Formosa spongicola sp. nov., isolated from the marine sponge *Hymeniacidon flavia*

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A Gram-negative, yellow-pigmented, rod-shaped, strictly aerobic, non-flagellated, oxidase- and catalase-positive, marine bacterium, designated A2^T, was isolated from a marine sponge, *Hymeniacidon flavia*, collected from the coast of Jeju Island, South Korea. Phylogenetic analysis based on nearly complete 16S rRNA gene sequences revealed that strain A2^T was a member of the family *Flavobacteriaceae*. Its closest relatives were *Formosa agariphila* KMM 3901^T and *Formosa algae* KMM 3553^T (96.99 and 96.98% 16S rRNA gene sequence similarity, respectively). DNA–DNA relatedness between strain A2^T and *F. agariphila* KMM 3901^T and *F. algae* KMM 3553^T was 14.1 and 26.8%, respectively. The dominant fatty acids (>5%) of strain A2^T were iso-C_{15:0} (33.9%), iso-C_{17:0} 3-OH (20.8%), iso-C_{15:1} G (10.5%) and iso-C_{15:0} 3-OH (6.1%). The DNA G+C content of strain A2^T was 36.0 mol% and the major respiratory quinone was MK-6. On the basis of phenotypic and phylogenetic analysis, strain A2^T represents a novel species of the genus *Formosa*, for which the name *Formosa spongicola* sp. nov. is proposed. The type strain is A2^T (=KCTC 22662^T =DSM 22637^T).

The genus *Formosa* was proposed by Ivanova *et al.* (2004) for heterotrophic, Gram-negative, motile by gliding, aerobic, halotolerant, saccharolytic, rod-shaped, yellow-pigmented strains isolated from an enrichment of a microbial community degrading the marine brown alga *Fucus evanescens*. The genus *Formosa* currently consists of two species, *Formosa algae* (Ivanova *et al.*, 2004) and *Formosa agariphila* (Nedashkovskaya *et al.*, 2006). In the present study, we report on the taxonomic characterization of a strain, designated A2^T, that displayed the basic characteristics of the genus *Formosa*.

Strain A2^T was isolated from a marine sponge, *Hymeniacidon flavia*, collected from the coast of Jeju Island, Republic of Korea. Homogenized tissues were serially diluted with sterile seawater, spread on marine agar 2216 (MA; Difco) and incubated at 30 °C for 7 days. Strain A2^T formed small, irregular, yellow colonies on MA after 4 days. The strain was preserved at –80 °C in marine broth 2216 (MB; Difco) supplemented with 20% (v/v) glycerol.

Genomic DNA of strain A2^T was extracted and purified using the Wizard Genomic DNA purification kit (Promega) according to the manufacturer's instructions and the nearly complete 16S rRNA gene (1394 bp) was amplified using bacterial universal primers (Johnson, 1994). The 16S rRNA gene fragment was sequenced directly using a BigDye Terminator version 3.1 cycle sequencing kit and an ABI 3730xl automated DNA

sequencer (Applied Biosystems). The 16S rRNA gene sequence was compared to those available in GenBank using BLAST (<http://www.ncbi.nlm.nih.gov/blast/>) to determine an approximate phylogenetic affiliation and then aligned with closely related sequences using CLUSTAL W (Thompson *et al.*, 1994). Sequence similarities were computed using Similarity Matrix version 1.1 [Ribosomal Database Project II (<http://rdp.cme.msu.edu/index.jsp>); Cole *et al.*, 2003] and the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. Phylogenetic trees were constructed using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms available in PHYLIP version 3.6 (Felsenstein, 2002). Evolutionary distance matrices were calculated according to the algorithm of Kimura's two-parameter model for the neighbour-joining method. Bootstrap analysis of the neighbour-joining tree was performed in PHYLIP with 1000 replications to evaluate the stability of the phylogenetic tree. The phylogenetic analysis indicated that strain A2^T formed a tight phyletic lineage with the type strains of species of the genus *Formosa* (Fig. 1). The distinct phylogenetic position of strain A2^T was confirmed in the maximum-likelihood and maximum-parsimony trees (not shown). Strain A2^T was most closely related to *F. agariphila* KMM 3901^T and *F. algae* KMM 3553^T (96.99 and 96.98% 16S rRNA gene sequence similarity, respectively).

DNA–DNA hybridization was carried out by the membrane filter technique using the DIG High Prime DNA Labelling and Detection Starter kit II (Roche Molecular

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain A2^T is FJ348469.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships of strain A2^T and related taxa. Bootstrap values (>50%) based on 1000 replicates are shown at branch nodes. The sequence of *Brumimicrobium glaciale* ACAM 644^T (GenBank accession no. AF521195) was used as an outgroup (not shown). Bar, 0.01 changes per nucleotide position.

Biochemicals). Genomic DNA was denatured by the alkaline method and immobilized on a nylon membrane (Hybond-N⁺; Amersham) by applying a vacuum. The denatured DNA samples were labelled according to the manufacturer's protocol. The membranes were prehybridized in hybridization solution at 43 °C for 30 min and hybridized in hybridization solution containing labelled DNA at 43 °C for 2 h. After hybridization, the membranes were washed twice in primary washing solution (2 × SSC, 0.1% SDS) and subsequently washed twice in secondary washing solution (0.5 × SSC, 0.1% SDS) at 43 °C. Detection reagents were added to the membranes for 5 min at room temperature and then excess liquid was removed. The membranes were exposed to autoradiography film (Hyperfilm-ECL; Amersham) for 15 min and signal intensities were determined using TINA version 2.0. The signal produced by self-hybridization was taken to be 100% and DNA–DNA relatedness was calculated from duplicate samples. Strain A2^T showed relatively low DNA–DNA relatedness with *F. agariphila* KMM 3901^T (14.1%) and *F. algae* KMM 3553^T (26.8%). It is clear from the 16S rRNA gene sequence analysis and the DNA–DNA hybridization data that strain A2^T represents a novel species in the genus *Formosa*.

Cell morphology and the presence of flagella were observed by light microscopy (FDX-35; Nikon) and transmission electron microscopy (JEM100; JEOL) using cells grown on MA at 30 °C for 4 days. Gliding motility was assessed on 1/10 MA (10% marine broth and 1% agar; Bowman, 2000) and with the hanging-drop method (Bernardet *et al.*, 2002). Gram staining was performed using the bioMérieux Gram-stain kit according to the manufacturer's instructions. Growth at 5–40 °C (in increments of 5 °C) was measured on MA and growth at pH 3, 4 and 5–11 (in increments of 0.5 pH units) and with 1, 1.5, 2, 2.5, 3, 3.5, 4,

5, 6 and 7% (w/v) NaCl was measured in MB. Growth with 0–12% (w/v) sea salts (Sigma) was determined on trypticase soy agar (TSA; Difco) and growth with NaCl as the sole salt was studied on TSA supplemented with 0–12% (w/v) NaCl. Anaerobic growth was assessed on MA in a GasPak anaerobic system (BBL) for 15 days at 30 °C. The presence of catalase activity was determined by the production of oxygen bubbles after mixing cells with 3% (v/v) H₂O₂ on a glass slide. The presence of oxidase activity was determined by oxidation of 1% (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine solution. DNase activity was examined on DNase test agar with methyl green (Difco) supplemented with 2% (w/v) sea salts. Hydrolysis of casein, starch and Tween 80 was tested on MA using the substrate concentrations given by Cowan & Steel (1965). Hydrolysis of agar and CM-cellulose was investigated as described by Cowan & Steel (1993). Hydrolysis of aesculin, gelatin and urea and reduction of nitrate were investigated as described by Lányi (1987) using media supplemented with 2% (w/v) sea salts. H₂S production was tested as described by Bruns *et al.* (2001). Other biochemical tests were performed using API 20NE and API 20E kits and enzyme activities were tested using an API ZYM kit (all from bioMérieux), following the manufacturer's instructions and using a heavy bacterial suspension in artificial seawater as inoculum (Yoon *et al.*, 2002). The biochemical, physiological and morphological characteristics of strain A2^T are given in the species description and Table 1.

The presence of flexirubin-type pigments was investigated as described by Bernardet *et al.* (2002). Flexirubin-type pigments were absent. For pigment characterization, strain A2^T was grown on MA at 30 °C for 4 days. Pigments were extracted in acetone/methanol (7:2, v/v) and the absorption spectrum between 300 and 700 nm was determined with a UV-visible spectrophotometer (Evolution 300;

Table 1. Phenotypic characteristics that differentiate strain A2^T from the type strains of species of the genus *Formosa*

Strains: 1, *Formosa spongicola* sp. nov. A2^T; 2, *F. agariphila* KMM 3901^T; 3, *F. algae* KMM 3553^T. All data were taken from this study. All strains grew optimally at pH 7 and were positive for gliding motility, oxidase, catalase and alkaline phosphatase activities, glucose fermentation, gelatin hydrolysis and acid formation from glucose. All strains were negative for flexirubin pigments, urease activity, production of H₂S, indole and acetoin, hydrolysis of casein, CM-cellulose, DNA and Tween 80, acid formation from rhamnose, sucrose, inositol and sorbitol and susceptibility to benzylpenicillin, gentamicin, kanamycin, neomycin, polymyxin B and streptomycin.

Characteristic	1	2	3
Temperature for growth (°C)			
Range	15–35	4–35	4–35
Optimum	30	21–23	23
pH range for growth	5–11	6–10	6–11
NaCl range for growth (%)	1–5	1–8	0–8
Nitrate reduction	+	–	+
β-Galactosidase	–	+	–
Acid production from mannitol	–	+	+
Hydrolysis of:			
Aesculin	–	+	+
Agar	–	+	–
Starch	–	–	+
Utilization of:			
Arabinose	+	+	–
Lactose	–	+	+
Susceptibility to tetracycline	–	–	+
DNA G + C content (mol%)	36	35–36	34–35

Thermo Scientific). Cells of strain A2^T produced yellow carotenoid pigments with maximum absorption at 433 nm.

Cellular fatty acid methyl esters were prepared from cultures grown on MA at 30 °C for 4 days and were determined using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's protocol. DNA G + C content was determined using the thermal denaturation method (Marmur & Doty, 1962). Isoprenoid quinones were analysed using HPLC (ZQ 4000; Waters) with a reversed-phase column (300 × 3.9 mm, Nova-Pak C18; Waters) as described by Komagata & Suzuki (1987). The dominant fatty acids of strain A2^T were iso-C_{15:0} (33.9%), iso-C_{17:0} 3-OH (20.8%), iso-C_{15:1} G (10.5%) and iso-C_{15:0} 3-OH (6.1%). The detailed fatty acid profile of strain A2^T is presented with those of closely related members of the genus *Formosa* in Table 2. The major respiratory quinone of strain A2^T was MK-6 and the DNA G + C content was 36.0 mol%.

On the basis of 16S rRNA gene sequence phylogenetic analysis and the above-mentioned phenotypic features, we conclude that strain A2^T represents a novel species within the genus *Formosa*, for which the name *Formosa spongicola* sp. nov., is proposed.

Table 2. Cellular fatty acid compositions of strain A2^T and the type strains of species of the genus *Formosa*

Strains: 1, *Formosa spongicola* sp. nov. A2^T; 2, *F. agariphila* KMM 3901^T; 3, *F. algae* KMM 3553^T. Values are percentages of total fatty acids and were obtained in this study. –, Not detected.

Fatty acid	1	2	3
iso-C _{14:0}	1.3	2.0	–
C _{15:0}	2.1	11.2	4.9
C _{15:0} 2-OH	–	2.8	–
C _{15:1} ω6c	1.3	14.0	12.2
iso-C _{15:0}	33.9	9.4	20.6
iso-C _{15:1} G	10.5	15.9	17.3
iso-C _{15:0} 3-OH	6.1	7.2	6.4
anteiso-C _{15:0}	4.5	2.4	4.7
C _{16:0} 3-OH	–	2.3	–
iso-C _{16:0}	–	1.5	–
iso-C _{16:1} H	–	2.2	–
iso-C _{16:0} 3-OH	3.3	8.1	4.7
C _{17:0} 2-OH	1.6	–	–
C _{17:1} ω6c	–	1.3	4.3
iso-C _{17:1} ω9c	1.8	–	–
iso-C _{17:0} 3-OH	20.8	4.3	7.2
Summed feature 3*	4.4	9.2	4.5

*Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of iso-C_{15:0} 2-OH and/or C_{16:1} ω7c.

Description of *Formosa spongicola* sp. nov.

Formosa spongicola [spon.gi'co.la. L. fem. *spongia* a sponge; L. masc./fem. suffix n. *-cola* (from *incola*) inhabitant; N.L. nom. n. (in apposition) *spongicola* inhabitant of sponges].

Cells are Gram-negative, yellow-pigmented rods (0.3–0.5 µm wide and 2.0–2.3 µm long). Strictly aerobic, non-flagellated and oxidase- and catalase-positive. After 4 days on MA, colonies are 1.0–1.5 mm in diameter, convex and circular with slightly irregular edges. Cells contain non-diffusible yellow carotenoid pigments with maximum absorption at 433 nm. Gliding motility is observed. Growth occurs at 15–35 °C (optimum 30 °C) and pH 5–11 (optimum pH 7–7.5). Growth requires sea salts or artificial seawater: NaCl alone does not support growth. Grows on TSA supplemented with 1–2 % (w/v) sea salt or 10–100 % (v/v) artificial seawater. Hydrolyses Tweens 40 and 60 and gelatin, but not agar, casein, CM-cellulose, DNA, starch, urea or Tweens 20 or 80. Does not produce H₂S, acetoin or indole. Reduces nitrate. Does not produce arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase or tryptophan deaminase. Acid is produced from glucose, but not from arabinose, melibiose, rhamnose, sucrose, inositol, mannitol, sorbitol or amygdalin. Assimilates arabinose, maltose, mannose, *N*-acetylglucosamine, gluconate, adipic acid and phenylacetate, but not caprate, citrate or malate. With API ZYM, alkaline phosphatase, esterase (C4), esterase lipase (C8), cystine arylamidase,

leucine arylamidase, valine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase and α -galactosidase activities are present, but lipase (C14), acid phosphatase, α -fucosidase, α - and β -glucosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, β -galactosidase and α -mannosidase activities are absent. Susceptible to ampicillin and oleandomycin, but resistant to benzylpenicillin, gentamicin, kanamycin, neomycin, polymyxin B, streptomycin and tetracycline. The major fatty acids are iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{15:1} G and iso-C_{15:0} 3-OH. The major respiratory quinone is MK-6. The DNA G + C content of the type strain is 36.0 mol%.

The type strain, A2^T (=KCTC 22662^T =DSM 22637^T), was isolated from the marine sponge *Hymeniacidon flavia* in Jeju, Republic of Korea.

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