Spongiibacterium flavum gen. nov., sp. nov., a member of the family *Flavobacteriaceae* isolated from the marine sponge *Halichondria oshoro*, and emended descriptions of the genera *Croceitalea* and *Flagellimonas*

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A Gram-negative, strictly aerobic, non-flagellated, non-gliding, oxidase- and catalase-positive, yellow-pigmented rod, designated A11^T, was isolated from a marine sponge, *Halichondria oshoro*, collected on the coastline of Jeju Island, Republic of Korea. Phylogenetic analysis based on nearly complete 16S rRNA gene sequences revealed that strain A11^T was a member of the family *Flavobacteriaceae*. Its closest relatives were members of the genera *Muricauda*, *Flagellimonas* and *Croceitalea* (94.4–94.8% 16S rRNA gene sequence similarity). The only polar lipid detected in strain A11^T was phosphatidylethanolamine. The dominant fatty acids were iso-C_{15:0} (30.4%), iso-C_{15:1} G (26.7%), iso-C_{17:0} 3-OH (12.4%) and iso-C_{15:0} 3-OH (7.3%). The DNA G + C content of strain A11^T was 41.7 mol% and its major respiratory quinone was MK-6. On the basis of combined data from phenotypic and phylogenetic analyses, strain A11^T represents a novel genus and species within the family *Flavobacteriaceae*, for which the name *Spongiibacterium flavum* gen. nov., sp. nov. is proposed. The type strain of the type species is A11^T (=KCTC 22665^T=DSM 22638^T). Emended descriptions of the genera *Croceitalea* and *Flagellimonas* are also given.

The family *Flavobacteriaceae* (Reichenbach, 1989; Bernardet *et al.*, 2002; Bernardet & Nakagawa, 2006; Bernardet, 2010) currently comprises 94 genera (<http://www.bacterio.cict.fr/>) and is one of the major phylogenetic lineages within the phylum ‘*Bacteroidetes*’ (Ludwig *et al.*, 2008). Marine sponges have attracted attention as they often harbour a diverse range of bacteria, which may be either resident in the mesohyl or found transiently within the channel system where rapid exchange of surrounding water occurs. Microbial communities have been analysed in a number of marine sponges originating mostly from Mediterranean, tropical and temperate regions (Webster & Hill, 2001; Taylor *et al.*, 2007).

While studying the microbial community of *Halichondria oshoro*, a common marine sponge of the phylum Porifera, of the South Sea in the Republic of Korea, a heterotrophic, Gram-negative, non-gliding and yellow-pigmented marine bacterium, designated A11^T, was isolated. The sponge was

collected on the coastal line of Jeju Island, Republic of Korea (33° 29.41′ N 126° 25.48′ E). Sections of the sponge were homogenized, serially diluted with filter-sterilized (pore size 0.2 µm) natural seawater, spread on marine agar 2216 (MA; Difco) and incubated at 30 °C for 7 days. Strain A11^T formed small irregular yellow-pigmented colonies on MA after 4 days of incubation. The strain was preserved at –80 °C in marine broth 2216 (MB; Difco) supplemented with 20% (v/v) glycerol.

Genomic DNA was extracted and purified by using the Wizard genomic DNA purification kit (Promega), according to the manufacturer’s instructions, and the nearly complete 16S rRNA gene (1382 bp) was amplified using the bacterial universal primers 27F and 1492R (Johnson, 1994). The 16S rRNA gene fragment was sequenced directly using a BigDye Terminator v3.1 Cycle sequencing kit and an ABI 3730xl automated DNA sequencer (Applied Biosystems). The 16S rRNA gene sequence of strain A11^T was compared with those available in GenBank using BLASTN (Altschul *et al.*, 1990) to determine an approximate phylogenetic affiliation and was then aligned with closely

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A11^T is FJ348473.

related sequences using CLUSTAL X (Thompson *et al.*, 1997). Sequence similarity was computed using 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. Sequences were assembled using the Bio-Edit program (Hall, 2007). Phylogenetic trees were constructed by the neighbour-joining method as described by Saitou & Nei (1987), the maximum-parsimony method as described by Felsenstein (1978) using MEGA version 4.0 (Tamura *et al.*, 2007) and the maximum-likelihood method (Felsenstein, 1981) using PHYLIP version 3.67 (Felsenstein, 2007). Evolutionary distance matrices were calculated according to the algorithm of Kimura's two-parameter model for the neighbour-joining method (Kimura, 1980). The topologies of the resultant trees were evaluated using bootstrap analysis (Felsenstein, 1985) based on 1000 replicates.

The phylogenetic analysis based on nearly complete 16S rRNA gene sequences revealed that strain A11^T was a member of the family *Flavobacteriaceae*. The isolate's closest relatives were *Muricauda olearia* CL-SS4^T (Hwang *et al.*, 2009), *Flagellimonas eckloniae* DOKDO 007^T (Bae *et al.*, 2007) and *Croceitalea eckloniae* DOKDO 025^T (Lee *et al.*, 2008), with which it shared 94.8, 94.5 and 94.4 % 16S rRNA gene sequence similarities, respectively. The neighbour-joining tree (Fig. 1) showed that strain A11^T formed a branch related to but distinct from members of the genera *Muricauda*, *Flagellimonas*, *Croceitalea*, *Pseudozobellia*, *Euzebyella*, *Kriegella*, *Maribacter* and *Robiginitalea*. The phylogenetic position of strain A11^T was confirmed in the maximum-likelihood and maximum-parsimony trees.

The following tests were performed on strain A11^T only. Cell morphology and the presence of flagella were assessed

by light and transmission electron microscopy (JEM100; JEOL) using cells grown on MA at 30 °C for 4 days. The following tests were performed on strain A11^T, *M. olearia* CL-SS4^T, *F. eckloniae* DOKDO 007^T and *C. eckloniae* DOKDO 025^T using the same culture conditions and methods. Gliding motility was assessed on 0.1 × MA (containing 10 % MB and 1 % agar) (Bowman, 2000) and by the hanging drop method as recommended by Bernardet *et al.* (2002). The Gram-reaction was performed using the bioMérieux Gram-stain kit, according to the manufacturer's instructions. The presence of flexirubin-type pigments was investigated as described by Bernardet *et al.* (2002). Growth at 5–45 °C (at intervals of 5 °C) was assessed on MA. Growth at pH 4.0–12 (at intervals of 0.5 pH unit) was tested in MB with the pH adjusted by the addition of 1 M HCl or 1 M NaOH, by assessing changes in OD₆₀₀ with time. Growth with 1–4 % (w/v) NaCl (at intervals of 0.5 %) and 5–10 % NaCl (at intervals of 1 %) was assessed in MB. Growth with 0–12 % (w/v) sea salts (at intervals of 1 %; Sigma) was determined in trypticase soy agar (TSA; Difco). Anaerobic growth was assessed on MA supplemented with 0.1 % NaNO₃ as an electron acceptor and incubation in a GasPak anaerobic system (BBL) for 15 days at 30 °C. The presence of catalase activity was revealed by the production of oxygen bubbles when cells were mixed 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. DNase activity was examined using DNase test agar with methyl green (Difco) supplemented with 2 % (w/v) sea salts. Hydrolysis of casein, starch, and Tweens 20, 40, 60 and 80 was tested on MA, using the substrate concentrations given by Cowan & Steel (1965). Hydrolysis of agar and carboxymethyl-cellulose was investigated as described

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships of strain A11^T and related taxa of the family *Flavobacteriaceae*. Bootstrap values (>70 %) based on 1000 replicates are shown at branch nodes. Asterisks indicate that the corresponding nodes were also recovered using the maximum-likelihood and maximum-parsimony methods. *Bacteroides fragilis* ATCC 25285^T (accession no. X83935) was used as an outgroup (not shown). Bar, 0.02 substitutions per nucleotide.

Table 1. Characteristics that differentiate strain A11^T from closely related members of the family *Flavobacteriaceae*

Strains: 1, *Spongiibacterium flavum* sp. nov. A11^T; 2, *Muricauda olearia* CL-SS4^T; 3, *Flagellimonas eckloniae* DOKDO 007^T; 4, *Croceitalea eckloniae* DOKDO 025^T. All data were from this study except for data in parentheses and DNA G+C contents in columns 2–4, which were taken from Hwang *et al.* (2009), Bae *et al.* (2007) and Lee *et al.* (2008), respectively. All strains are positive for respiratory metabolism, catalase, alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -glucosidase, utilization of glucose and susceptibility to polymyxin B. All strains are negative for gliding motility, H₂S production, nitrate reduction, lipase (C14), β -galactosidase, β -glucuronidase, α -fucosidase, hydrolysis of agar and carboxymethyl-cellulose, production of flexirubin pigments, acid production from arabinose, inositol, mannitol, melibiose, rhamnose, sorbitol and sucrose, utilization of arabinose, citrate and gluconate and susceptibility to chloramphenicol. +, Positive; –, negative.

Characteristic	1	2	3	4
Presence of:				
Flagella	–	–	+	–
Cellular appendages	–	+	–	–
Temperature range (°C)	20–35	15–40	15–35 (17–36)	10–35 (10–34)
NaCl range (%)	1–6	1–5 (0.8–4.8)	1.5–6 (1.9–5.4)	1–5.5 (0.4–5.4)
NaCl optimum (%)	2–3	2–3 (1.6–2.4)	2–3 (2.7–3.1)	1.5–2 (1.6)
Seawater requirement	+	+	+	–
Oxidase activity	+	+	–	–
Hydrolysis of:				
Casein	–	–	+	–
Gelatin	–	– (+)	–	+
DNA	+	–	+	+
Starch	–	+	–	+
Urea	+	–	–	–
Aesculin	+	+	+	– (+)
Tween 20	–	– (+)	+	–
Tweens 40, 60, 80	–	+	+	–
Acid production from glucose	+	+	–	–
Utilization of:				
Mannose	+	– (+)	– (+)	+
Mannitol	+	–	–	+
N-Acetylglucosamine	+	+	–	– (+)
Maltose	–	+	–	+
Capric acid	–	–	–	+
Adipic acid	+	–	–	–
Malic acid	+	–	–	+
Phenylacetic acid	+	–	–	+
Enzyme activities (API ZYM)				
Esterase (C4)	+	– (+)	–	–
Esterase lipase (C8)	+	+	–	–
α -Galactosidase	+	–	–	–
β -Glucosidase	+	–	–	–
N-Acetyl- β -glucosaminidase	+	– (+)	–	–
α -Mannosidase	+	–	–	–
Susceptibility to:				
Ampicillin	–	+	–	+
Carbenicillin	+	+	–	+
Gentamicin	–	–	–	+
Neomycin	–	–	–	+
Kanamycin	–	–	+	–
Penicillin G	+	–	+	+
Oleandomycin	–	+	–	+
Streptomycin	–	–	+	–
Tetracycline	–	+	+	+
DNA G+C content (mol%)	41.7	50.7	56.3	35.8

Fig. 2. Polar lipid profiles of strain A11^T and closely related members of the family *Flavobacteriaceae*. (a) Standard mixture of phospholipids, (b) *Songiibacterium flavum* sp. nov. A11^T, (c) *Muricauda olearia* CL-SS4^T, (d) *Flagellimonas eckloniae* DOKDO 007^T, (e) *Croceitalea eckloniae* DOKDO 025^T. The reagent used to spray the plates was 5% ethanolic molybdatophosphoric acid. DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

by Barrow & Feltham (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 the API 20 NE and API 20 E kits, and enzyme activities were tested using the API ZYM kit (bioMérieux), according to the manufacturer's instructions, except that the strips were inoculated with a heavy bacterial suspension in artificial seawater (Yoon *et al.*, 2002) and incubated at 30 °C for 4 days. Susceptibility to antibiotics was determined by the disc-diffusion method with commercial antibiotic-impregnated discs (BBL Becton Dickinson) on MA. Results were interpreted according to the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI, 2003) after 4 days at 30 °C. The main physiological, biochemical and morphological characteristics of strain A11^T are given in the genus and species descriptions and Table 1.

The polar lipids of strain A11^T, *M. olearia* CL-SS4^T, *F. eckloniae* DOKDO 007^T and *C. eckloniae* DOKDO 025^T were extracted according to the procedures described by

Minnikin *et al.* (1984), separated by two-dimensional TLC using silica gel 60 F254 plates, and identified by spraying with ninhydrin reagent (Kates, 1985), Zinzadze reagent (Dittmer & Lester, 1964), α -naphthol reagent (Jacin & Mishkin, 1965) and molybdatophosphoric acid stain (Gunstone & Jacobsberg, 1972) and by co-migration with authentic standards (Sigma) (Komagata & Suzuki, 1987). The cellular fatty acid methyl esters of strain A11^T, *M. olearia* CL-SS4^T, *F. eckloniae* DOKDO 007^T and *C. eckloniae* DOKDO 025^T were prepared from cultures grown on MA at 30 °C for 4 days and determined using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's protocol (Instant FAME method, version 4.5, TSBA 50 database). The following tests were performed on strain A11^T only. DNA was isolated by the method of Marmur (1961) and the G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962). The isoprenoid quinones were analysed by reversed-phase HPLC (ZQ 4000; Waters) with a 300 × 3.9 mm Nova-Pak C18 column (Waters) as described by Komagata & Suzuki (1987).

Strain A11^T and the three reference strains displayed similar polar lipid profiles, with phosphatidylethanolamine

Table 2. Cellular fatty acid compositions of strain A11^T and closely related members of the family *Flavobacteriaceae*

Strains: 1, *Spongiibacterium flavum* sp. nov. A11^T; 2, *Muricauda olearia* CL-SS4^T; 3, *Flagellimonas eckloniae* DOKDO 007^T; 4, *Croceitalea eckloniae* DOKDO 025^T. All data were taken from this study except for data in parentheses in columns 2–4, which were taken from Hwang *et al.* (2009), Bae *et al.* (2007) and Lee *et al.* (2008), respectively. Fatty acids amounting to <1% in all strains are omitted. tr, Trace (<1.0%); –, not detected.

Fatty acid (%)	1	2	3	4
Saturated				
C _{15:0}	4.2	5.9 (10.7)	3.3 (7.0)	2.9 (8.7)
C _{16:0}	1.4	tr (tr)	tr (–)	1.3 (2.6)
Branched saturated				
iso-C _{13:0}	1.9	tr	–	–
iso-C _{15:0}	30.4	19.4 (18.7)	24.5 (41.8)	14.6 (13.0)
anteiso-C _{15:0}	2.9	2.1 (1.4)	tr (–)	tr (tr)
Branched monounsaturated				
iso-C _{15:1} G	26.7	23.1 (17.1)	28.4 (–)	24.5 (16.0)
iso-C _{17:1} ω9c	–	1.8 (3.6)	2.3 (–)	1.4 (4.3)
Hydroxy				
C _{15:0} 3-OH	–	1.4 (tr)	– (–)	– (tr)
C _{16:0} 3-OH	tr	1.1 (tr)	1.0 (tr)	2.1 (2.7)
C _{17:0} 3-OH	1.9	2.4 (1.2)	2.2 (–)	2.6 (tr)
iso-C _{15:0} 3-OH	7.3	6.4 (4.4)	8.8 (–)	6.8 (5.0)
iso-C _{16:0} 3-OH	1.3	1.0 (1.4)	1.1 (–)	1.5 (tr)
iso-C _{17:0} 3-OH	12.4	13.7 (20.5)	18.9 (–)	12.4 (18.6)
Summed feature 3*	6.5	8.8 (7.1)	6.6 (–)	13.9 (14.3)

*Summed features represent 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} ω9c.

as the only polar lipid detected (Fig. 2). The dominant fatty acids of strain A11^T were iso-C_{15:0} (30.4%), iso-C_{15:1} G (26.7%), iso-C_{17:0} 3-OH (12.4%) and iso-C_{15:0} 3-OH (7.3%). The fatty acid composition of strain A11^T is compared with those of the reference strains in Table 2. The overall fatty acid compositions were similar, with only minor differences in the proportions of some fatty acids. The DNA G+C content of strain A11^T was 41.7 mol%, a value significantly lower than those reported for the reference strains (Table 1). The major respiratory quinone of strain A11^T was menaquinone MK-6, which is in line with that found in all other members of the family *Flavobacteriaceae*.

On the basis of 16S rRNA gene sequence phylogenetic inference and the above-mentioned phenotypic features, we conclude that strain A11^T represents a novel genus and species within the family *Flavobacteriaceae*, for which the name *Spongiibacterium flavum* gen. nov., sp. nov. is proposed. On the basis of data obtained in this study, we also give emended descriptions of the genera *Croceitalea* and *Flagellimonas*.

Emended description of the genus *Croceitalea* Lee *et al.* 2008

The description is as given by Lee *et al.* (2008) with the following amendments. The only polar lipid detected in the type strain of the type species is phosphatidylethanolamine. Characteristics of the type strain of the type species that are either in addition to or at variance with published data are shown in Table 1.

Emended description of the genus *Flagellimonas* Bae *et al.* 2007

The description is as given by Bae *et al.* (2007) with the following amendments. The only polar lipid detected in the type strain of the type species is phosphatidylethanolamine. Characteristics of the type strain of the type species that are either in addition to or at variance with published data are shown in Table 1.

Description of *Spongiibacterium* gen. nov.

Spongiibacterium (Spon.gi.i.bac.te'ri.um. L. fem. n. *spongia* sponge; L. neut. n. *bacterium* a rod; N.L. neut. n. *Spongiibacterium* a rod-shaped bacterium isolated from a sponge).

Cells are strictly aerobic, Gram-negative, yellow-pigmented, non-flagellated rods. Gliding motility is absent. Oxidase- and catalase-positive. Cells produce non-diffusible pigments that do not belong to the flexirubin type. The major respiratory quinone is MK-6. The cellular fatty acid composition is dominated by iso-C_{15:0}, iso-C_{15:1} G, iso-C_{17:0} 3-OH and iso-C_{15:0} 3-OH. Phosphatidylethanolamine is the only polar lipid detected. As determined by 16S rRNA gene sequence analysis, the genus is a member of the family *Flavobacteriaceae*. The type species is *Spongiibacterium flavum*.

Description of *Spongiibacterium flavum* gen. nov., sp. nov.

Spongiibacterium flavum (fla'vum. L. neut. adj. *flavum* yellow, the colour of colonies or pigment).

Displays the following properties in addition to those listed in the genus description. Cells are 0.3–0.5 µm wide and 2.0–2.5 µm long and are devoid of appendages. 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 pigments. Growth occurs at 20–35 °C (optimum 30 °C) and pH 6.0–10.0 (optimum pH 7.0–8.0). Na⁺ alone does not support growth; sea salts are required for growth. Grows with 1–6% (w/v) NaCl (optimum 2–3%). Aesculin, arginine, DNA and urea are hydrolysed, but agar, gelatin, carboxymethyl-cellulose, casein, starch, and Tweens 20, 40, 60 and 80 are not. Acetoin is produced, but indole and H₂S are not. Nitrate is not reduced. Lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. Acid is produced

from glucose, but not from amygdalin, arabinose, inositol, mannitol, melibiose, rhamnose, sorbitol or sucrose. Glucose, mannose, mannitol, *N*-acetylglucosamine, adipic acid, malate and phenylacetic acid are assimilated, but arabinose, maltose, gluconate, capric acid and citrate are not. With API ZYM, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase and α -mannosidase are present, but lipase (C14), β -galactosidase, β -glucuronidase and α -fucosidase are absent. Susceptible to (μ g per disc, unless otherwise stated) carbenicillin (100), chloramphenicol (100) and penicillin G (10 U), but resistant to ampicillin (10), gentamicin (10), kanamycin (30), neomycin (30), oleandomycin (15), polymyxin B (30), streptomycin (10) and tetracycline (30). The fatty acid composition is given in Table 2.

The type strain, A11^T (=KCTC 22665^T =DSM 22638^T), was isolated from a marine sponge on Jeju Island, Republic of Korea. The DNA G+C content of the type strain is 41.7 mol%.

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