Emendation of the genus *Flammeovirga* and *Flammeovirga aprica* with the proposal of *Flammeovirga arenaria* nom. rev., comb. nov. and *Flammeovirga yaeyamensis* sp. nov.

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The taxonomic positions of five bacterial strains isolated from the Yaeyama Islands of Japan and '*Microscilla arenaria*' NBRC 15982 were determined using a polyphasic taxonomic approach. 16S rRNA gene sequence analyses placed all of the strains close to the genus *Flammeovirga*. DNA–DNA hybridization studies, biochemical and physiological characterizations and chemotaxonomic analyses suggested that '*M. arenaria*' NBRC 15982 and the five novel isolates represented two separate species of the genus *Flammeovirga*. Emendation of the genus *Flammeovirga* Nakagawa *et al.* 1997 and the species *Flammeovirga aprica* (Reichenbach 1989) Nakagawa *et al.* 1997 is proposed. In addition, '*Microscilla arenaria*' Lewin 1969 is proposed as *Flammeovirga arenaria* nom. rev., comb. nov. (with the type strain NBRC 15982^T = CIP 109101^T) and the novel isolates are proposed as *Flammeovirga yaeyamensis* sp. nov. (type strain IR25-3^T = NBRC 100898^T = CIP 109099^T).

The genus *Flammeovirga*, a member of the phylum *Bacteroidetes*, was created to accommodate the misclassified species [*Cytophaga*] *aprica* (Nakagawa *et al.*, 1997). It currently consists of a single species, *Flammeovirga aprica*, a Gram-negative, aerobic, heterotrophic, gliding, orange-pigmented marine bacterium possessing menaquinone-7 (MK-7). It has been reported that the genus *Microscilla* is very heterogeneous and that '*Microscilla arenaria*' Lewin 1969 is also related to the genus *Flammeovirga* (Nakagawa *et al.*, 2002), however, to date, this species name has not been validly published.

During a survey of microbial communities within subtropical zones of Japan (Nakagawa, 2004), five strains, IR25-3^T, IR27-3, IR28-1, IS22-1 and IS26-1, were isolated from seaweeds, coastal sands and dead leaves. These samples were collected along the seashores of Iriomote and Ishigaki Islands in October 2001 (see Supplementary Table S1 in IJSEM Online). These islands are located at 24° 20' N 123° 45' E and 24° 20' N 124° 9' E, respectively and belong to the Yaeyama Island group. In this paper, we describe taxonomic

studies on the five novel strains and '*M. arenaria*' NBRC 15982.

Genomic DNA extraction and 16S rRNA gene sequencing were conducted according to previously published procedures (Nakagawa *et al.*, 2002). Sequence data obtained were aligned with those of representative members of the phylum *Bacteroidetes* by using CLUSTAL_X (Thompson *et al.*, 1997) and then manually aligned following the 16S rRNA secondary structure of *Escherichia coli* (Gutell *et al.*, 1994) by using the Se-Al v2.0 alignment editor (available at <http://evolve.zoo.ox.ac.uk/>). A phylogenetic tree was constructed on the basis of the neighbour-joining method (Saitou & Nei, 1987) and K_{nuc} values (Kimura, 1980) (Fig. 1). The topology of the tree was evaluated by bootstrap analysis (Felsenstein, 1985) using 1000 replicates. The almost-complete 16S rRNA gene sequences, ranging from *E. coli* equivalent positions (Brosius *et al.*, 1978) 28 to 1494, were determined for the five novel isolates and for *F. aprica* NBRC 15941^T. All of the novel isolates showed identical 16S rRNA gene sequences. Phylogenetic analysis of strain IR25-3^T and '*M. arenaria*' NBRC 15982 revealed that they were closely related to the genus *Flammeovirga*. 16S rRNA gene sequence similarities between *F. aprica* NBRC 15941^T and strain IR25-3^T and '*M. arenaria*' NBRC 15982 were 95.2 and 98.1 %, respectively. Gene sequence similarity between strain IR25-3^T and '*M. arenaria*' NBRC 15982 was 95.9 %.

DNA–DNA hybridizations were performed with photo-biotin-labelled probes in microplate wells as described by

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains NBRC 15941^T, IR25-3^T, IR27-3, IR28-1, IS22-1 and IS26-1 are AB247553–AB247558, respectively.

Tables detailing the sources of the isolates, their cellular fatty acid contents and variable phenotypic characteristics between isolates are available as supplementary material in IJSEM Online.

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of '*M. arenaria*' NBRC 15982 and strain IR25-3^T based on 16S rRNA gene sequences. Bootstrap values greater than 700 in 1000 replicates are shown. Bar, 0.02 K_{nuc} .

Ezaki *et al.* (1989). The hybridization temperature was 48.7 °C in 2 × SSC buffer containing 25 % formamide. The five novel isolates constituted a single species as their DNA–DNA relatedness values ranged from 73 to 110 % when IR25-3^T was used as the probe. Strain IR25-3^T exhibited 12–16 % DNA–DNA relatedness with *F. aprica* NBRC 15941^T and 18–27 % relatedness with '*M. arenaria*' NBRC 15982. The DNA–DNA binding value between *F. aprica* NBRC 15941^T and '*M. arenaria*' NBRC 15982 was 11–21 %. These results indicate that *F. aprica* NBRC 15941^T, '*M. arenaria*' NBRC 15982 and the five novel isolates constitute three separate species.

The G + C content of DNA was determined according to Mesbah *et al.* (1989). The DNA G + C contents of the novel isolates were 33.4–35.7 mol%. The value for '*M. arenaria*' NBRC 15982 was 31.8 mol%. These values were similar to the value of 34.2 mol% obtained in this study for *F. aprica* NBRC 15941^T. To determine whole-cell fatty acid content, all strains were grown on Bacto marine agar 2216 (Difco) at 25 °C for 24 h. Fatty acid methyl esters were prepared and analysed according to the standard protocol of the Microbial Identification System (Microbial ID). *F. aprica* NBRC 15941^T, '*M. arenaria*' NBRC 15982 and the novel isolates had similar fatty acid contents in which the dominant fatty acids were iso-C15:0, C20:4ω6,9,12,15c and C16:0 3-OH

(see Supplementary Table S2 in IJSEM Online). The presence of arachidonic acid (C20:4ω6,9,12,15c) was confirmed by using a GC-MS (5973 Network MSD; Agilent) with commercially available standards. Respiratory quinones were extracted according to Nakagawa & Yamasato (1993) and determined by using a LC-MS (8000x; Shimadzu). MK-7 was found to be the major respiratory quinone in all strains. Using the bathochromic shift test with 20 % (w/v) KOH (Fautz & Reichenbach, 1980), no flexirubin-type pigments were detected in any of the strains. Carotenoids were extracted as described by Schmidt *et al.* (1994) and their light absorption spectra were investigated by using a UV-visible spectrophotometer (UV-1650PC; Shimadzu). It has been previously reported that *F. aprica* NBRC 15941^T and '*M. arenaria*' NBRC 15982 contained sproxanthin (Reichenbach, 1989a, b). In this study, the carotenoids of the novel isolates were identified as sproxanthin as they showed identical spectra to those of *F. aprica* NBRC 15941^T and '*M. arenaria*' NBRC 15982. Cellular polyamines were prepared and analysed according to Hamana *et al.* (1995). In addition to cadaverine, spermidine and agmatine were detected for the novel isolates when they were cultivated on Bacto marine agar. It has been previously reported that putrescine, cadaverine, spermidine and agmatine are present in '*M. arenaria*' NBRC 15982 (Hosoya & Hamana, 2003).

Gliding motility was determined as described by Perry (1973). Anaerobic growth was examined by using the Anaeropack system (Mitsubishi Gas Chemical). The ability of strains to degrade agar, alginic acid, casein, cellulose, carboxymethylcellulose, chitin, DNA, inulin, aesculin, gelatin, starch, Tween 80, tyrosine and urea was investigated according to the methods of Lewin & Lounsbury (1969), Smibert & Krieg (1981) and Barrow & Feltham (1993) with media prepared with Daigo's artificial seawater SP (Wako Pure Chemicals). Production of indole, H₂S and the activities of oxidase, urease and nitrate reductase were also examined as described by Smibert & Krieg (1981). Oxidation or fermentation of glucose (O–F test; Hugh & Leifson, 1953) was investigated by using OF basal media (Eiken Kizai) prepared with artificial seawater. For growth responses at different temperatures (5–45 °C) and pH (3–11), one-fifth strength LBM medium was used (Suzuki *et al.*, 2001) for cultivation. The potential of the strains to metabolize 95 different carbon sources was examined by using GN2 Microplates (Biolog) with the method modified for marine bacteria as described by Rüger & Krambeck (1994). Acid production from various carbon sources was determined by using the API 50CH test (bioMérieux) with cells grown on a medium composed of 50 % CHB/E medium with artificial seawater (Lau *et al.*, 2005). The phenotypic characteristics of *F. aprica* NBRC 15941^T, '*M. arenaria*' NBRC 15982 and the novel isolates are given in the genus and species descriptions and in Table 1 (see also Supplementary Table S3 in IJSEM Online).

According to a phylogenetic analysis of 16S rRNA gene sequences, we have concluded that '*M. arenaria*' NBRC 15982 and the novel isolates should be classified in the genus *Flammeovirga*. In the original description of the genus *Flammeovirga*, it was stated that oxidase activity and degradation of gelatin were positive and that catalase activity was negative. However, we found that '*M. arenaria*' NBRC 15982 did not produce oxidase and *F. aprica* NBRC 15941^T and the novel isolates showed catalase activity. In addition, degradation of gelatin was not detected in *F. aprica* NBRC 15941^T or '*M. arenaria*' NBRC 15982. Thus, we propose the emendation of the genus *Flammeovirga* to include '*Microscilla arenaria*' NBRC 15982 and our novel isolates. *F. aprica* NBRC 15941^T, '*Microscilla arenaria*' NBRC 15982 and the five novel isolates can be distinguished from each other by the phenotypic characteristics summarized in Table 1. These results suggest that '*Microscilla arenaria*' NBRC 15982^T and the novel isolates represent two separate species of the genus *Flammeovirga*. We propose to transfer '*Microscilla arenaria*' to the genus *Flammeovirga* as *Flammeovirga arenaria* nom. rev., comb. nov. Strains IR25-3^T, IR27-3, IR28-1, IS22-1 and IS26-1 are proposed as members of a novel species, *Flammeovirga yaeyamensis* sp. nov. According to the rules of Latin and latinization, the original spelling of *Flammeovirga* should be corrected to *Flammeivirga*, however, the original version of the name, *Flammeovirga*, has been retained according to the new Rule 61 of the Bacteriological Code (Euzéby, personal communication).

Emended description of the genus

Flammeovirga

Flammeovirga (Flam.me.o.vir'ga. L. adj. *flammeus* fire-coloured; L. fem. n. *virga* rod; L. fem. n. *Flammeovirga* fire-coloured rod).

Cells are Gram-negative, aerobic, chemo-organotrophic, asporogenic, long rods that are 0.4–0.9 µm wide and 1.7–96 µm long or longer. Motile by gliding. Cell mass is orange to reddish orange. Oxidase and catalase activities are variable. Major respiratory quinone is MK-7. Saproxanthin is present as the major carotenoid pigment. Flexirubin-type pigments are absent. Predominant cellular fatty acids are iso-C15:0, C20:4ω6,9,12,15c and C16:0 3-OH. Members of the genus are marine organisms that require NaCl or seawater for growth. Growth is supported by NaCl alone. Nitrate is reduced. Agar, alginic acid, aesculin and starch are degraded. The G+C content of DNA is 31–36 mol%. The type species of the genus is *Flammeovirga aprica*.

Emended description of *Flammeovirga aprica*

Flammeovirga aprica (a'pri.ca. L. fem. adj. *aprica* sunlit, sun-loving).

Displays the following properties in addition to those given in the genus description. Cells are long rods, 0.5–0.9 µm wide and 1.7–96 µm long or longer. Colonies spread and produce large gelase fields and deep craters in agar plates. Growth is detected at 15–30 °C, with the optimum growth yield at 25 °C. The pH range for growth is 6–8 with the optimum growth yield at pH 7. Growth occurs at 1–5 % NaCl with the optimum growth yield at 3 %. Oxidase and catalase activities are positive. Urease activity is negative. Agar, alginic acid, carboxymethylcellulose, DNA, aesculin and starch are degraded, but cellulose, chitin, inulin, gelatin, Tween 80 and tyrosine are not degraded. Casein is weakly degraded. H₂S is produced, but indole is not. O–F test of glucose is fermentative. Utilization (Biolog GN2 microplate data) is observed for α-cyclodextrin, dextrin, L-fucose, glycogen, D-galactose, gentiobiose, α-D-glucose, DL-lactic acid, α-D-lactose, D-trehalose, lactulose, maltose, glucose 1-phosphate, cellobiose, D-mannose, monomethyl succinate, glycyl L-glutamic acid and glucose 6-phosphate, but not for D-melibiose, urocanic acid, L-ornithine, L-rhamnose, L-alanyl glycine, N-acetyl-D-glucosamine or L-threonine. Acid production (API 50CH data) is positive for D-xylose, galactose, N-acetylglucosamine, amygdalin, aesculin, salicin, cellobiose, maltose, lactose, starch, glycogen and gentiobiose and weakly positive for ribose, glucose, mannose and L-fucose. Acid is not produced from rhamnose, mannitol, sorbitol, methyl α-D-glucoside, arbutin, melibiose, melezitose, raffinose or xylitol. The major cellular fatty acids are iso-C15:0, C20:4ω6,9,12,15c, C16:0 3-OH and C14:0. The DNA G+C content of the type strain is 34.2 mol%.

The type strain is NBRC 15941^T (= ATCC 23126^T = CIP 104807^T).

Table 1. Phenotypic differentiation of *Flammeovirga* species

Taxa: 1, *Flammeovirga aprica* NBRC 15941^T; 2, *Flammeovirga* ('*Microscilla*') *arenaria* NBRC 15982^T; 3, *Flammeovirga yaeyamensis* sp. nov. (n=5). All strains are negative for the utilization of i-erythritol, *p*-hydroxyphenylacetic acid, bromosuccinic acid, D-fructose, methyl β -D-glucoside, *cis*-aconitic acid, itaconic acid, succinamic acid, hydroxy L-proline, inosine, D-psicose, citric acid, glucuronamide, L-leucine, D-raffinose, formic acid, α -ketoglutaric acid, Tween 40, D-galactonic acid lactone, α -ketovaleric acid, D-alanine, L-phenylalanine, phenylethylamine, Tween 80, D-sorbitol, D-galacturonic acid, putrescine, *N*-acetyl-D-galactosamine, *myo*-inositol, sucrose, D-gluconic acid, malonic acid, L-pyrogutamic acid, 2-aminoethanol, D-glucosaminic acid, D-serine, 2,3-butanediol, adonitol, turanose, D-glucuronic acid, quinic acid, glycerol, L-arabinose, xylitol, α -hydroxybutyric acid, D-saccharic acid, L-glutamic acid, DL- α -glycerol phosphate, D-arabitol, D-mannitol, methyl pyruvate, β -hydroxybutyric acid, sebacic acid, DL-carnitine, γ -hydroxybutyric acid, succinic acid and γ -aminobutyric acid with the Biolog GN2 Microplate. All strains are negative for acid production from glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, adonitol, methyl β -D-xyloside, dulcitol, inositol, inulin, D-turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate and 2-ketogluconate with the API 50CH test. The optimum pH for growth was 7 for all species. +, Positive; –, negative; V, variable results among strains; W, weakly positive; +/W, positive or weakly positive.

Characteristic	1	2	3
Growth:			
Temperature range (°C)	15–30	10–30	15–35
Optimum temperature (°C)	25	25	30
pH	6–8	6–8	6–10
Degradation of:			
Carboxymethylcellulose	+	–	+
Casein	W	W	–
Gelatin	–	–	+
Inulin	–	–	+
Tween 80	–	–	+
DNase	+	–	+
Oxidase	+	–	+ /W
Catalase	+	–	V
O–F test (glucose)	Fermentation	Oxidation	Fermentation
Assimilation of:			
<i>N</i> -Acetyl-D-glucosamine	–	+	+
L-Alanyl glycine	–	W	+ /W
Glucose 6-phosphate	W	+	–
Glucose 1-phosphate	+	+	–
D-Melibiose	–	+	+
Monomethyl succinate	W	–	–
L-Ornithine	–	W	+ /W
L-Rhamnose	–	–	+
L-Threonine	–	W	+ /W
Urocanic acid	–	–	+
Acid production from:			
Arbutin	–	+	+
Mannitol	–	W	–
Melezitose	–	W	–
Melibiose	–	+	+
Methyl α -D-glucoside	–	W	W
Raffinose	–	W	+ /W
Rhamnose	–	–	+
Ribose	W	W	–
Sorbitol	–	W	–
Xylitol	–	W	+ /W
D-Xylose	+	–	+
DNA G + C content (mol%)	34.2	31.8	33.4–35.7

Description of *Flammeovirga arenaria* nom. rev., comb. nov.

Synonym: '*Microscilla arenaria*' Lewin 1969

Flammeovirga arenaria (a.re.na'ria. L. fem. adj. *arenarius* pertaining to sand, referring to the source of the organism).

Displays the following properties in addition to those given in the genus description. Cells are long rods, 0.5–0.9 µm wide and 2.0–40 µm long or longer. Cell mass is orange. Colonies spread and produce large gelase fields and deep craters in agar plates. Growth is detected at 10–30 °C with the optimum growth yield at 25 °C. The pH range for growth is 6–8 with the optimum growth yield at pH 7. Growth occurs at 1–5 % NaCl with the optimum growth yield at 3 %. Oxidase, catalase and urease activities are negative. Agar, alginic acid, aesculin and starch are degraded, but carboxymethylcellulose, cellulose, chitin, DNA, inulin, gelatin, Tween 80 and tyrosine are not degraded. Casein is weakly degraded. H₂S is produced, but indole is not. O–F test of glucose is oxidative. Utilization (Biolog GN2 microplate data) is observed for D-melibiose, α-cyclodextrin, dextrin, L-fucose, glycogen, D-galactose, gentiobiose, α-D-glucose, N-acetyl-D-glucosamine, α-D-lactose, lactulose, maltose, L-glutamic acid, glycyl L-aspartic acid, glucose 1-phosphate, cellobiose, D-mannose, glycyl L-glutamic acid and glucose 6-phosphate. Weakly positive for utilization of alaninamide, L-ornithine, DL-lactic acid, L-alanine, L-alanyl glycine, L-aspartic acid and L-threonine, but negative for urocanic acid, L-rhamnose and monomethyl succinate. Acid production (API 50CH data) is positive for galactose, glucose, mannose, N-acetylglucosamine, amygdalin, arbutin, aesculin, cellobiose, maltose, lactose, melibiose, trehalose, starch, glycogen, gentiobiose and L-fucose and weakly positive for ribose, fructose, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, salicin, sucrose, melezitose, raffinose, xylitol and 5-ketogluconate. Acid is not produced from D-xylose or rhamnose. The major cellular fatty acids are iso-C15:0, C14:0, C16:0 3-OH, C20:4ω6,9,12,15c, C16:1ω5c and iso-C15:0 3-OH. The DNA G+C content of the type strain is 31.8 mol%.

The type strain is NBRC 15982^T (= CIP 109101^T).

Description of *Flammeovirga yaeyamensis* sp. nov.

Flammeovirga yaeyamensis (ya.e.ya.men'sis. N.L. fem. adj. *yaeyamensis* referring to the Yaeyama Islands, from where the organisms were isolated).

Displays the following properties in addition to those given in the genus description. Cells are long rods, 0.4–0.9 µm wide and 1.7–90 µm long or longer. Cell mass is orange. Colonies spread and produce large gelase fields and deep craters in agar plates. Growth is detected at 15–35 °C with the optimum growth yield at 30 °C. The pH range for growth is 6–10 with the optimum growth yield at pH 7. Growth occurs at 1–5 % NaCl with the optimum growth

yield at 3 %. Oxidase activity is positive. Catalase activity is variable. Urease activity is negative. Agar, alginic acid, carboxymethylcellulose, DNA, aesculin, inulin, gelatin, starch and Tween 80 are degraded, but casein, cellulose, chitin and tyrosine are not degraded. H₂S is produced, but indole is not. O–F test of glucose is fermentative. Utilization (Biolog GN2 microplate data) is observed for D-melibiose, urocanic acid, D-galactose, gentiobiose, L-rhamnose, N-acetyl-D-glucosamine, cellobiose and glycyl L-glutamic acid. Positive or weakly positive for L-ornithine, α-D-glucose, L-alanyl glycine, maltose and L-threonine, but negative for glucose 1-phosphate, monomethyl succinate and glucose 6-phosphate. Acid production (API 50CH data) is positive for D-xylose, galactose, glucose, mannose, rhamnose, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, starch, glycogen and gentiobiose. Positive or weakly positive for methyl α-D-glucoside, raffinose, xylitol and L-fucose. Acid is not produced from ribose, mannitol, sorbitol or melezitose. The major cellular fatty acids are iso-C15:0, C20:4ω6,9,12,15c, C16:0 3-OH, iso-C15:0 3-OH, C16:0 and C14:0. The G+C content of DNA is 33.4–35.7 mol%.

The type strain is IR25-3^T (=NBRC 100898^T=CIP 109099^T). Four other strains, IR27-3 (=NBRC 100899), IR28-1 (=NBRC 100900), IS22-1 (=NBRC 100901) and IS26-1 (=NBRC 100902), are available as reference strains.

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