 mm i.d.) at 200 °C. The FID was operated at 240 °C. Helium was used as the carrier gas (Carreau & Dubacq, 1978; Christie, 1988). Catalytic hydrogenation of fatty acid methyl esters was carried out as described by Appelquist (1972).

Genetic analysis. DNA was isolated following the method of Marmur (1961) and the DNA 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).

Phylogenetic analysis. The 16S rRNA gene was amplified and sequenced by MIDI Labs (Newark, DE, USA). Briefly, primers used for amplification corresponded to *Escherichia coli* positions 5 and 1540. Amplification products were purified using Microcon 100 molecular mass cut-off membranes (Millipore) and checked for quality and quantity on an agarose gel. Cycle sequencing of the 16S rRNA amplification products was carried out using AmpliTaq ES DNA polymerase and Rhodamine dye terminators. Samples were electrophoresed on an ABI Prism 377 DNA sequencer.

Related sequences were selected according to previous phylogenetic analyses of a database of 62 000 previously aligned bacterial 16S rRNA gene sequences and BLAST searches against the latest release of the EBI (European Bioinformatic Institute). In a preliminary analysis, 150 sequences were selected according to the result of a BLAST query. An initial tree was built that allowed 27 closely related sequences to be selected from reference strains when available. When several sequences were available for a type species, the sequence with the fewest ambiguities was selected. Phylogenetic trees were constructed using three different methods (bioNJ, maximum-likelihood and maximum-parsimony). For the bioNJ analysis, distance matrices were calculated using Kimura's two-parameter correction. bioNJ analysis was performed according to Gascuel (1997). Maximum-likelihood and maximum-parsimony were from PHYLIP (Felsenstein, 1985, 1993). Phylogenetic trees were drawn using NJPLOT (Perrière & Gouy, 1996). Because of the genetic distance between the 27 strains, homoplasy was detected between aligned sequences. These domains were not used for phylogenetic analysis, and the tree shown was obtained using positions 91–208, 212–827 and 835–1444 of the KMM 3553^T sequence. The topology shown is that of the bootstrap tree, as it has been demonstrated that this topology is often better than that of a simple neighbour-joining or maximum-parsimony analysis (Berry & Gascuel, 1996). There is no scale bar in Fig. 2 as this would be meaningless because the distances are corrected (see above) and this is a bootstrap tree.

RESULTS AND DISCUSSION

Phenotypic characteristics of the isolates

The novel organisms, isolated from a microbial community formed during degradation of the thallus of a brown alga

(*Fucus evanescens*), were subjected to detailed characterization. The isolates were Gram-negative and aerobic, although anaerobic growth was observed due to fermentation of D-glucose by anaerobic respiration of nitrate. Cells were gliding rods, slightly pointed, 0·4–0·9 μm in diameter and 0·8–1·8 μm long, according to AFM investigations (Fig. 1a–c). AFM imaging enables high-resolution imaging of the native bacterial cell surface in three dimensions, without staining or shadowing, by mechanically scanning a tip mounted on a flexible cantilever over the sample surface, and allows accurate investigation of cellular morphology. Gliding was probably facilitated by production of slime, as shown by AFM (Fig. 1b, c). All strains showed remarkable resistance to the 10 antibiotics tested. Other phenotypic characteristics are given in Tables 1 and 3 and the species description.

Polar lipid and fatty acid composition

Phosphatidylethanolamine (PE) was the only phospholipid identified in this group of isolates. Amino-containing lipids that were detected on TLC plates were ninhydrin-positive. The predominant cellular fatty acids were mainly branched-chain saturated and unsaturated, namely i15 : 0, ai15 : 0, 15 : 0, i15 : 1 and 15 : 1ω6 fatty acids (Table 2). In spite of the intraspecific heterogeneity of the fatty acid content of micro-organisms belonging to the family, high levels of i15 : 0 and i17 : 0 3-OH are often observed (Holmes *et al.*, 1984; Holmes, 1993; Bernardet *et al.*, 1996, 2002). The novel isolates contained a high proportion of branched-chain saturated and unsaturated cellular fatty acids, which is a characteristic feature of the family.

DNA base composition and DNA relatedness

The DNA G+C content was in the range 34·0–34·4 mol% (thermal denaturation method). Levels of DNA relatedness between the isolates were 89–98 %. Since the level of DNA similarity of the four strains was greater than 70 %, all of them were assigned to a single species (Wayne *et al.*, 1987).

Phylogeny

To establish the precise phylogenetic affiliation of strain KMM 3553^T, the almost entire 16S rRNA gene sequence was determined. Phylogenetic analysis was carried out including all known taxa from the phylum *Bacteroidetes*. According to 16S rRNA gene sequence analysis, a consensus of all three methods (bioNJ, maximum-parsimony and maximum-likelihood) showed that KMM 3553^T does not form a robust clade with any recognized species and/or genus (Fig. 2). The level of 16S rDNA sequence similarity to members of the two most closely related genera phylogenetically, *Psychroserpens* and *Gelidibacter*, was almost equal, i.e. 94·2 % or less, suggesting that the strains isolated in this study represent a novel genus. The most closely related species, *Psychroserpens burtonensis*, had 94·2 % sequence similarity and 82 sequence differences, whereas the most closely related sequence, from an uncultured species from the toxic dinoflagellate *Alexandrium tamarense* (Groben *et al.*, 2000), was 97·2 % similar and had 27 differences. Because the 16S rRNA gene sequence of KMM 3553^T does not form a clade with any recognized species and differs greatly from sequences of closely related species, phylogenetic analysis suggests that strain KMM 3553^T should be classified in a new genus.

Taken together, all of these features allow us to conclude that the isolates bear sufficient resemblance to species and genera included in the emended family *Flavobacteriaceae* (Bernardet *et al.*, 2002) to allow their inclusion in the family. The novel isolates can be easily differentiated from previously described genera (Table 3). One of two *Flavobacterium* taxa described recently for marine bacteria related to the [*Flexibacter*] *maritimus* rRNA branch, *Psychroserpens burtonensis* (Bowman *et al.*, 1997), has characteristic cell morphology, non-saccharolytic metabolism, requires Na^+ ions for growth and has a low DNA G+C content. Strains of the second taxon, *Gelidibacter algens*, have different cell morphology, require Na^+ ions for growth and have significant levels of characteristic ai15 : 1ω10*c* and ai17 : 1ω7*c* fatty acids. Consequently, it is proposed that the four strains isolated from a brown alga be classified as representatives of a new genus, as *Formosa algae* gen. nov., sp. nov.

Description of *Formosa* gen. nov.

Formosa (For.mo′sa. L. fem. n. *Formosa* beautiful, finely formed).

Rod-shaped cells with slightly irregular sides and pointed ends, approx. 0·8–1·8 × 0·4–0·9 μm. Gram-negative. Does not form endospores or resting stages. Does not accumulate poly-β-hydroxybutyrate as an intracellular reserve product and does not have an arginine dihydrolase system. Aerobic. Anaerobic growth occurs by fermentation of D-glucose by anaerobic respiration of nitrate. Chemo-organotroph. Cytochrome oxidase-negative, catalase-positive. Phylogenetically, *Formosa* is a member of the family *Flavobacteriaceae*, class *Flavobacteria*, phylum *Bacteroidetes*. The type species is *Formosa algae*.

Description of *Formosa algae* sp. nov.

Formosa algae (al′gae. L. fem. gen. n. *algae* of an alga, pertaining to the source of isolation, brown algae).

Description is as for the genus plus the following. Exhibits gliding motility. Colonies are circular, 1–3 mm in diameter, low-convex on solid media containing high nutrient concentrations. Colonies are light yellow in colour. Produces carotenoid pigments with absorbance peaks at 455 and 480 nm. Flexirubin pigments are absent. No growth detected at 4 or 37 °C; optimum growth at 23 °C. Grows at pH 5·0–10·0; optimum growth at pH 8·0–8·5. Alkalitolerant. Grows in 0–6 % NaCl. Does not decompose agar or chitin. Starch and gelatin are hydrolysed weakly. Utilizes Tween 40, D-galactose, gentiobiose, α-D-glucose, monosuccinate, citric acid, D-glucuronic acid, succinamic acid,

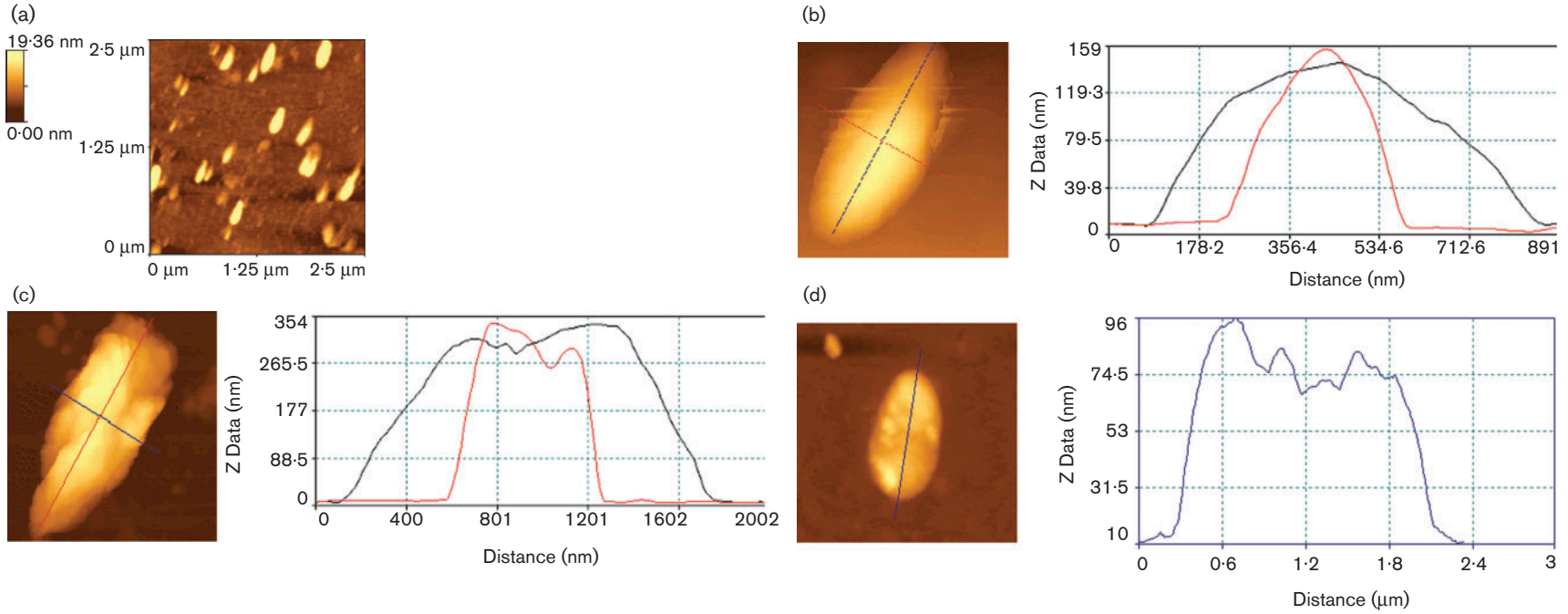

Fig. 1. AFM of cells of *Formosa algae* KMM 3553^T. (a) Deflection image of cells deposited on mica. (b) Deflection image and line profile of a cell freshly deposited on mica (30 min); the two curves correspond to the longitudinal and transverse profiles of the cell. Note the occurrence of noise along the edges of the bacterium. Closer investigation revealed a fine layer of conditioning film (extracellular polymeric substances), secreted by the bacterium to promote gliding and/or attachment. (c) Deflection image and line profile of a cell deposited on mica and visualized after 2 days. (d) Deflection image and line profile of a deflated cell deposited on mica and visualized after 2 days.

Table 1. Major phenotypic characteristics of *Formosa algae* gen. nov., sp. nov.

Values for other strains are the number positive/number tested. W, Weakly positive.

Characteristic	KMM 3553^T	Other strains
Flagellation	−	0/3
Gliding motility	+	3/3
Oxidase, arginine dihydrolase, phenylalanine deaminase	−	0/3
Catalase	+	3/3
Na^+ required for growth	−	0/3
Organic growth factors required for growth	−	0/3
Denitrification	+	3/3
Indole production	−	0/3
Urease	+	3/3
Haemolytic activity	−	0/3
Hydrolysis of gelatin and starch	W	2/3
Degradation of agar, cellulose and chitin	−	0/3
Growth in/at:		
5–35 °C	+	3/3
0–6 % NaCl	+	3/3
Utilization of:		
Melibiose, citrate, trehalose, L-arginine, L-tyrosine	−	0/3
Lactose, glycerol	+	3/3

Table 2. Cellular fatty acid composition of *Formosa algae* gen. nov., sp. nov.

Values are percentages of total fatty acids for strain KMM 3553^T; ranges for the other three strains are given in parentheses.

Fatty acid	Content
Saturated acids	
12 : 0	0·5 (0·4–0·6)
14 : 0	1·03 (0·4–0·5)
15 : 0	27·2 (17·0–18·0)
16 : 0	1·0 (0·9–1·1)
17 : 0	0·6 (0·8–0·9)
18 : 0	0·4 (0·0)
Branched-chain acids	
i14 : 0	0·3 (0·4–0·5)
i15 : 0	13·3 (20·0–22·0)
ai15 : 0	7·2 (8·1–9·2)
i16 : 0	2·3 (1·4–1·5)
Unsaturated acids	
15 : 1ω8	1·0 (0·4–0·5)
15 : 1ω6	13·0 (9·2–10·1)
16 : 1ω7	3·3 (5·7–6·7)
17 : 1ω8	2·2 (0·8–1·2)
18 : 1ω9	0·5 (0·5–0·8)
Branched-chain unsaturated acids	
i15 : 1	15·3 (19·5–20·9)
a16 : 1	1·0 (1·2–1·7)
i17 : 1	1·4 (2·1–2·6)
a17 : 1	0·4 (0·9–1·0)
Hydroxy fatty acids	
14 : 0 3-OH	1·1 (2·0–3·1)
Cyclo fatty acids	
17 : 0 cy	7·4 (2·7–3·9)
19 : 0 cy	0·4 (0·4–0·9)

succinic acid, alaninamide, glycyl L-aspartic acid, hydroxy-L-proline, L-ornithine, L-pyroglutamic acid, urocanic acid, thymidine, 2-aminoethanol and glycerol. Does not utilize α-cyclodextrin, dextrin, glycogen, Tween 80, *N*-acetyl D-glucosamine, adonitol, D-cellobiose, L-arabinose, D-arabitol, i-erythritol, D-fructose, L-fucose, *m*-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, methyl β-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, acetic acid, *cis*-aconitic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketoglutaric acid, α-ketobutyric acid, α-ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, glucuronamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine,

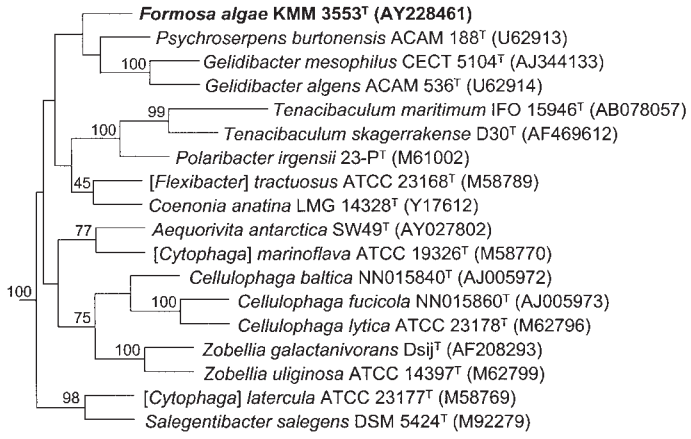

Fig. 2. Phylogenetic position of *Formosa algae* KMM 3553^T according to 16S rRNA gene sequence analysis.

Table 3. Differential characteristics of *Formosa algae* gen. nov., sp. nov. and other phylogenetically related halophilic genera in the family *Flavobacteriaceae*

Genera/species: 1, *Formosa*; 2, *Aequorivita*; 3, *Cellulophaga*; 4, [*Cytophaga*] *latercula*; 5, [*Cytophaga*] *marinoflava*; 6, [*Flexibacter*] *tractuosus*; 7, *Psychroflexus*; 8, *Salegentibacter*; 9, *Gelidibacter*; 10, *Polaribacter*; 11, *Psychroserpens*; 12, *Tenacibaculum*; 13, *Zobellia*. Data from Bernardet *et al.* (1996), Bowman *et al.* (1997, 1998), Bowman & Nichols (2002), Barbeyron *et al.* (2001), Vandamme *et al.* (1999), McGuire *et al.* (1987), Suzuki *et al.* (2001), Macián *et al.* (2002) and this study. −, Negative; +, positive; V, variable; ND, not determined; ±, previously reported results differ.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Habitat*	M	M	M	M	M	F	H	H	M	M	M	M	M
Flexirubin	−	−	−	−	−	ND	−	−	−	−	−	−	+
Gliding motility	+	−	+	−	+	+	V	−	+	−	−	−	+
Sea water requirement	−	±	V	+	−	−	−	−	+	+	+	+	+
Amino acids requirement	−	+	−	−	−	+	±	−	+	−	+	+	−
Carbohydrate utilization	+	±	+	+	+	+	+	+	−	+	−	+	+
Starch hydrolysis	V	V	+	−	+	V	+	+	+	+	−	−	+
DNA G+C content (mol%)	34	33–39	32–42	34	37	37	32–39	37–38	36–38	31–33	27–29	29–33	32

*M, Marine; F, freshwater/marine shore; H, hypersaline lake.

L-proline, D-serine, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, inosine, uridine, phenylethylamine, 2,3-butanediol, DL-α-glycerol phosphate, α-D-glucose 1-phosphate or D-glucose 6-phosphate according to the Biolog system. Not susceptible to ampicillin, lincomycin, benzylpenicillin, kanamycin, oleandomycin, tetracycline, neomycin, streptomycin, gentamicin or polymyxin B. PE is the only phospholipid identified. Sphingophospholipids are absent. The predominant cellular fatty acids are odd-numbered: 15 : 0, i15 : 0, ai15 : 0, i15 : 1 and 15 : 1(n-6). DNA G+C content is 34·0–34·4 mol%.

The type strain is KMM 3553^T (=CIP 107684^T); its DNA G+C content is 34·0 mol%.

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