Limibacter armeniacum gen. nov., sp. nov., a novel representative of the family 'Flammeovirgaceae' isolated from marine sediment

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Two strictly aerobic, Gram-negative, gliding, apricot- to pale-orange-pigmented, rod-shaped bacteria, strains YM11-185^T and YM11-159, were isolated from marine sediments in the Republic of Palau. Preliminary analysis based on the 16S rRNA gene sequence revealed that the novel isolates could be affiliated with the family 'Flammeovirgaceae' of the phylum *Bacteroidetes*. Strains YM11-185^T and YM11-159 shared 100% sequence similarity and showed less than 90% similarity to cultivated representatives of the family 'Flammeovirgaceae'. The novel isolates are phenotypically and physiologically different from strains described previously. The G+C content of DNA was 27.8–27.9 mol%, MK-7 was the major menaquinone and iso-C_{15:0} and C_{16:1ω7c} were the major fatty acids. On the basis of polyphasic taxonomic studies, it was concluded that strains YM11-185^T and YM11-159 represent a novel species in a new genus of the family 'Flammeovirgaceae', for which the name *Limibacter armeniacum* gen. nov., sp. nov. is proposed. The type strain of *Limibacter armeniacum* is YM11-185^T (=MBIC08286^T =KCTC 22132^T).

Members of the phylum *Bacteroidetes* (Ludwig & Klenk, 2001), also known as the *Cytophaga*–*Flavobacterium*–*Bacteroides* group, are ubiquitous in aquatic and marine environments (DeLong *et al.*, 1993; Bowman *et al.*, 1997; Pinhassi *et al.*, 1997; Glöckner *et al.*, 1999; Cottrell & Kirchman, 2000; O'Sullivan *et al.*, 2002). According to several past reports, they play significant roles as decomposers of organic matter in nature (Cottrell & Kirchman, 2000; Nedashkovskaya *et al.*, 2003). However, the majority of representatives of the phylum *Bacteroidetes* remain uncultivated (O'Sullivan *et al.*, 2004). At the time of writing, the family 'Flammeovirgaceae' within the phylum *Bacteroidetes* incorporates the genera *Flammeovirga*, *Flexithrix*, *Perexilibacter*, *Persicobacter*, *Rapidithrix* and *Thermonema* (Lewin, 1970; Hudson *et al.*, 1989; Nakagawa *et al.*, 1997; Takahashi *et al.*, 2006; Yoon *et al.*, 2007; Srisukchayakul *et al.*, 2007).

Strains YM11-185^T and YM11-159 were isolated from sediment samples respectively collected from the shore of Carp Island (GPS location 07° 05' 22.9" N 134° 16' 46.4" E) and Jellyfish marine lake (GPS location 07° 09' 41.2" N 134° 22' 30.5" E) in the Republic of Palau in March 2004.

The GenBank/EMBL/DBJ accession number for 16S rRNA gene sequence of strain YM11-185^T is AB359907.

The composition of medium AnsV is detailed in a supplementary table with the online version of this paper.

Each sample (0.5–1 cm³) was homogenized with a glass rod in 5 ml sterile seawater. A 50 µl sample of the homogenate was applied to the surface of AnsV agar medium (the composition of medium AnsV is provided in Supplementary Table S1 in IJSEM Online). Strains YM11-185^T and YM11-159 appeared after incubation for 30 days at 25 °C. The apricot- to pale-orange-pigmented colonies were purified on marine 2216 broth (Difco) containing 1.5% agar after being cultivated for 7–10 days.

In the present study, we attempted to elucidate the phylogenetic position of strains YM11-185^T and YM11-159 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 isolates represent a novel genus of the family 'Flammeovirgaceae' in the phylum *Bacteroidetes*.

The temperature and pH range for growth were determined utilizing 1/10-strength marine 2216 agar (MA; Difco). Salt tolerance was tested on 1/2-strength R2A agar (Difco) 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). Gliding motility was determined as described by Perry (1973). Cells of strains YM11-185^T and YM11-159 were mostly flexible and straight rod-shaped. Cells of both

Fig. 1. Phase-contrast micrograph of cells of strain YM11-185^T. Bar, 10 μm.

strains were 0.3–0.5 μm wide and 15–40 μm long (Fig. 1). Gliding motility was observed. Flagella were not seen by electron microscopy. Growth under anaerobic conditions was determined after 2 weeks of incubation in an AnaeroPack (Mitsubishi Gas Chemical Co.) on 1/10-strength MA. Catalase activity was determined by bubble formation in a 3 % H₂O₂ solution. Oxidase activity was determined by cytochrome oxidase paper (Nissui Pharmaceutical Co.). API 20E, API 50CH and API ZYM strips (bioMérieux) were used to determine physiological and biochemical characteristics. The API 20E and API 50CH tests were read after 72 h incubation at 30 °C and API ZYM tests were read after 4 h incubation at 37 °C. The substrate oxidation ranges of strains YM11-185^T and YM11-159 were determined using Biolog MicroPlates. Determination of the respiratory quinone system was carried out as described previously (Xie & Yokota, 2003). GC analysis of cellular fatty acid methyl esters was performed using a culture grown on 1/10-strength MA at 30 °C for 48 h according to the instructions of the Microbial Identification System (MIDI; Microbial ID). DNA was prepared according to the method of Marmur

(1961) from cells grown on 1/10-strength MA 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 27F and 1492R specific to the 16S rRNA gene (*Escherichia coli* numbering system; Weisburg *et al.*, 1991). To ascertain the phylogenetic position of the new isolates, the 16S rRNA gene sequences of strains YM11-185^T and YM11-159 were compared with sequences obtained from GenBank (National Center for Biotechnology Information; <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 1340 bases of the 16S rRNA gene sequence were compared. Aligned sequences were analysed by the MEGA3.1 software (Kumar *et al.*, 2004). Evolutionary distances [distance options according to the Kimura two-parameter model (Kimura, 1983)] and clustering with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods were determined by using bootstrap values based on 1000 replications (Felsenstein 1985). Similarity values were calculated using the same software.

Comparative analysis of the 16S rRNA gene sequences revealed that strains YM11-185^T and YM11-159 were affiliated phylogenetically with the family ‘*Flammeovirgaceae*’ within the phylum *Bacteroidetes* with bootstrap confidence value of 100 % in both the neighbour-joining (Fig. 2) and maximum-parsimony (not shown) methods. Analysis of the 16S rRNA gene sequences also indicated that the sequences of strains YM11-185^T and YM11-159 were identical and that no other sequence showed more than 90 % similarity; related sequences were those of *Flammeovirga arenaria* NBRC 15982^T (84.9 %; Takahashi *et al.*, 2006), *Rapidithrix thailandica* TISTR 1750^T (84.5 %; Srisukchayakul *et al.*, 2007), *Perexilibacter aurantiacus* MBIC06993^T (84.4 %; Yoon *et al.*, 2007), *Flexithrix dorotheae* NBRC 15987^T (84.2 %; Lewin, 1970) and *Flammeovirga kamogawensis* NCIMB 14281^T (84.2 %; Hosoya & Yokota, 2007). All other species of the family ‘*Flammeovirgaceae*’ with currently published names were

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequence similarity, showing the phylogenetic position of strains YM11-185^T and YM11-159 and representatives of closely related and other more distantly related genera of the family ‘*Flammeovirgaceae*’. The tree was rooted using *Flavobacterium aquatile* ATCC 11947^T as the outgroup. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees. Bar, 2 % sequence divergence.

related more distantly, showing less than 84.0% 16S rRNA gene sequence similarity. Thus, on the basis of phylogenetic data presented, strains YM11-185^T and YM11-159 should be classified within a novel genus and species of the family 'Flammeovirgaceae' within the phylum Bacteroidetes.

As shown in Table 1, the predominant cellular fatty acids of strains YM11-185^T and YM11-159 differentiate them from recognized species of the genera *Flammeovirga*, *Perexilibacter* and *Rapidithrix* based on the proportions of iso-C_{15:0} (26.9–28.1%) and C_{16:1}ω7c (16.8–23.2%). Furthermore, these strains could also be distinguished from the neighbouring taxa by the presence of C_{16:1}ω7c alcohol (2.3–4.2%) and iso-C_{17:1}ω9c (1.2–1.6%). Additionally, the two novel isolates showed distinct phenotypic features that distinguished them from the closest described relatives (Table 2).

Based on the results of the phylogenetic analysis and their biochemical and physiological properties, the two novel strains YM11-185^T and YM11-159 isolated from marine sediments in the Republic of Palau should be considered as members of a novel independent genus and species of the family 'Flammeovirgaceae' within the phylum Bacteroidetes. We propose the name *Limibacter armeniacum* gen. nov., sp. nov. for these organisms.

Description of *Limibacter* gen. nov.

Limibacter [Li.mi.bac'ter. L. adj. *limus* mud; N.L. masc. n. *bacter* (equivalent of Gr. neut. n. *baktron*) rod or staff; N.L.

Table 1. Fatty acid contents of members of the genus *Limibacter* gen. nov. (strains YM11-185^T and YM11-159) and related taxa of the family 'Flammeovirgaceae'

Taxa: 1, strain YM11-185^T; 2, strain YM11-159; 3, *Flammeovirga* species (data from Takahashi *et al.*, 2006; Hosoya & Yokota, 2007); 4, *Perexilibacter aurantiacus* MBIC06993^T (Yoon *et al.*, 2007); 5, *Rapidithrix thailandica* TISTR 1750^T (Srisukchayakul *et al.*, 2007). Values are percentages of total fatty acid content. —, Not detected; tr, trace.

Fatty acid	1	2	3	4	5
C _{13:1} at 12–13	1.6	1	0.8–2.9	1.6	—
C _{14:0}	1.1	1	3.7–9	1.9	1.2
iso-C _{15:0}	28.1	26.9	21.2–54.3	50.6	6.3
C _{15:0}	1.7	tr	tr–7.1	1.4	tr
C _{16:1} ω7c alcohol	2.3	4.2	—	—	—
iso-C _{16:1} H	2.2	3.1	tr–3.2	—	—
C _{16:0} N alcohol	3.2	10	tr–1.8	—	—
C _{16:1} ω7c	23.2	16.8	—	16.7	1.5
C _{16:1} ω5c	9.9	7.9	1.8–4.7	4.9	44.8
C _{16:0}	3.5	4.4	2.8–4.8	3.76	4
iso-C _{15:0} 3-OH	3.7	2.4	3.9–5.5	2.2	4.3
iso-C _{17:1} ω9c	1.6	1.2	—	—	—
C _{17:1} ω6c	1	tr	—	tr	—
C _{16:0} 3-OH	4.9	2.8	4.1–14.6	2.2	3.8
iso-C _{17:0} 3-OH	4.5	3.5	2–2.4	2.4	10.8

masc. n. *Limibacter* rod from mud, referring to the isolation source of the first strains].

Cells are rod-shaped, Gram-negative and strictly aerobic. Motile by gliding. Do not form endospores. Catalase- and oxidase-positive. Nitrate and nitrite are not reduced. The major respiratory menaquinone is MK-7. The G+C content of the genomic DNA of the type strain of the type species is 27.9 mol%. Predominant cellular fatty acids are iso-C_{15:0} and C_{16:1}ω7c. The type species is *Limibacter armeniacum*.

Description of *Limibacter armeniacum* sp. nov.

Limibacter armeniacum [ar.me'ni.a.cum. L. neut. n. *armeniacum* (nominative in apposition) an apricot, a rod called the apricot, intended to mean apricot-coloured].

Main characteristics are the same as those given for the genus. In addition, cells are long rods, 0.3–0.5 μm wide and 15–40 μm long. Colonies grown on 1/10-strength MA are circular, convex and apricot- to pale-orange-pigmented. The temperature range for growth is 17–37 °C; optimal growth occurs at 27–30 °C and no growth occurs at 4 or 45 °C. The pH range for growth is 7–9, with an optimum around neutral pH. NaCl is required for growth and can be tolerated up to 6.5% (w/v). Seawater is not required for growth. Gelatin is hydrolysed but agar and starch are not. Positive for *o*-nitrophenyl β-D-galactosidase (ONPG) and production of tryptophan deaminase. Negative for the Voges–Proskauer reaction, citrate utilization and production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide and indole. Acid is produced from galactose, glucose, aesculin ferric citrate, melibiose, sucrose, trehalose, turanose, D-lyxose, D-tagatose and 5-ketogluconate, but not from D- or L-arabinose, ribose, D- or L-xylose, methyl β-D-xylopyranoside, fructose, mannose, rhamnose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melezitose, inulin, raffinose, gentiobiose, D- or L-fucose, glycerol, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, sorbitol, starch, glycogen, xylitol, D- or L-arabitol, gluconate or 2-ketogluconate. Alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase tests are positive, but esterase (C4), esterase lipase (C8), lipase (C4), cystine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase tests are negative. Cyclodextrin, dextrin, glycogen, N-acetyl-D-glucosamine, L-arabinose, cellobiose, D-galactose, gentiobiose, maltose, D-lactose, D-mannose and trehalose are oxidized, but D-fructose, L-fucose, D-glucose, D-mannitol, melibiose, D-sorbitol, acetic acid, *cis*-aconitic acid, α-ketobutyric acid, α-ketoglutaric acid, inosine, glucose 1-phosphate, Tween 40, Tween 80, N-acetyl-D-galactosamine, adonitol, D-arabitol, i-erythritol, myo-inositol, lactulose, methyl β-D-glucoside, D-psicose,

Table 2. Characteristics that differentiate members of the genus *Limibacter* gen. nov. (strains YM11-185^T and YM11-159) from related taxa of the family 'Flammeovirgaceae'

Taxa: 1, strain YM11-185^T; 2, strain YM11-159; 3, *Flammeovirga aprica* NBRC 15941^T (data from Takahashi *et al.*, 2006); 4, *Flammeovirga arenaria* NBRC 15982^T (Takahashi *et al.*, 2006); 5, *Flammeovirga yaeyamensis* NBRC 100898^T (Takahashi *et al.*, 2006); 6, *Flammeovirga kamogawensis* NCIMB 14281^T (Hosoya & Yokota, 2007); 7, *Perexilibacter aurantiacus* MBIC06993^T (Yoon *et al.*, 2007); 8, *Rapidithrix thailandica* TISTR 1750^T (Srisukchayakul *et al.*, 2007); 9, *Flexithrix dorotheae* ATCC 23163^T (Lewin, 1970); 10, *Persicobacter diffluens* (Nakagawa *et al.*, 1997). +, Positive; w, weakly positive; v, variable; –, negative; ND, no data.

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

raffinose, L-rhamnose, sucrose, furanose, xylitol, methyl pyruvate, monomethyl succinate, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α -, β - and γ -hydroxybutyric acids, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, alaninamide, D- and L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D- and L-serine, L-threonine, DL-carnitine, γ -aminobutyric acid, urocanic acid, uridine, thymidine, phenylethylamine, putrescine, 2-amino-ethanol, 2,3-butanediol, glycerol, DL- α -glycerol phosphate and D-glucose 6-phosphate are not oxidized. Major fatty acid components (>1.0%) include C_{13:1} at 12–13 (1.6%), C_{14:0} (1.1%), iso-C_{15:0} (28.1%), C_{15:0} (1.7%), C_{16:1} ω 7c alcohol (2.3%), iso-C_{16:1} H (2.2%), C_{16:0} N

alcohol (3.2%), C_{16:1} ω 7c (23.2%), C_{16:1} ω 5c (9.9%), C_{16:0} (3.5%), iso-C_{15:0} 3-OH (3.7%), iso-C_{17:1} ω 9c (1.6%), C_{17:1} ω 6c (1.0%), C_{16:0} 3-OH (4.9%) and iso-C_{17:0} 3-OH (4.5%).

The type strain is YM11-185^T (=MBIC08286^T =KCTC 22132^T), which was isolated from sediment samples collected from the shore of Carp Island, Republic of Palau.

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