Perexilibacter aurantiacus gen. nov., sp. nov., a novel member of the family 'Flammeovirgaceae' isolated from sediment

Jaewoo Yoon,¹ Shu Ishikawa,² Hiroaki Kasai² and Akira Yokota¹

Correspondence

Jaewoo Yoon

aa57058@mail.ecc.u-tokyo.ac.jp

¹Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-Ku, Tokyo 113-0032, Japan

²Marine Biotechnology Institute Co. Ltd, 3-75-1, Heita, Kamaishi, Iwate 026-0001, Japan

A strictly aerobic, Gram-negative, gliding, dull-orange-pigmented, rod-shaped bacterium, designated strain Shu-F-UV2-2^T, was isolated from sediment (Carp Island, Republic of Palau) and was the focus of a polyphasic taxonomic study. Phylogenetic analyses based on the 16S rRNA gene sequence revealed that the novel isolate was affiliated to the family 'Flammeovirgaceae' of the phylum *Bacteroidetes* and that it showed highest sequence similarity (85.5%) to *Flammeovirga yaeyamensis* NBRC 100898^T. The novel isolate could be differentiated phenotypically and physiologically from recognized members of the family 'Flammeovirgaceae'. The G + C content of the DNA was 43.0 mol%, MK-7 was the major menaquinone and iso-C_{15:0}, C_{16:1}ω7c and C_{16:1}ω5c were the major fatty acids. On the basis of this polyphasic evidence, it was concluded that strain Shu-F-UV2-2^T represents a novel species in a new genus of the family 'Flammeovirgaceae', for which the name *Perexilibacter aurantiacus* gen. nov., sp. nov. is proposed. The type strain is Shu-F-UV2-2^T (=MBIC06993^T=IAM 15413^T=KCTC 12867^T).

The *Cytophaga*–*Flavobacterium*–*Bacteroides* (CFB) group is also known as the phylum *Bacteroidetes* (Ludwig & Klenk, 2001). Molecular phylogenetic studies based on 16S rRNA gene sequence analysis have revealed that members of the phylum *Bacteroidetes* are ubiquitous in aquatic environments (DeLong *et al.*, 1993; Bowman *et al.*, 1997; Glöckner *et al.*, 1999; Cottrell & Kirchman, 2000; O'Sullivan *et al.*, 2002). They play a significant role in the decomposition of organic matter and in carbon cycling within the global ecosystem (Cottrell & Kirchman, 2000; Nedashkovskaya *et al.*, 2003). However, despite the significance of their ecological niche, the majority of members of this phylogenetic group remain unknown and as yet uncultured (O'Sullivan *et al.*, 2004). At the time of writing, genera such as *Flammeovirga*, *Flexithrix*, *Persicobacter* and *Thermonema* (Lewin, 1970; Hudson *et al.*, 1989; Nakagawa *et al.*, 1997; Takahashi *et al.*, 2006) are incorporated within the family 'Flammeovirgaceae'.

Strain Shu-F-UV2-2^T was isolated from a sandy sediment, collected in August 2003 at a shallow beach 1 m deep on Carp Island, Republic of Palau (7° 5.25' N 134° 16.75' E). Surface sediment plus seawater from the same locality were collected by using a spatula. They were kept in a 15 ml tube

for 5 h at 15 °C. For microbiological analyses, 1 g sediment was suspended in 15 ml autoclaved artificial seawater (Lyman & Fleming, 1940). A 50 µl sample of this suspension was applied to marine agar 2216 (Difco) and UV-irradiated for 2 min in a laminar flow cabinet. Dull-orange-coloured colonies appeared after incubation at 25 °C. We investigated the phylogenetic position of strain Shu-F-UV2-2^T by using a polyphasic taxonomic approach, including 16S rRNA gene sequence analysis, fatty acid composition analysis, quinone analysis and genotypic and physiological comparisons. Based on these data, it is proposed that the new isolate represents a novel species in a new genus of the family 'Flammeovirgaceae' in the phylum *Bacteroidetes*.

The temperature and pH range for growth were determined by incubating on ten-fold diluted marine agar 2216 (MA). Salt tolerance was tested on R2A agar (Difco) with artificial seawater containing 0–5% (w/v) NaCl. Gram staining was performed as described by Murray *et al.* (1994). Cell morphology was observed by 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. In the course of TEM, cells of various sizes were observed. Cells were mostly straight and rod-shaped. Cells varied between 0.3 and 0.5 µm in width and 10

Abbreviation: TEM, transmission electron microscopy.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain Shu-F-UV2-2^T is AB276355.

Fig. 1. Transmission electron micrograph of negatively stained cells of strain Shu-F-UV2-2^T. Bar, 2 µm.

and 20 µm in length (Fig. 1). Cells were motile by gliding and no flagella were seen by electron microscopy. Growth under anaerobic conditions was determined after 2 weeks incubation in an AnaeroPack (Mitsubishi Gas Chemical Co., Inc.) on MA. Catalase activity was determined by bubble formation in a 3% H₂O₂ solution. Oxidase activity was determined by use of cytochrome oxidase paper (Nissui Pharmaceutical Co., Ltd). API 20E, API 50CH and API ZYM strips (bioMérieux) were used to determine physiological and biochemical characteristics. The API 20E results were read after 48 h incubation at 30 °C, and the API 50CH and API ZYM results after 4 h incubation at 37 °C. Determination of the respiratory quinone system and cellular fatty acid content (MIDI system) were carried out as described by Xie & Yokota (2003). DNA was prepared according to the method of Marmur (1961) from cells grown on MA medium and the DNA base composition was determined by using the HPLC method of Mesbah *et al.* (1989). An approximately 1500 bp fragment of the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers specific to the 16S rRNA gene: 27F and 1492R (Weisburg *et al.*, 1991). To ascertain the phylogenetic position of the novel isolate, the 16S rRNA gene sequence of strain Shu-F-UV2-2^T was compared with sequences obtained from GenBank (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>). Multiple alignments of the sequences were performed by using CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997). Alignment gaps and ambiguous bases were not taken into consideration when the 1383 bases of 16S rRNA gene nucleotides were compared. Phylogenetic relationships were analysed by using the same software. Distances were calculated by using the Kimura two-parameter model (Kimura, 1980). Clustering with the neighbour-joining method

(Saitou & Nei, 1987) was determined by using bootstrap values based on 1000 replications (Felsenstein, 1985). Similarity values were calculated via MEGA3 (Kumar *et al.*, 2004).

Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain Shu-F-UV2-2^T belonged to the family ‘*Flammeovirgaceae*’ and showed highest sequence similarity (85.5%) to *Flammeovirga yaeyamensis* NBRC 100898^T (Takahashi *et al.*, 2006), followed by *Flammeovirga arenaria* NBRC 15982^T (84.9%; Takahashi *et al.*, 2006) and *Flammeovirga aprica* NBRC 15941^T (84.7%; Takahashi *et al.*, 2006). All other recognized species of the family ‘*Flammeovirgaceae*’ were more distantly related, showing 16S rRNA gene sequence similarity to strain Shu-F-UV2-2^T of less than 84%. Thus, on the basis of the phylogenetic data presented, strain Shu-F-UV2-2^T should be identified as representing a novel species in a new genus of the family ‘*Flammeovirgaceae*’ within the phylum *Bacteroidetes* (Fig. 2).

The fatty acid profile of strain Shu-F-UV2-2^T differentiated it from recognized species of the genus *Flammeovirga* based on the presence of C_{12:0} 3-OH and iso-C_{15:1} G. In particular, strain Shu-F-UV2-2^T could clearly be distinguished from recognized species of the genus *Flammeovirga* by the absence of C_{20:4} ω6 (arachidonic acid) (Table 1).

Strain Shu-F-UV2-2^T also showed distinct phenotypic features that distinguished it from its closest recognized relatives of the family ‘*Flammeovirgaceae*’: colonies were dull-orange-pigmented and cells were straight rods with a length of 10–20 µm (Fig. 1). Gliding motility was observed via phase-contrast microscopy.

The physiological and biochemical features further supported the phylogenetic results. The major quinone system was menaquinone MK-7. The G + C content of the DNA of strain Shu-F-UV2-2^T was 43.0 mol%, a value different from those for members of the genus *Flammeovirga* (Table 2).

Strain Shu-F-UV2-2^T was strictly aerobic, was isolated from seawater and was able to grow only in artificial seawater medium containing 0–3.5% NaCl. In contrast, its closest recognized relatives within the genus *Flammeovirga* do not require seawater for growth and can tolerate 1–5% NaCl. Furthermore, strain Shu-F-UV2-2^T could also be distinguished based on its highest growth temperature and inability to hydrolyse agar, starch or DNA (Table 2).

Based on the results of phylogenetic analysis and its physiological and biochemical properties, strain Shu-F-UV2-2^T is considered to represent a novel species in a new genus of the family ‘*Flammeovirgaceae*’, phylum *Bacteroidetes*. We propose the name *Perexilibacter aurantiacus* gen. nov., sp. nov. for this organism.

Description of *Perexilibacter* gen. nov.

Perexilibacter [Pe.rex.i.li.bac'ter. L. adj. *perexilis* very slender; N.L. masc. n. *bacter* (equivalent of Gr. neut. n. *baktron*) rod

Fig. 2. Neighbour-joining tree of 16S rRNA gene sequence similarity values showing the phylogenetic position of strain Shu-F-UV2-2^T and representatives of closely related genera of the family ‘*Flammeovirgaceae*’. The tree was rooted by using *Flavobacterium aquatile* ATCC 11947^T as the outgroup. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values >70% are shown. Bar, 1% sequence divergence.

or staff; N.L. masc. n. *Perexilibacter* very slender rod, referring to its cell shape].

Cells are straight and rod-shaped, Gram-negative and strictly aerobic. Motile by gliding. Do not form endospores. Catalase- and oxidase-positive. Nitrate and nitrite reduction are negative. The major respiratory menaquinone is MK-7. The G + C content of the genomic DNA is 43.0 mol%. Predominant cellular fatty acids are iso-C_{15:0}, C_{16:1}ω7c and C_{16:1}ω5c. The genus *Perexilibacter* is a member of the family ‘*Flammeovirgaceae*’, phylum *Bacteroidetes*. The type species is *Perexilibacter aurantiacus*.

Table 1. Fatty acid content of strain Shu-F-UV2-2^T and species of the genus *Flammeovirga*

Values are percentages of the total fatty acid content. Data are from this study and Takahashi *et al.* (2006). –, Not detected.

Fatty acid	Shu-F-UV2-2 ^T	<i>Flammeovirga</i>
C _{12:0} 3-OH	1	–
C _{14:0}	1.9	3.7–12.4
iso-C _{15:1} G	2.9	–
iso-C _{15:0}	50.6	30.5–53.3
C _{16:1} ω7c	16.7	0.5–1.4
C _{16:1} ω5c	4.9	2.3–8.2
C _{16:0}	3.8	0.8–4.8
iso-C _{15:0} 3-OH	2.2	3.9–5.4
iso-C _{17:0} 3-OH	2.4	0.3–2.4
C _{20:4} ω6	–	8.6–24.2

Description of *Perexilibacter aurantiacus* sp. nov.

Perexilibacter aurantiacus (au.ran’ti.a.cus. N.L. masc. adj. *aurantiacus* orange-coloured).

Main characteristics are as given for the genus. In addition, cells are 0.3–0.5 μm in width and 10–20 μm in length. Swarming growth is not observed. Colonies grown on ten-fold diluted marine agar are circular, convex and dull orange-pigmented. The optimum temperature range for growth is 30–37 °C; no growth occurs at 4 or 45 °C. The pH range for growth is 5–10, with an optimum around neutral pH. Seawater is required for growth. NaCl alone does not support growth. Growth occurs in artificial seawater medium containing 0–3.5% NaCl. Gelatin is hydrolysed but agar and starch are not. Negative for the Voges–Proskauer reaction, ONPG, citrate utilization and production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide and indole. Acid is produced from aesculin ferric citrate and 5-keto-gluconate, but not from D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, methyl β-D-xylopyranoside, galactose, glucose, fructose, mannose, rhamnose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, inulin, raffinose, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, glycerol, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, sorbitol, starch, glycogen, xylitol, D-arabitol, L-arabitol, gluconate or 2-keto-gluconate. Positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase, but negative for esterase (C4), esterase lipase (C8),

Table 2. Characteristics that differentiate strain Shu-F-UV2-2^T from related taxa

Taxa: 1, strain Shu-F-UV2-2^T; 2, *Flammeovirga aprica* NBRC 15941^T; 3, *Flammeovirga arenaria* NBRC 15982^T; 4, *Flammeovirga yaeyamensis* NBRC 100898^T; 5, *Flexithrix dorotheae* ATCC 23163^T; 6, *Persicobacter diffluens* ATCC 49458^T. Data are from this and earlier studies (Lewin, 1970; Nakagawa *et al.*, 1997; Takahashi *et al.*, 2006). +, Positive; w, weakly positive; v, variable; –, negative; ND, no data.

Characteristic	1	2	3	4	5	6
Isolation source	Seawater	Marine	Marine	Marine	Seashore silt	Marine
Colony colour	Dull orange	Orange to reddish orange	Orange to reddish orange	Orange to reddish orange	Bright golden yellow	Pink to orange
Cell morphology	Rods	Long rods	Long rods	Long rods	Rods	Rods
Cell length (µm)	10–20	1.7–96	2–40	1.7–90	5–15	4–30
Cell width (µm)	0.3–0.5	0.5–0.9	0.5–0.9	0.4–0.9	0.7	0.5
Catalase	w	+	–	v	–	–
Hydrolysis of:						
Agar	–	+	+	+	–	+
Starch	–	+	+	+	–	+
DNA	–	+	–	+	ND	ND
Highest growth temperature (°C)	43	30	30	35	35	30
Growth in NaCl alone	–	+	+	+	+	ND
NaCl range for growth (% w/v)	0–3.5	1–5	1–5	1–5	1–6	ND
DNA G+C content (mol%)	43	34.2	31.8	33.4–35.7	37	40–42

lipase (C4), cystine arylamidase, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Major fatty acid components (>2.0% of the total) are iso-C_{15:0} (50.6%), C_{16:1} ω 7c (16.7%), C_{16:1} ω 5c (4.9%), C_{16:0} (3.8%), iso-C_{15:1} G (2.9%), iso-C_{17:0} 3-OH (2.4%), iso-C_{15:0} 3-OH (2.2%) and C_{16:0} 3-OH (2.2%).

The type strain, Shu-F-UV2-2^T (=MBIC06993^T=IAM 15413^T=KCTC 12867^T), was isolated from sediment from Carp Island, Republic of Palau.

Acknowledgements

We thank Atsuko Katsuta, Chiaki Komukai, Ayako Matsuzaki, Tomomi Haga and Yukiko Itazawa for technical assistance. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO).

References

- Bowman, J. P., McCammon, S. A., Brown, M. V., Nichols, D. S. & McMeekin, T. A. (1997). Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Appl Environ Microbiol* **63**, 3068–3078.
- Cottrell, M. T. & Kirchman, D. L. (2000). Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. *Appl Environ Microbiol* **66**, 5116–5122.
- DeLong, E. F., Franks, D. G. & Alldredge, A. L. (1993). Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnol Oceanogr* **38**, 924–934.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Glöckner, F. O., Fuchs, B. M. & Amann, R. (1999). Bacterioplankton composition of lakes and oceans: a first comparison based on fluorescence in situ hybridization. *Appl Environ Microbiol* **65**, 3721–3726.

Hudson, J. A., Schofield, K. M., Morgan, H. W. & Daniel, R. M. (1989). *Thermonema lapsum* gen. nov., sp. nov., a thermophilic gliding bacteria. *Int J Syst Bacteriol* **39**, 485–487.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Lewin, R. A. (1970). *Flexithrix dorotheae* gen. et sp. nov. (Flexibacterales); and suggestions for reclassifying sheathed bacteria. *Can J Microbiol* **16**, 511–515.

Ludwig, W. & Klenk, H.-P. (2001). Overview: a phylogenetic backbone and taxonomic framework for prokaryotic systematics. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 49–65. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York: Springer.

Lyman, J. & Fleming, R. H. (1940). Composition of sea water. *J Mar Res* **3**, 134–146.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Murray, R. G. E., Doetsch, R. N. & Robinow, C. F. (1994). Determinative and cytological light microscopy. In *Methods for General and Molecular Bacteriology*, pp. 21–41. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Nakagawa, Y., Hamana, K., Sakane, T. & Yamasato, K. (1997). Reclassification of *Cytophaga aprica* (Lewin 1969) Reichenbach 1989 in *Flammeovirga* gen. nov. as *Flammeovirga aprica* comb. nov. and of

Cytophaga diffuens (ex Stanier 1940; emend. Lewin 1969) Reichenbach 1989 in *Persicobacter* gen. nov. as *Persicobacter diffuens* comb. nov. *Int J Syst Bacteriol* **47**, 220–223.

Nedashkovskaya, O. I., Suzuki, M., Vysotskii, M. V. & Mikhailov, V. V. (2003). *Reichenbachia agariperforans* gen. nov., sp. nov., a novel marine bacterium in the phylum *Cytophaga–Flavobacterium–Bacteroides*. *Int J Syst Evol Microbiol* **53**, 81–85.

O'Sullivan, L. A., Weightman, A. J. & Fry, J. C. (2002). New degenerate *Cytophaga–Flexibacter–Bacteroides*-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in River Taff epilithon. *Appl Environ Microbiol* **68**, 201–210.

O'Sullivan, L. A., Fuller, K. E., Thomas, E. M., Turley, C. M., Fry, J. C. & Weightman, A. J. (2004). Distribution and culturability of the uncultivated 'AGG58 cluster' of the Bacteroidetes phylum in aquatic environments. *FEMS Microbiol Ecol* **47**, 359–370.

Perry, L. B. (1973). Gliding motility in some non-spreading flexi-bacteria. *J Appl Bacteriol* **36**, 227–232.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Takahashi, M., Suzuki, K. & Nakagawa, Y. (2006). Emendation of the genus *Flammeovirga* and *Flammeovirga aprica* with the proposal of *Flammeovirga arenaria* nom. rev., comb. nov. and *Flammeovirga yaeyamensis* sp. nov. *Int J Syst Evol Microbiol* **56**, 2095–2100.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

Xie, C. & Yokota, A. (2003). Phylogenetic analysis of *Lampropedia hyalina* based on the 16S rRNA gene sequence. *J Gen Appl Microbiol* **49**, 345–349.