**Formosa agariphila** sp. nov., a budding bacterium of the family *Flavobacteriaceae* isolated from marine environments, and emended description of the genus *Formosa*

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Two marine, heterotrophic, aerobic, yellow-pigmented, agarolytic bacterial strains that are motile by means of gliding were isolated from the green alga *Acrosiphonia sonderi* and from sea water. Comparative 16S rRNA gene sequence analysis revealed an affiliation between the strains studied and the genus *Formosa*, a member of the family *Flavobacteriaceae*. The level of sequence similarity between strain KMM 3901T and *Formosa algae* KMM 3553T was 99.1%. The results of DNA–DNA hybridization experiments and phenotypic analysis indicated that the strains represent a novel species of the genus *Formosa*, for which the name *Formosa agariphila* sp. nov. is proposed, with KMM 3901T (=KCTC 12365T =LMG 23005T =DSM 15362T) as the type strain. The description of the genus *Formosa* is emended with newly obtained data.

The genus *Formosa* was proposed by Ivanova *et al.* (2004) for heterotrophic, Gram-negative, motile (by means of gliding), aerobic, halotolerant, saccharolytic, rod-shaped, yellow-pigmented strains isolated from an enrichment community degrading the brown alga *Fucus evanescens*. According to phylogenetic analysis, the genus was distantly related to the genera *Psychroserpens* and *Gelidibacter* within the family *Flavobacteriaceae*. Here, we report the establishment of the taxonomic position of two novel bacteria, and emend the description of the genus *Formosa*, as a result of the new data obtained in this work.

Strain KMM 3901T was isolated in Troitsa Bay, Gulf of Peter the Great, East Sea (also known as the Sea of Japan) from the green alga *Acrosiphonia sonderi*. Strain KMM 3962 (=LMG 23006) was isolated from sea water in Amursky Bay (Gulf of Peter the Great). All samples were collected in June 2000. For isolation, 0.1 ml algal homogenates or 0.1 ml sea water was transferred on marine agar 2216 (Difco) at 28°C; the isolates were then purified and cultivated for further experiments under the same conditions and stored at −80°C in marine broth (Difco) supplemented with 20% (v/v) glycerol.
The phylogenetic position of strains KMM 3901T and KMM 3962 was determined by means of complete 16S rRNA gene sequence analysis. For strain KMM 3901T, genomic DNA extraction, PCR and 16S rRNA gene sequencing were performed according to the procedures of Kim et al. (1998). Genomic DNA of strain KMM 3962 was prepared according to the protocol of Niemann et al. (1997). The 16S rRNA gene was amplified using oligonucleotide primers complementary to highly conserved regions of the 16S rRNA genes. The forward primer was 5'-AGAGTTTGATCCTGGCTCAG-3' (hybridizing at positions 8–27, according to the Escherichia coli numbering system) and the reverse primer was 5'-AAGGAGGTGATCCAGCCGCA-3' (hybridizing at positions 1541–1522). PCR products were purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions. Purified PCR products were sequenced by using the ABI Prism BigDye terminator cycle sequencing ready reaction kit and an Applied Biosystems 3100 DNA sequencer, using the protocols of the manufacturer (Applied Biosystems). The eight sequencing primers used are listed by Coenye et al. (1999). Sequence assembly was performed using the program AutoAssembler (Applied Biosystems). The 16S rRNA gene sequence data obtained were aligned together with those of representative members of selected genera belonging to the family Flavobacteriaceae by using PHYDIT, version 3.2 (http://plaza.snu.ac.kr/~jchun/phyditi). Phylogenetic trees were inferred using suitable programs of the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated from the 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 the almost-complete 16S rRNA gene sequences of strains KMM 3901T (1432 bp) and KMM 3962 (1472 bp) revealed that the marine isolates are members of the family Flavobacteriaceae and form a distinct lineage within the genus Formosa (Fig. 1). The topologies of phylogenetic trees constructed using neighbour-joining, maximum-likelihood and maximum-parsimony algorithms were similar, and the stability of the trees was supported by high bootstrap values. The level of sequence similarity between the strains studied and Formosa algae KMM 3553T was 98.9–99.1 % Strains KMM 3901T and KMM 3962 shared 99.6 % sequence similarity.

For determination of the DNA G+C contents of strains KMM 3901T and KMM 3962, DNA was extracted using the protocol described by Marmur (1961) and was analysed by the thermal denaturation method (Marmur & Doty, 1962). The DNA G+C contents of the strains studied and Formosa algae KMM 3553T were also determined by HPLC (Mesbah et al., 1989), with DNA preparation according to the protocol used for DNA–DNA hybridizations (see below). For the HPLC experiments, the nucleoside mixture was separated by using a Waters SymmetryShield C8 column maintained at a temperature of 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated λ-phage DNA (Sigma) was used as the calibration reference. The DNA G+C contents of strains KMM 3901T and KMM 3962 were 35·9 (Tₘ) and 33·6 mol% (HPLC) and 35·7 (Tₘ) and 34·1 mol% (HPLC), respectively. The value for Formosa algae KMM 3553T was 34·3 mol% (HPLC), which is similar to the value [34·0 mol% (Tₘ)] from the literature (Ivanova et al., 2004).

For DNA–DNA hybridization experiments among strains KMM 3901T, KMM 3962 and Formosa algae KMM 3553T, cells were cultivated on marine agar for 24 h at 30 °C. DNA was extracted from 0.75–1.25 g cells (wt weight) by using the DNA extraction protocol of Wilson (1987), as modified by Cleenwerck et al. (2002). Cells were lysed in a Tris/EDTA buffer (10 mM Tris/HCl with up to 200 mM EDTA, pH 8·0) containing RNase A (Sigma), SDS (Serva) and proteinase K (Merck) to final concentrations of 400 µg ml⁻¹, 2 % (w/v) and 200 µg ml⁻¹, respectively. NaCl (5 M stock solution) and CTAB/NaCl solution (10 % w/v CTAB in 0·7 M NaCl) were added to final concentrations of 1 M and 13·3 % (v/v), respectively. For hybridization experiments, the microplate method was carried out as described by Ezaki et al. (1989) and Goris et al. (1998), using an HTS7000 BioAssay Reader (Perkin Elmer) for fluorescence measurements. Biotinylated
DNA was hybridized with unlabelled ssDNA, which was bound non-covalently to microplate wells. Hybridizations were performed at 37°C in a hybridization mixture [2× SSC, 5× Denhardt’s solution, 2.5% dextran sulfate, 50% formamide, denatured salmon sperm DNA (100 µg ml⁻¹) and biotinylated probe DNA (1250 ng ml⁻¹)]. Hybridization was carried out with three replications for each sample.

The value for DNA–DNA binding between strains KMM 3901T and KMM 3962 was 97%; this indicates that the strains represent a single species (Wayne et al., 1987). DNA–DNA hybridizations performed between KMM 3901T, KMM 3962 and Formosa alga KMM 3553T revealed binding levels of 27 and 29%, respectively. These data clearly demonstrate separate species status within the genus Formosa for the strains under study.

Isoprenoid quinones were extracted from lyophilized cells of strains KMM 3901T, KMM 3962 and Formosa alga KMM 3553T and analysed as described by Akagawa-Matsushita et al. (1992). The isoprenoid quinone composition was characterized by HPLC (Shimadzu instruments) using a reverse-phase type Zorbax ODS column (250 × 6.0 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 using monitoring at 270 nm and were identified by comparison with known quinones from reference strain Salegentibacter salgenses DSM 5424T. The major lipoquinone was MK-6. Polar lipids were determined as described previously (Nedashkovskaya et al., 2004b). The only phospholipid of KMM 3901T and KMM 3962 was phosphatidylethanolamine, in accordance with the results reported for Formosa alga by Ivanova et al. (2004).

For fatty acid methyl ester analysis, a loopful of well-grown cells of strains KMM 3901T, KMM 3962 and Formosa alga KMM 3553T were harvested. The fatty acid methyl esters were prepared as described previously (Vandamme et al., 1992) and were separated and identified using the Sherlock Microbial Identification System (version 3.0; MIDI). The predominant cellular fatty acids for all strains analysed were C₁₅:₀, iso-C₁₅:₁, C₁₅:₁ (v/t)c, iso-C₁₅:₀ (3-OH), iso-C₁₇:₀ 3-OH and summed feature 3 (comprising any combination of C₁₆:₁ (v/t)c, C₁₆:₁ (t)r/t and iso-C₁₅:₀ 2-OH) (Table 1).

For pigment characterization, strains KMM 3901T and Formosa alga KMM 3553T were grown on marine agar for 48 h at 28°C. The absorption spectrum of pigments extracted using acetone/methanol (7:2, v/v) was determined between 300 and 700 nm with a UV spectrophotometer (CE 7250, 7000 series; CECIL Instruments). Cells of strains KMM 3901T and KMM 3553T produced yellow carotenoid pigments with maximum absorption at 443.8 and 446.9 nm, respectively.

The cell morphology of strain KMM 3901T was observed by using scanning and transmission electron microscopy as described by Bruns et al. (2001). These studies demonstrated that cells of strain KMM 3901T show budding fission (Fig. 2). To study the cell morphology of KMM 3962 and Formosa alga KMM 3553T, samples were fixed in a 2% paraformaldehyde/glutaraldehyde mixture buffered with 0.1 M phosphate (pH 7.2) for 2 h, fixed in 1% osmium tetroxide in the same buffer for 1 h and then dehydrated in graded ethanol and substituted by isooamyl acetate. They were then dried at the critical point in CO₂. Finally, the samples were sputtered with gold in a sputter coater (SC502; Poloron) and observed using the scanning electron microscope (SEM 515; Philips).

The strains isolated in this study were Gram-negative, chemo-organotrophic, aerobic and motile by gliding. They comprised short rod-shaped cells, 0.5–0.6 µm in diameter and 0.8–1.0 µm long (Fig. 2a, b). Buds formed on mature cells of strain KMM 3901T (Fig. 2). This is the first report of a budding process in marine flavobacteria. On marine agar, colonies formed by strain KMM 3901T were round, flat, 3–4 mm in diameter, translucent, yellow in colour and sunken into the agar, while colonies of strain KMM 3962 were 2–3 mm in diameter, pale yellow in colour and only slightly sunken into the agar. In addition to producing buds, cells of KMM 3901T formed threads that connected them to colonies.

### Table 1. Fatty acid content (percentage of total) of whole-cell hydrolysates of the Formosa strains

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>F. agariphila KMM 3901T</th>
<th>F. agariphila KMM 3962</th>
<th>F. alga KMM 3553T</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₅:₀</td>
<td>8-7</td>
<td>11-4</td>
<td>15-5</td>
</tr>
<tr>
<td>C₁₅:₀ 2-OH</td>
<td>1-8</td>
<td>1-6</td>
<td>1-5</td>
</tr>
<tr>
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<td>4-0</td>
<td>2-3</td>
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<td>6-0</td>
<td>11-8</td>
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<td>17-2</td>
<td>17-1</td>
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<td>6-5</td>
<td>11-4</td>
<td>9-9</td>
</tr>
<tr>
<td>iso-C₁₅:₀ 3-OH</td>
<td>7-7</td>
<td>10-5</td>
<td>6-7</td>
</tr>
<tr>
<td>anteiso-C₁₅:₀ 3</td>
<td>3-4</td>
<td>1-6</td>
<td>4-7</td>
</tr>
<tr>
<td>anteiso-C₁₅:₁ A</td>
<td>tr</td>
<td>–</td>
<td>1-0</td>
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<tr>
<td>C₁₆:₀</td>
<td>1-6</td>
<td>–</td>
<td>1-1</td>
</tr>
<tr>
<td>C₁₆:₀ 3-OH</td>
<td>2-1</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>iso-C₁₆:₀ 1</td>
<td>2-1</td>
<td>–</td>
<td>1-1</td>
</tr>
<tr>
<td>iso-C₁₆:₁ 3</td>
<td>2-5</td>
<td>–</td>
<td>tr</td>
</tr>
<tr>
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<td>8-9</td>
<td>3-1</td>
<td>4-0</td>
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<tr>
<td>C₁₇:₀ 2-OH</td>
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<td>1-5</td>
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<td>3-5</td>
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<td>iso-C₁₇:₁ 3-OH</td>
<td>8-5</td>
<td>10-7</td>
<td>9-6</td>
</tr>
<tr>
<td>Summed feature 3</td>
<td>15-8</td>
<td>12-1</td>
<td>5-9</td>
</tr>
</tbody>
</table>

Values below 1% for all strains are not shown. Summed feature 3 consisted of one or more of the following fatty acids, which could not be separated by the Microbial Identification System: iso-C₁₅:₀ 2-OH, C₁₆:₁ (t)7r and C₁₆:₁ (t)7t. Trace amount (<1% of total); –, not detected.
each other. These structures were not observed for KMM 3962 or the type strain of Formosa algae. The formation of fibrillar networks connecting cells of flavobacteria was reported by Reichenbach (1989), and similar features were previously observed in strains of the genus Winogradskyella, another member of the family Flavobacteriaceae (Nedashkovskaya et al., 2005). The ability of cells of strain KMM 3901T to form fibrillar threads correlates well with results reported in the literature (Cooksey & Wigglesworth-Cooksey, 1995; Heissenberger et al., 1996; Romanenko et al., 2003). Cells of strain KMM 3901T also produced a dense translucent slime on solid media. The extracellular fibrillar structures observed in this study could be involved in the attachment of the cells to suitable substrates and attachment to other cells, which are probably responsible for the formation of biofilms on the surface of algae and for the small aggregates observed in liquid cultures, respectively.

Biochemical tests and carbohydrate-oxidation tests were carried out as described previously (Nedashkovskaya et al., 2003, 2004a), except that 50% natural sea water (v/v) was added to the base medium used for testing for acid production from carbohydrates. Additionally, the type strain of Formosa algae and the two isolates were tested using API 20E and API 20 NE galleries (bioMérieux). The ability to grow under anaerobic conditions was observed using the Oxoid Anaerobic System. Gliding motility was determined as described by Bowman (2000).

The phenotypic characteristics of the two strains studied are listed in Table 2 and summarized below in the species description. The strains shared a number of similar properties, but KMM 3901T and KMM 3962 differed in maximum growth temperature (33 and 31°C, respectively) and NaCl concentration (8 and 7%, respectively). Also, strain KMM 3901T did not oxidize N-acetylglucosamine or hydrolyse starch or Tween 40, in contrast to KMM 3962. While strain KMM 3901T was susceptible to ampicillin, carbenicillin, lincomycin and oleandomycin, KMM 3962 was resistant to all antibiotics tested. Both strains studied can hydrolyse agar, form acid from D-galactose and utilize L-arabinose and D-lactose, in contrast with Formosa algae.
Table 2. Phenotypic characteristics of the Formosa strains

All strains were positive for the following characteristics: gliding motility, oxidase, catalase and alkaline phosphatase activities, glucose fermentation, growth at 1–6% NaCl and at 4–30 °C, hydrolysis of gelatin and ascinulin, acid formation from L-fucose, D-glucose, D-maltose, DL-xylene and mannitol and utilization of D-mannose. All strains were negative for the following characteristics: D-glucose, D-maltose, DL-xylose and mannitol and utilization of glucose fermentation, growth at 1–6% NaCl and at 4–30 °C.

Table 3. Differential characteristics of the genus Formosa and other allied genera of the family Flavobacteriaceae

Genera: 1, Formosa; 2, Algibacter; 3, Gaetbulibacter; 4, Gelidibacter; 5, Lacinutrix; 6, Olleya; 7, Psychrospermus; 8, Subsaxibacter; 9, Subsaximicrobium. Data are from Bowman et al. (1997), Macián et al. (2002), Ivanova et al. (2004), Nedashkovskaya et al. (2004b), Bowman & Nichols (2005), Nichols et al. (2005), Jung et al. (2005) and this study. Abbreviations: −, negative; +, positive; v, variable; ND, not determined.

KMM 3553? Moreover, the latter strain differs from KMM 3901T and KMM 3962 in its ability to produce acid from glycerol and its susceptibility to tetracycline. The genomic data obtained here, in combination with discriminative phenotypic findings (Table 2), support the conclusion that strains KMM 3901T and KMM 3962 represent a separate species.

Although Formosa algae KMM 3553? had originally been described as oxidase-negative and urease- and nitrate reductase-positive, in the course of the comparative phenotypic study of Formosa algae KMM 3553? and the two novel isolates, we detected oxidase activities and the absence of urease and nitrate reductase activities for all strains, using the same methods (Ivanova et al., 2004). As all strains studied fermented glucose, their ability to grow under anaerobic conditions was examined, but no growth was observed. The whole-cell fatty acid profiles of strains KMM 3901T, KMM 3962 and Formosa algae KMM 3553? were determined under the same conditions. The menaquinone composition, which was not reported by Ivanova et al. (2004) for Formosa algae strains, was also determined in this study. Differential phenotypic features for the genus Formosa and its close relatives are presented in Table 3. For most flavobacteria, generic affiliation on the basis of phenotypic traits is difficult. For instance, although the genera Algibacter, Formosa and Gaetbulibacter occupy distinct phylogenetic positions within the family Flavobacteriaceae, they share many similar phenotypic features (Table 3). Finding differential characteristics is especially difficult when most of them are variable between species; see, for instance, the genus Gelidibacter in Table 3.
Consequently, a polyphasic taxonomic approach is necessary to distinguish different members of the family Flavobacteriaceae.

Taking together the phenotypic and phylogenetic data described here, we conclude that the marine bacteria KMM 3901T and KMM 3962 represent a novel species of the genus *Formosa*, for which the name *Formosa agariphila* sp. nov. is proposed. On the basis of the novel phenotypic and chemotaxonomic data obtained in this study, we also propose an emended description for the genus *Formosa*.

**Emended description of the genus *Formosa* Ivanova et al. 2004**

As the description given by Ivanova et al. (2004), with the following changes. Cells are motile by gliding. Buds may be produced. Do not form endospores or resting stages. Facultative anaerobes. Cytochrome oxidase-, catalase- and alkaline phosphatase-positive. May require Na+ ions for growth. Produces non-diffusible carotenoid pigments. The main cellular fatty acids are iso-C15:0, C15:0, iso-C15:1, G, C15:096c, iso-C15:03-OH, iso-C17:0 3-OH and summed feature 3 (comprising any combination of C16:1ω7t, C16:1ω7t and iso-C15:0 2-OH). The major isoprenoid quinone is MK-6. Phosphatidylethanolamine is the only phospholipid identified. The G+C content of the DNA is 34–36 mol%. The type species is *Formosa alga*.

**Emended description of *Formosa algae* Ivanova et al. 2004**

The description is as given by Ivanova et al. (2004) with the following additions: the strains decompose aesculin and Tween 40 and can hydrolyse urea, grow in 0–8 % NaCl and at 4–34 °C. Strains form acid from L-fucose, D-glucose, D-maltose, DL-xylate and mannotet, but not from L-arabinose, D-cellobiose, D-lactose, D-melibiose, L-rhamnose, l-sorbose, D-sucrose, acetate, citrate, fumarate, malate, adonitol, inositol or sorbitol. Utilizes D-arabinose, D-lactose, D-mannose and D-sucrose, but not inositol, sorbitol, malonate or citrate. Produces β-galactosidase. Nitrate is not reduced to nitrite. H2S, indole and acetoin (Voges-Proskauer reaction) production are negative. Some strains are susceptible to ampicillin, carbenicillin, lincomycin and oleandomycin. Resistant to benzylpenicillin, gentamicin, kanamycin, neomycin, polymyxin B, tetracycline and streptomycin. The predominant fatty acids are C15:0 (8–7–11·4 %), iso-C15:1 (6–5–11·4 %), C15:096c (6–11·8 %), iso-C15:0 (12–7–17·2 %), iso-C15:0 3-OH (7–7–10·5 %), iso-C17:0 3-OH (8–5–10·7 %) and summed feature 3 (15–8–12·1 %, comprising any combination of C16:1ω7t, C16:1ω7t and iso-C15:0 2-OH). The G+C content of the DNA is 35–36 mol% (Tm).

The type strain, KMM 3901 T (=KCTC 12365T =LMG 23005T =DSM 15362T), was isolated from the green alga *Acrosiphonia sonderi*, collected in Troitsa Bay, Gulf of Peter the Great, East Sea (Sea of Japan). Strain KMM 3962 (=LMG 23006) was isolated from sea water collected in Amursky Bay, Gulf of Peter the Great.

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