Carboxylicivirga linearis sp. nov., isolated from a sea cucumber culture pond

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A yellow-pigmented, Gram-stain-negative and facultatively anaerobic bacterium, designated FB218^T, was isolated from a sediment sample collected from a sea cucumber culture pond in Rongcheng, China (36° 54′ 36″ N 122° 14′ 34″ E). Cells of strain FB218^T were slender, gliding, catalase-positive and oxidase-negative. Optimal growth occurred at 30 °C, pH 6.5–7.0 and in medium containing 2–3 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain FB218^T belonged to the genus *Carboxylicivirga*, family *Marinilabiliaceae*. The predominant fatty acids were iso-$C_{15:0}$ and anteiso-$C_{15:0}$. MK-7 was the main respiratory quinone. The major polar lipids of strain FB218^T were two unidentified lipids and a phospholipid. The genomic DNA G+C content was 40.0 mol%. Based on the distinct phylogenetic position and the combination of physiological and phenotypic characteristics, strain FB218^T represents a novel species of the genus *Carboxylicivirga*, for which the name *Carboxylicivirga linearis* sp. nov. is proposed. The type strain is FB218^T (=KCTC 42254^T= MCCC 1H00106^T). An emended description of the genus *Carboxylicivirga* is also provided.

In recent years, many novel species of the family *Marinilabiliaceae* have been described, which includes, at the time of writing, 10 genera. Seven of these genera, *Alkaliflexus*, *Alkalitalea*, *Anaerophaga*, *Mangroviflexus*, *Marinilabilia*, *Natronoflexus* and *Thermophagus*, are anaerobic (Zhilina *et al.*, 2004; Zhao *et al.*, 2012; Denger *et al.*, 2002; Suzuki, 2011; Sorokin *et al.*, 2011; Gao *et al.*, 2013); the other three, *Geofilum*, *Saccharicrinis* and *Carboxylicivirga*, described more recently, are facultatively anaerobic. The genus *Geofilum* was created by Miyazaki *et al.* (2012) following the description of *Geofilum rubicundum*; the genus *Saccharicrinis* was ascertained by reclassification of [*Cytophaga*] *fermentans* as *Saccharicrinis fermentans* (Yang *et al.*, 2014); and the genus *Carboxylicivirga* was created by Yang *et al.* (2014) following description of two novel species, *Carboxylicivirga mesophila* and *Carboxylicivirga taeanensis*. Bacterial cells of the genus *Carboxylicivirga* are facultatively anaerobic, rod-shaped, Gram-stain- and oxidase-negative and catalase-positive. The major respiratory quinone of the genus *Carboxylicivirga* is MK-7. Members of the genus *Carboxylicivirga* can utilize carboxylic acids as metabolic substrates. Here, we describe the phenotypic and genotypic characterization of strain FB218^T, and on the basis of a taxonomic study using a polyphasic approach, we propose that this strain should be included in the genus *Carboxylicivirga* as a representative of a novel species.

Strain FB218^T was isolated from a sample of sediment from a sea cucumber culture pond in Rongcheng, China (36° 54′ 36″ N 122° 14′ 34″ E) in September 2013. The sediment sample was processed with an enrichment culture technique (Du *et al.*, 2014). A yellow-pigmented, circular and slightly ropy colony was isolated after incubation for 5 days on 2216E agar (Hopebio) at 28 °C. Strain FB218^T was routinely incubated at 30 °C for physiological, biochemical and chemical analysis after purification, and was stored at −80 °C in sterile distilled water supplemented with 1 % (w/v) NaCl and 15 % (v/v) glycerol. *C. mesophila* JCM 18290^T and *C. taeanensis* JCM 19490^T were obtained from the Japan Collection of Micro-organisms (JCM) and were cultured on 2216E agar at 30 °C, studied in parallel with strain FB218^T and used as reference strains for physiological and chemotaxonomic comparisons.

Colony morphology was examined on 2216E agar after incubation for 60 h at 30 °C. Cell size, morphology and motility were observed by using light microscopy (Ci-L; Nikon).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain FB218^T is KP172527.

A supplementary table and a supplementary figure are available with the online Supplementary Material.

Gliding motility was assessed according to the methods of Perry (1973) and Bowman (2000). Procedures for the Gram reaction and hydrolysis of agar, starch, gelatin and alginate were performed as described by Smibert & Krieg (1994). The temperature range for growth was determined on 2216E agar and in 2216E liquid medium (Hopebio) from 4 to 45 °C for 2–14 days. Tolerance to NaCl was tested on 2216E agar and in 2216E liquid medium [seawater in 2216E agar and 2216E liquid medium was replaced by artificial seawater containing ($g\,l^{-1}$): $MgSO_4$ (3.2), $MgCl_2$ (2.2), $CaCl_2$ (1.2), KCl (0.7) and $NaHCO_3$ (0.2)] with 0–10 % (w/v) NaCl at 30 °C for 10 days. The effect of pH on growth was investigated in 2216E liquid medium from pH 5.5 to 10.0, adding the following buffers at a concentration of 20 mM for pH adjustment: MES (for pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5) and CAPSO (pH 9.0, 9.5 and 10.0). Acid production from carbohydrates was detected using an API 50CHB fermentation kit (bioMérieux). Oxidation of carbohydrates, alcohols, organic acids, amino acids and nucleosides as sole carbon sources was checked in Biolog GEN III MicroPlates. Constitutive enzyme activities were assayed in API ZYM kits (bioMérieux). Additional physiological and biochemical parameters were assessed in API 20E and API 20NE kits (bioMérieux). All API tests were performed according to the manufacturer's instructions, except that the inoculum was prepared by suspending cells in 3 % (w/v) NaCl solution.

Anaerobic growth was checked on 2216E agar with or without 0.1 % (w/v) $NaNO_3$ in an anaerobic jar at 30 °C for 2 weeks. Oxidase activity was evaluated using an oxidase reagent (bioMérieux). Catalase activity was confirmed by bubble production after application of 3 % (v/v) hydrogen peroxide solution. As strain FB218^T was difficult to grow on Iso-Sensitest agar (Oxoid) and Mueller–Hinton agar, susceptibility to antibiotics was tested on 2216E agar according to Du *et al.* (2014) and procedures outlined by the Clinical and Laboratory Standards Institute (CLSI, 2012).

Two universal primers, 27f and 1492r, were used for PCR amplification (Jordan *et al.*, 2007). The PCR product was ligated into the pGM-T vector (Tiangen) for cloning, as described by Liu *et al.* (2014). Sequencing reactions were carried out using an ABI BigDye 3.1 sequencing kit (Applied Biosystems) and an automated DNA sequencer (model ABI 3730; Applied Biosystems). A near-complete sequence (1438 bp) was submitted to the GenBank database to determine the taxonomic position of strain FB218^T. The EzTaxon server (http://ezbiocloud.net/eztaxon; Kim *et al.*, 2012) was used to obtain sequences of the type strains. Alignment of sequences was carried out using the alignment program, CLUSTAL X (version 1.81) (Thompson *et al.*, 1997). A phylogenetic tree based on almost-complete 16S rRNA gene sequences of strain FB218^T and members of the family *Marinilabiliaceae* was reconstructed using the neighbour-joining method implemented in the software package MEGA (version 6.0) (Tamura *et al.*, 2013).

For further genotypic analyses, genomic DNA was extracted using a commercial genomic DNA extraction kit (TaKaRa MiniBEST Bacteria Genomic DNA Extraction kit version 3.0). The DNA G+C content was determined using HPLC according to methods described by Tamaoka & Komagata (1984) and Mesbah *et al.* (1989). The DNA G+C content determined for strain FB218^T was 40.0 mol%.

In order to determine the major menaquinone, whole-cell fatty acid and polar lipid profiles, cells shaken (120 r.p.m.) in 2216E liquid medium at 30 °C for 60 h were harvested and subjected to freeze-drying. The major menaquinones were detected according to the method described by Minnikin *et al.* (1984). A microbial identification system (MIDI; Microbial ID) was used to analyse fatty acid compositions, according to procedures described by Sasser (1990). Polar lipids were extracted from 100 mg freeze-dried cell material using a chloroform/methanol/0.3 % (w/v) aqueous NaCl mixture (1 : 2 : 0.8, by vol.), modified after Bligh & Dyer (1959), recovered into the chloroform phase by adjusting the chloroform/methanol/0.3 % (w/v) aqueous NaCl mixture to a ratio of 1 : 1 : 0.9 (by vol.), and separated by two-dimensional silica gel TLC (Macherey-Nagel Art. No. 818 135). The first direction was developed in chloroform/methanol/water (65 : 25 : 4, by vol.), and the second in chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.). Total lipid material was detected using molybdatophosphoric acid, and specific functional groups were detected using spray reagents for defined functional groups (Tindall *et al.*, 2007). Analysis of polar lipids was performed by the Identification Service of the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

Strain FB218^T was slender, 0.3–0.4 μm in width and 4.0–25.5 μm in length. The major menaquinone was MK-7. The predominant (>10 %) fatty acids were iso-$C_{15:0}$ (37.7 %) and anteiso-$C_{15:0}$ (14.3 %). Detailed cellular fatty acid compositions of strain FB218^T, *C. mesophila* JCM 18290^T and *C. taeanensis* JCM 19490^T are shown in Table S1 (available in the online Supplementary Material). The major polar lipids of strain FB218^T were two unidentified lipids (L3 and L4) and a phospholipid. Two additional unknown lipids (L1 and L2), an aminophospholipid and a glycolipid were present in moderate to minor amounts in the polar lipid profile (see Fig. S1). Detailed data are given in the species description.

The 16S rRNA gene sequence of strain FB218^T was 94.9 % similar to that of *C. mesophila* MEBiC 07026^T (=JCM 18290^T) and 94.8 % similar to that of *C. taeanensis* MEBiC 08903^T (=JCM 19490^T) of the family *Marinilabiliaceae*. These values are lower than the threshold value (97 %) used to define separate bacterial species (Stackebrandt & Goebel, 1994). Likewise, the topological structure of phylogenetic neighbour-joining tree (Fig. 1) clearly illustrated that strain FB218^T clustered with species of the genus

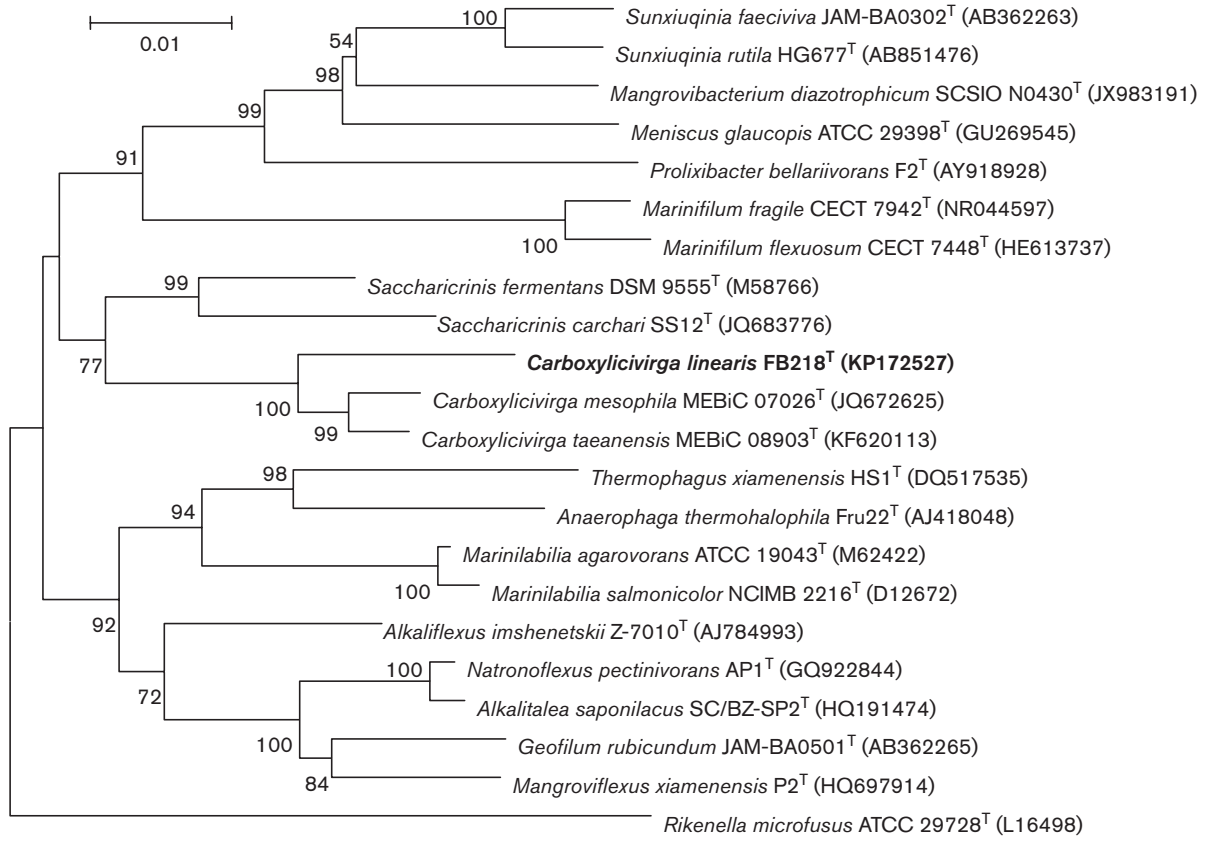

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the taxonomic status of strain FB218^T, type strains of other species of the genus *Carboxylicivirga* and some related taxa. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at branching nodes. Bar, 0.01 substitutions per nucleotide position.

Carboxylicivirga, and was distinctly separated from members of other genera.

The main respiratory quinone of strain FB218^T was MK-7, which is characteristic of the genus *Carboxylicivirga*. The major cellular fatty acids were iso-$C_{15:0}$ and anteiso-$C_{15:0}$, consistent with the genus *Carboxylicivirga*. On the whole, the chemotaxonomic characteristics of strain FB218^T were typical for members of the genus *Carboxylicivirga*, although some differences in the proportions of minor fatty acids were found. The novel isolate may also differ from closely related species based on a combination of phenotypic, genotypic and phylogenetic evidence. The ability to grow at 15 °C and capacity for nitrate reduction may provide sufficient justification for distinguishing strain FB218^T from *C. mesophila* JCM 18290^T. In addition, strain FB218^T differed from *C. taeanensis* JCM 19490^T in their capacities for hydrolysis of gelatin and indole production. Strain FB218^T could also be differentiated from the reference strains based on variations in enzyme activities. The differential characteristics of strain FB218^T, *C. mesophila* JCM 18290^T and *C. taeanensis* JCM 19490^T are shown in Table 1. Based on the results of this taxonomic study using a polyphasic approach, strain FB218^T is considered to represent a novel species of the genus *Carboxylicivirga*, for which we propose the name *Carboxylicivirga linearis* sp. nov. An emended description of the genus *Carboxylicivirga* is also provided.

Description of *Carboxylicivirga linearis* sp. nov.

Carboxylicivirga linearis (li.ne.a′ris. L. fem. adj. *linearis* linear, pertaining to the cell shape of the type strain).

Cells are Gram-stain-negative, slender, 0.3–0.4 μm in width and 4.0–25.5 μm in length. Colonies on 2216E agar are yellow-pigmented, circular with entire edges, smooth, slightly viscous and 0.8–1.2 mm in diameter after 60 h of incubation at 30 °C. Growth was observed at 15–40 °C (optimum 30 °C), pH 6.0–9.0 (optimum pH 6.5–7.0) and in the presence of 1–7 % (w/v) NaCl (optimum 2–3 %). Facultatively anaerobic, gliding, catalase-positive and oxidase-negative. Indole is produced but H_2S is not produced. Nitrate is reduced to nitrite. Starch, gelatin and agar are hydrolysed, but alginate is not. Acid is produced from D-mannose, D-galactose, α-D-glucose, D-fructose, aesculin, maltose, α-lactose, melibiose, sucrose,

Table 1. Differential characteristics of strain FB218^T and type strains of species of the genus *Carboxylicivirga*

Strains: 1, FB218^T; 2, *C. mesophila* JCM 18290^T; 3, *C. taeanensis* JCM 19490^T. All data are from this study except where indicated otherwise. All strains were positive for catalase, alkaline phosphatase and acid phosphatase activities, and acid production from D-galactose, α-D-glucose, aesculin, maltose and starch. All strains were negative for H_2S production and enzyme activities of lipase (C14), valine arylamidase, cystine arylamidase, α-mannosidase and β-fucosidase. +, Positive; W, weakly positive; −, negative; V, variable.

Characteristic	1	2	3
Cell size (μm)	0.3–0.4 × 4.0–25.5	0.4–0.7 × 8.2–11.6	0.3–0.6 × 6.1–18.7
Nitrate reduction	+	−	+
Indole production	+	V	−
Urease	+	+	−
Hydrolysis of:			
Agar	W	−	W
Gelatin	W	+	−
Enzyme activities			
Esterase (C4)	−	−	+
Trypsin	+	+	−
β-Galactosidase	−	+	−
α-Glucosidase	+	−	+
Acid production from:			
D-Fructose	+	−	−
N-Acetyl-D-glucosamine	−	+	+
Melibiose	+	−	−
Sucrose	+	−	−
Cellobiose	−	+	−
DNA G+C content (mol%)	40.0	44.2*	44.1*

*Data from Yang *et al.* (2014).

raffinose, D-inulin, starch, D-gentiobiose and potassium 5-ketogluconate (variable results are obtained for D-xylose and trehalose). Alkaline phosphatase, trypsin, α-chymotrypsin, acid phosphatase, α-glucosidase, β-glucosidase and *N*-acetyl-glucosaminidase activities are detected in API ZYM tests, but not esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase or β-fucosidase activities. In Biolog GEN III MicroPlates, the following compounds are used as the sole carbon and energy sources: maltose, trehalose, raffinose, α-lactose, melibiose, α-D-glucose, D-mannose, D-fructose, D-galactose, L-aspartic acid, D-galacturonic acid, D-glucuronic acid, glucuronamide, β-hydroxy-DL-butyric acid, α-ketobutyric acid, acetoacetic acid, stachyose, L-fucose, D-fructose 6-phosphate, L-histidine, acetic acid, and L-malic acid. Resistant to gentamicin, kanamycin, nalidixic acid, streptomycin, neomycin, tobramycin and tetracycline, but susceptible to vancomycin, penicillin, latamoxef, ceftriaxone, erythromycin, ofloxacin, lincomycin, cefotaxime sodium, clindamycin, acetylspiramycin, rifampicin, and chloramphenicol. The major fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. Major polar lipids include two unidentified lipids and a phospholipid, and the main respiratory quinone is MK-7.

The type strain, FB218^T (=KCTC 42254^T=MCCC 1H00106^T), was isolated from sediment collected from a sea cucumber culture pond in Rongcheng, China. The genomic DNA G+C content of the type species is 40.0 mol%.

Emended description of the genus *Carboxylicivirga* Yang *et al.* 2014

The description of the genus *Carboxylicivirga* is as given by Yang *et al.* (2014) with the following amendments. Some members of the genus *Carboxylicivirga* are motile by gliding. Membrane polar lipids include two unidentified lipids as major polar lipids. The genomic DNA G+C content is 40.0–44.0 mol%.

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