Aureibacter tunicatorum gen. nov., sp. nov., a marine bacterium isolated from a coral reef sea squirt, and description of *Flammeovirgaceae* fam. nov.

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Two aerobic, Gram-reaction-negative, golden-yellow pigmented and rod-shaped bacteria, designated strains A5Q-118^T and A5Q-27, were isolated from an unidentified sea squirt that thrives in the coral reefs off the coast of Okinawa, Japan. Phylogenetic analyses based on the 16S rRNA gene sequence revealed that the novel isolates were affiliated with the family 'Flammeovirgaceae' of the phylum *Bacteroidetes*. Strains A5Q-118^T and A5Q-27 shared 100% sequence similarity with each other and showed <92% similarity with other cultivated members of the family 'Flammeovirgaceae'. The novel isolates were phenotypically and physiologically different from strains described previously. The DNA G + C content was 35.5–36.2 mol%, MK-7 was the major menaquinone and iso-C_{15:0} and C_{16:1}ω5c were the major fatty acids. Based on the results of this polyphasic taxonomic study, it was concluded that strains A5Q-118^T and A5Q-27 represent a novel species in a new genus of the family 'Flammeovirgaceae', for which the name *Aureibacter tunicatorum* gen. nov., sp. nov. is proposed. Proposal for designation of the *Flammeovirgaceae* fam. nov. is also presented. The type strain of *Aureibacter tunicatorum* is A5Q-118^T (=KCTC 23232^T =NBRC 107587^T).

Members of the phylum *Bacteroidetes* (Ludwig & Klenk, 2001), also known as the *Bacteroides*–*Cytophaga*–*Flexibacter* group, are widely distributed over a diverse range of ecological niches (Bowman *et al.*, 1997; DeLong *et al.*, 1993; Glöckner *et al.*, 1999; O'Sullivan *et al.*, 2002; Pinhassi *et al.*, 1997). According to several reports, members of the phylum *Bacteroidetes* have been implicated as being major utilizers of high-molecular-mass organic matter dissolved in marine ecosystems (Cottrell & Kirchman, 2000) and are often abundant in nutrient-rich waters where biomacromolecules accumulate (Reichenbach, 1989). In spite of their wide ecological distribution in nature, the classification of the

phylum *Bacteroidetes* is still in a state of flux. For formal classification of the phylum *Bacteroidetes*, representatives that thrive in a wide range of terrestrial, aquatic and marine habitats need to be isolated, cultivated and taxonomically investigated. At the time of writing, the family 'Flammeovirgaceae' within the phylum *Bacteroidetes*, incorporated the genera *Flammeovirga*, *Flexithrix*, *Limibacter*, *Perexilibacter*, *Persicobacter*, *Rapidithrix* and *Sedimentimix*. An emended description of the genus *Persicobacter* was also recently published (Muramatsu *et al.*, 2010).

Two strains, designated A5Q-118^T and A5Q-27, were isolated from an unidentified sea squirt collected near Iriomote Island, Okinawa, Japan. A piece of sea squirt (~1 cm³) was gently rinsed in sterile artificial seawater and then homogenized with a sterilized glass rod. The suspension was plated onto marine agar 2216 (Difco) adjusted to pH 11 with Na₂CO₃ and NaHCO₃. A colony was transferred onto a 1/3 strength marine agar 2216 plate to purify the isolate for use in this study. In the present study, we determined the phylogenetic position of strains A5Q-118^T and A5Q-27 and used a polyphasic taxonomic

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Abbreviations: MP, maximum-parsimony; NJ, neighbour-joining; TEM, transmission electron microscopy.

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain A5Q-118^T is AB572584.

approach, including analysis of 16S rRNA gene sequences, fatty acid compositions and quinones as well as genotypic and physiological properties, to characterize the organisms. Based on these data, it is proposed that the isolates represent a novel genus of the family 'Flammeovirgaceae' in the phylum *Bacteroidetes*.

Temperature and pH ranges for growth were determined on marine agar 2216. Salt tolerance was tested on marine agar 2216 containing 0–10 % (w/v) NaCl. Gram-staining was performed as described by Murray *et al.* (1994). Cell morphology was observed using light microscopy (BX60; Olympus) and transmission electron microscopy (TEM). Gliding motility was determined as described by Perry (1973). For the TEM observations, cells were mounted on Formvar-coated copper grids and negatively stained with 1 % (w/v) aqueous uranyl acetate. Grids were observed in a JEOL 1010 transmission electron microscope (JEOL) operated at 100 kV. Growth under anaerobic conditions was determined after 2 weeks of incubation on marine agar 2216 in an AnaeroPack (Mitsubishi Gas Chemical). Catalase activity was determined by bubble formation in a 3 % hydrogen peroxide solution. Oxidase activity was determined by using cytochrome oxidase paper (Nissui Pharmaceutical). API 20 E, API 50 CH and API ZYM strips (bioMérieux) were used to determine physiological and biochemical characteristics. All suspension media for API test strips were supplemented with 0.85 % (w/v) NaCl (final concentration). The API 20 E and API 50 CH tests were read after 72 h of incubation at 30 °C and API ZYM tests were read after 4 h of incubation at 37 °C. Preparation and identification of isoprenoid quinones was carried out at the TechnoSuruga Lab as described previously (Nishijima *et al.*, 1997). GC analysis of cellular fatty acid methyl esters was performed using a culture grown on marine agar 2216 at 30 °C for 48 h according to the instructions of the Microbial Identification System (MIDI). DNA was prepared according to the method of Marmur (1961) from cells grown on marine agar 2216 medium and the DNA base composition was determined at the TechnoSuruga Lab by using the HPLC method of Katayama-Fujimura *et al.* (1984). A ~1500 bp fragment of the 16S rRNA gene was amplified from the extracted DNA by using the bacterial universal primers 27F and 1492R, which are specific to the 16S rRNA gene (based on the *Escherichia coli* numbering system; Weisburg *et al.*, 1991). To ascertain the phylogenetic position of the new isolates, the 16S rRNA gene sequences of strains A5Q-118^T and A5Q-27 were compared with the sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov>). Multiple alignments of the sequences were performed using CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997). Alignment gaps and ambiguous bases were not taken into consideration when the 1322 bases of 16S rRNA gene nucleotides were compared. Aligned sequences were analysed by using MEGA4.0 software (Tamura *et al.*, 2007). Evolutionary distances (distance options according to the Kimura two-parameter model; Kimura, 1983) and

clustering, using the neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-parsimony (MP) (Fitch, 1971) methods, were determined by using bootstrap values based on 1000 replications (Felsenstein, 1985). Similarity values were calculated using the same software.

In the course of TEM, cells of various sizes were observed. Cells were mostly straight and rod-shaped. Cells were 0.4–0.5 × 3.5 and 4.5 in size, gliding motility was not observed and flagella were not seen (Fig. 1).

Comparative phylogenetic analysis of 16S rRNA gene sequences revealed that strains A5Q-118^T and A5Q-27 were affiliated with the family 'Flammeovirgaceae' within the phylum *Bacteroidetes* with a bootstrap confidence value of 100 % using the NJ method (Fig. 2). Analysis of 16S rRNA gene sequences indicated that the sequences of strains A5Q-118^T and A5Q-27 were identical to one another and showed the highest sequence similarity (91.4 %) to *Persicobacter psychrovivids* Asr22-19^T (Muramatsu *et al.*, 2010), followed by *Persicobacter diffluens* NBRC 15940^T (90.8 %; Nakagawa *et al.*, 1997), *Flexithrix dorotheae* NBRC 15987^T (84.9 %; Lewin, 1970), *Flammeovirga aprica* NBRC 15941^T (84.4 %; Takahashi *et al.*, 2006), *Sedimentomix flava* NBRC 101625^T (83.6 %; Khan *et al.*, 2007) and *Limibacter armeniacum* KCTC 22132^T (83.2 %; Yoon *et al.*, 2008). All other species of the family 'Flammeovirgaceae' with validly published names were more distantly related, showing 16S rRNA gene sequence similarity values <83.5 %.

As shown in Table 1, the predominant cellular fatty acids of strains A5Q-118^T and A5Q-27 differentiate it from recognized species of the genera *Flammeovirga*, *Limibacter*, *Perexilibacter*, *Rapidithrix* and *Sedimentomix* based on the proportions of C_{13:1ω1c} (1–6.8 %), iso-C_{15:0} (26.9–33.3 %), C_{16:1ω5c} (11–17.3 %), C_{16:0} 3-OH (7.6–16.1) and iso-C_{17:0} 3-OH (5–11 %). In addition to this, the two novel isolates showed distinct phenotypic features that distinguished them from their closest relatives (Table 2).

Based on the results of the phylogenetic analysis and on their biochemical and physiological properties, strains A5Q-118^T and A5Q-27, isolated from an unidentified sea squirt collected

Fig. 1. Transmission electron micrograph of a negatively stained cell of strain A5Q-118^T. Bar, 1 µm.

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strains A5Q-118^T and A5Q-27 and representatives of closely related and other more distantly related taxa. Bootstrap values >50% (based on 1000 replications) from both neighbour-joining and maximum-parsimony analyses are shown, respectively; '-' indicates a branch not found or sufficiently supported by bootstrap analysis. The tree was rooted using *Flavobacterium aquatile* ATCC 11947^T as an outgroup. Bar, 2% sequence divergence.

near Iriomote Island in Japan, represent a novel species of a new genus in the family 'Flammeovirgaceae' within the phylum *Bacteroidetes*, for which the name *Aureibacter tunicatorum* gen. nov., sp. nov. is proposed.

Description of *Flammeovirgaceae* fam. nov.

Flammeovirgaceae (Flam.me.o.vir.ga'ce.ae. N.L. fem. n. *Flammeovirga* type genus of the family; -aceae ending to

denote a family; N.L. fem. pl. n. *Flammeovirgaceae* the *Flammeovirga* family).

Encompasses Gram-negative bacteria retrieved mainly from soil and marine environments. At the time of writing, the family comprises the genera *Aureibacter*, *Flammeovirga*, *Flexithrix*, *Limibacter*, *Perexilibacter*, *Persicobacter*, *Rapidithrix* and *Sediminitomix*. Delineation of the family is determined primarily by phylogenetic information from

Table 1. Fatty acid contents of strains A5Q-118^T and A5Q-27 and members of related taxa

Taxa: 1, strain A5Q-118^T; 2, strain A5Q-27; 3, *Persicobacter* (data from Muramatsu *et al.*, 2010); 4, *Flammeovirga* (Takahashi *et al.*, 2006; Hosoya & Yokota, 2007); 5, *Sediminitomix flava* NBRC 101625^T (Khan *et al.*, 2007); 6, *Limibacter armeniacum* YM11-185^T (Yoon *et al.*, 2008); 7, *L. armeniacum* YM11-159 (Yoon *et al.*, 2008); 8, *Perexilibacter aurantiacus* MBIC06993^T (Yoon *et al.*, 2007); 9, *Rapidithrix thailandica* TISTR 1750^T (Srisukchayakul *et al.*, 2007). Values are percentages of total fatty acid contents. -, Not detected; tr, trace.

Fatty acid	1	2	3	4	5	6	7	8	9
C _{13:1} ω1c	6.8	1.0	—	0.8–2.9	—	1.6	1.0	1.6	—
C _{14:0}	1.9	1.6	1.0–2.0	3.7–9.0	5.0	1.1	1.0	1.9	1.2
iso-C _{15:0}	33.3	26.9	47.7–55.6	21.2–54.3	23.0	28.1	26.9	50.6	6.3
C _{15:0}	tr	tr	—	tr–7.1	—	1.7	tr	1.4	tr
C _{16:1} ω7c alcohol	—	—	—	—	—	2.3	4.2	—	—
iso-C _{16:1} H	—	—	—	tr–3.2	—	2.2	3.1	—	—
C _{16:0} N alcohol	—	—	—	tr–1.8	—	3.2	10.0	—	—
C _{16:1} ω7c	—	—	—	—	—	23.2	16.8	16.7	1.5
C _{16:1} ω5c	11	17.3	1.3–4.2	1.8–4.7	21.0	9.9	7.9	4.9	44.8
C _{16:0}	10.6	5.0	4.6–9.4	2.8–4.8	6.0	3.5	4.4	3.76	4.0
iso-C _{15:0} 3-OH	1.0	1.2	3.2–7.6	3.9–5.5	2.0	3.7	2.4	2.2	4.3
iso-C _{17:1} ω9c	—	—	—	—	—	1.6	1.2	—	—
C _{17:1} ω6c	—	tr	—	—	—	1.0	tr	tr	—
C _{16:0} 3-OH	7.6	16.1	3.9–6.5	4.1–14.6	18.0	4.9	2.8	2.2	3.8
iso-C _{17:0} 3-OH	5.0	11.0	6.6–16.8	2.0–2.4	2.0	4.5	3.5	2.4	10.8

Table 2. Characteristics that differentiate strains A5Q-118^T and A5Q-27 from members of related taxa

Taxa: 1, strain A5Q-118^T; 2, strain A5Q-27; 3, *Persicobacter psychrovidus* (data from Muramatsu *et al.*, 2010); 4, *P. diffluens* (Muramatsu *et al.*, 2010); 5, *Flexithrix dorothea* ATCC 23163^T (Lewin, 1970); 6, *Flammeovirga aprica* NBRC 15941^T (Takahashi *et al.*, 2006); 7, *F. arenaria* NBRC 15982^T (Yoon *et al.*, 2008); 8, *F. yaeyamensis* NBRC 100898^T (Takahashi *et al.*, 2006); 9, *F. kamogawensis* NCIMB 14281^T (Hosoya & Yokota, 2007); 10, *Sedimentomix flava* NBRC 101625^T (Khan *et al.*, 2007); 11, *Limibacter armeniacum* YM11-185^T (Yoon *et al.*, 2008); 12, *L. armeniacum* YM11-159 (Yoon *et al.*, 2008); 13, *Perexilibacter aurantiacus* MBIC06993^T (Yoon *et al.*, 2007); 14, *Rapidithrix thailandica* TISTR 1750^T (Srisukchayakul *et al.*, 2007). +, Positive; w, weakly positive; v, variable; –, negative; ND, no data.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Isolation source	Sea squirt	Sea squirt	Shellfish	Mud	Seashore silt	Marine	Marine	Marine	Coastal seawater	Marine sediment	Marine sediment	Marine sediment	Marine sediment	Marine algae
Colony colour	Golden yellow	Golden yellow	Orange	Pink to orange	Bright golden yellow	Orange to reddish orange	Orange to reddish orange	Orange to reddish orange	Reddish orange	Orange	Apricot to pale-orange	Apricot to pale-orange	Dull orange	Light olive grey
Cell morphology	Rods	Rods	Rods	Rods	Rods	Long rods	Long rods	Long rods	Long rods	Long rods	Long rods	Long rods	Rods	Flexible filaments
Cell length (µm)	3.5–4.5	3.5–4.5	0.9–6	0.9–30	5–15	1.7–96	2–40	1.7–90	2.0–40	10–35	15–40	15–40	10–20	20–100
Cell width (µm)	0.4–0.5	0.4–0.5	0.4–0.6	0.4–0.5	0.7	0.5–0.9	0.5–0.9	0.4–0.9	0.6–1.0	0.5–0.7	0.3–0.5	0.3–0.5	0.3–0.5	0.7
Catalase	+	+	+	+	–	+	–	v	+	+	+	+	w	–
Hydrolysis of:														
Agar	–	–	+	+	–	+	+	+	+	w	–	–	–	ND
Starch	–	+	w	w	–	+	+	+	+	+	–	–	–	ND
DNA	–	–	ND	+	ND	+	–	+	+	+	–	–	–	ND
Max. temperature for growth (°C)	37	35	45	45	35	30	30	35	30	40	37	45	43	ND
NaCl range for growth (% w/v)	1–5	1–7	7	7	1–6	1–5	1–5	1–5	2–4	1–4	1–6.5	1–6.5	0–3.5	ND
DNA G + C content (mol%)	36.2	35.5	42–42.7	42.6–43.8	37	34.2	31.8	33.4–35.7	32–33	38	27.9	27.8	43	40

16S rRNA gene sequences. Cells are rod-shaped, chemoheterotrophic and aerobic. The DNA G+C contents of members of the family are 27–43 mol%. The type genus of the family is *Flammeovirga* Takahashi *et al.* 2006.

Description of *Aureibacter* gen. nov.

Aureibacter (Au.re.i.bac'ter. L. adj. *aureus* golden; N.L. masc. n. *bacter* rod; N.L. masc. n. *Aureibacter* golden rod).

Cells are rod-shaped, Gram-reaction-negative, aerobic and do not form endospores. Catalase- and oxidase-positive. Nitrate and nitrite reduction are negative. The major respiratory menaquinone is MK-7. Predominant cellular fatty acids are iso-C_{15:0} and C_{16:1ω5c}.

The type species of the genus is *Aureibacter tunicatorum*.

Description of *Aureibacter tunicatorum* sp. nov.

Aureibacter tunicatorum (tu.ni.ca'to.rum. N.L. gen. pl. n. *tunicatorum* of Tunicata, isolated from tunicates).

Characteristics are the same as those given in the genus description with the following additions. Cells are long rods, 0.4–0.5 × 3.5–4.5 µm. Non-motile. No gliding motility is observed. Colonies grown on marine agar 2216 are circular, convex and golden-yellow. Grows at 20–37 °C (optimum ~30 °C) but not 4 or 45 °C, at pH 6–9 (optimum ~pH 7) and in up to 7 % (w/v) NaCl. NaCl is required for growth but seawater is not. Gelatin and starch are hydrolysed but agar and DNA are not. Positive for *o*-nitrophenyl-β-D-galactosidase (ONPG) and the production of lysine decarboxylase and tryptophan deaminase. Negative for the Voges–Proskauer reaction, citrate utilization and production of arginine dihydrolase, ornithine decarboxylase, hydrogen sulfide and indole. Acid is produced from galactose, glucose, aesculin ferric citrate, fructose, melibiose, mannose, sucrose, trehalose, turanose, D-lyxose, D-tagatose and 5-ketoglucuronate but not from D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, methyl-β-D-xylopyranoside, rhamnose, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melezitose, inulin, raffinose, gentiobiose, D- and L-fucose, glycerol, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, sorbitol, starch, glycogen, xylitol, D- and L-arabitol, gluconate or 2-ketogluconate. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase activities are positive but lipase (C4), α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, α-mannosidase and α-fucosidase activities are negative. Major fatty acid components (>1.0 %) include C_{10:0} (3.8 %), C_{13:1ω1c} (6.8 %), C_{14:0} (1.9 %), iso-C_{15:0} (33.3 %), C_{16:1ω5c} (11 %), C_{16:0} (10.6 %), iso-C_{15:0} 3-OH (1 %), anteiso-C_{17:1ω9c} (3.3 %), iso-C_{16:0} 3-OH (4 %), C_{16:0} 3-OH (7.6 %), iso-C_{17:0} 3-OH (5 %) and C_{20:4ω6}, 9, 12, 15c (2.2 %).

The type strain, A5Q-118^T (=KCTC 23232^T =NBRC 107587^T), was isolated from an unidentified sea squirt collected near Iriomote Island, Okinawa, Japan. The G+C content of the genomic DNA of the type strain is 36.2 mol%.

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