Acanthopleuribacter pedis gen. nov., sp. nov., a marine bacterium isolated from a chiton, and description of *Acanthopleuribacteraceae* fam. nov., *Acanthopleuribacterales* ord. nov., *Holophagaceae* fam. nov., *Holophagales* ord. nov. and *Holophagae* classis nov. in the phylum 'Acidobacteria'

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Strain FYK2218^T was isolated from a specimen of the chiton *Acanthopleura japonica*, which had been collected from a beach on the Boso peninsula in Japan. Phylogenetic analyses based on 16S rRNA gene sequences revealed that the strain belonged to the phylum 'Acidobacteria'. The most closely related type strains to strain FYK2218^T were *Holophaga foetida* TMBS4^T (83.6% 16S rRNA gene sequence similarity) and *Geothrix fermentans* H-5^T (83.6%) in subdivision 8 of the 'Acidobacteria'. Cells of FYK2218^T were motile, rod-shaped, Gram-negative, mesophilic and strictly aerobic. The G+C content of the strain was 56.7 mol%. The strain had isoprenoid quinones MK-6 and MK-7 as major components. Major fatty acids of the strain were iso-C_{15:0}, iso-C_{17:0}, C_{16:0} and C_{20:5ω3c} (*cis*-5,8,11,14,17-eicosapentaenoic acid). From the taxonomic data obtained in this study, it is proposed that the new marine isolate be placed into a novel genus and species named *Acanthopleuribacter pedis* gen. nov., sp. nov. within the new family, order and class *Acanthopleuribacteraceae* fam. nov., *Acanthopleuribacterales* ord. nov. and *Holophagae* classis nov. The family *Holophagaceae* fam. nov. is also described. The type strain of *Acanthopleuribacter pedis* is FYK2218^T (=NBRC 101209^T =KCTC 12899^T).

Culture-independent studies based on the detection of 16S rRNA gene sequences have shown that bacteria belonging to the phylum 'Acidobacteria' are ubiquitous in various environments including fresh water, a hot spring microbial mat (Barns *et al.*, 1999), sewage sludge (Layton *et al.*, 2000) and desert soils (Chanal *et al.*, 2006). The range of divergence in 16S rRNA gene sequences among members of the 'Acidobacteria' was almost 20% (Quaiser *et al.*, 2003), indicating that this phylum is as diverse as *Proteobacteria* (Barns *et al.*, 1999). In terms of validly published names, the phylum 'Acidobacteria' contains a

single class, *Acidobacteria*, and a single order, *Acidobacteriales* (Cavalier-Smith, 2002), consisting of the genera *Acidobacterium* (Kishimoto *et al.*, 1991), *Holophaga* (Liesack *et al.*, 1994) and *Geothrix* (Coates *et al.*, 1999). However, eight monophyletic subdivisions are currently recognized in the phylum 'Acidobacteria' based on rRNA sequence approaches (Hugenholtz *et al.*, 1998). Thus, although a few strains belonging to the phylum 'Acidobacteria' have since been isolated from soils (Janssen *et al.*, 2002; Joseph *et al.*, 2003), several groups in the phylum 'Acidobacteria' remain uncultured.

We have reported that marine invertebrates are interesting sources for the discovery of novel lineages of bacteria (Fukunaga *et al.*, 2006; Kurahashi & Yokota, 2002, 2004). Here, we describe a novel strain isolated from a chiton

The DDBJ/GenBank/EMBL accession number for the 16S rRNA gene sequence of *Acanthopleuribacter pedis* FYK2218^T is AB303221.

Transmission electron micrographs of strain FYK2218^T, a 16S rRNA gene sequence-based tree and a detailed fatty acid profile of strain FYK2218^T are available as supplementary material with the online version of this paper.

which was classified by its 16S rRNA gene sequence to be a member of the phylum 'Acidobacteria'.

In 2003, a chiton (*Acanthopleura japonica*) was collected from a Pacific Ocean beach near the Natural History Museum and Institute at Katsuura, located on the Boso peninsula, Chiba, Japan. The chiton was washed with sterile artificial seawater (ASW; Naigai Chemical Products) and dissected. The dissected tissues were homogenized separately using a hand-held homogenizer. Subsequently, the chiton homogenates were serially diluted 10-fold in sterile ASW and suitable dilutions were plated onto 1/5-strength marine agar 2216 (Difco). The plates were incubated at 20 °C for 8 days and colonies that grew were purified on the same medium. Strain FYK2218^T was isolated from the foot tissue of the chiton.

Colonies of the strain were circular, entire, smooth and yellow in colour. Phase-contrast microscopy showed that cells of the strain grown on marine broth 2216 (Difco) are motile. Cells were negatively stained with phosphotungstic acid and observed under a transmission electron microscope (Hitachi H7600). Electron micrographs showed that the cells were rod-shaped (0.7–1.0 × 2.4–4.7 µm) and peritrichously flagellated (Supplementary Fig. S1a, available in IJSEM Online). For the preparation of ultrathin sections, cells of FYK2218^T were processed by rapid freezing in liquid propane cooled with liquid nitrogen, cryosubstituted with 2 % (w/v) OsO₄ in acetone for 3 days and embedded in epoxy resin. Ultrathin sections were double-stained with a platinum-blue staining solution (Inaga *et al.*, 2007) (using threefold-diluted stock solution) for 10 min and a lead staining solution (Reynolds, 1963) for 1 min. Ultrathin sectioning revealed a typical Gram-negative cell wall structure (Supplementary Fig. S1b), which was further confirmed with the 3 % (w/v) KOH reaction (Buck, 1982).

The DNA G+C content of strain FYK2218^T was determined by HPLC analysis (Mesbah *et al.*, 1989) to be 56.7 mol%. Extraction of cellular fatty acids from cells grown for 48 h at 25 °C on marine agar and determination of the fatty acid content by GC were carried out using the Microbial Identification (MIDI) System (MIDI Laboratories) according to the manufacturer's instructions. Fatty acids not identified by the MIDI System were further analysed using GC-MS (Agilent). The cellular fatty acid profile of strain FYK2218^T is shown in Supplementary Table S1. The major fatty acids in FYK2218^T were iso-C_{15:0} (19.0 % of the total fatty acids), iso-C_{17:0} (14.1 %), C_{16:0} (12.5 %) and C_{20:5ω3c} (7.9 %). Hydroxy fatty acids in FYK2218^T were iso-C_{13:0} 3-OH (5.7 %) and iso-C_{17:0} 3-OH (3.4 %). Isoprenoid quinones were extracted from dry cells with chloroform/methanol (2:1, v/v), dried under a nitrogen stream and analysed by LC-MS [Shimadzu model LC-5A equipped with an ODS-II column (2.0 × 150 mm; Shinwa Chemical Industries)] using methanol/isopropyl ether (2:1, v/v) as mobile phase. The respiratory quinones were menaquinones MK-6 (57.8 %) and MK-7 (42.2 %).

Physiological and biochemical tests were performed by using API 20NE and API ZYM kits (both from bioMérieux) and Biolog GN2 microtitre plates according to the manufacturers' specifications except that cells of FYK2218^T were suspended in a sterile solution as suggested by Rüger & Krambeck (1994). In the Biolog GN2 tests, 8 of 95 carbon sources were oxidized by strain FYK2218^T. The results of the API ZYM, API20NE and Biolog GN2 tests are listed in the description of the novel species.

Tests for anaerobic growth were conducted on either M9 minimal medium plates (Sambrook & Russell, 2001) containing 0.2 % (w/v) glucose and 1.5 % (w/v) NaCl or marine agar at 25 °C in a GasPak anaerobic jar (Becton Dickinson). Growth of strain FYK2218^T on M9 or marine agar plates was not observed under anaerobic conditions.

Salt tolerance was tested with R2A agar (Difco) suspended in ASW (0–200 %, v/v) and the plates were incubated at 25 °C. Growth was observed on R2A agar containing 50–150 % ASW, with optimal growth at 70–120 % ASW. To test the pH range for growth, 20 µl cell suspension was transferred to test tubes containing 5 ml filter-sterilized TYSW (2 g tryptone and 1 g yeast extract dissolved in 1 l ASW) at pH 1–11 and incubated at 25 °C. Strain FYK2218^T grew in TYSW at initial pH between 5.0 and 9.0. The optimum pH for growth was pH 7.0–8.0. To test the temperature range for growth, marine agar plates were incubated at 4, 10, 15, 20, 25, 30, 35, 37, 40 or 45 °C for 7 days. Strain FYK2218^T grew at 15–30 °C, but did not grow at 10 or 35 °C. Optimum growth occurred at 30 °C.

The 16S rRNA gene was analysed as described by Fukunaga *et al.* (2006). The 16S rRNA gene sequence of strain FYK2218^T was aligned with related sequences using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were inferred by using neighbour-joining (NJ; Saitou & Nei, 1987), maximum-parsimony (MP; Swofford, 2000) and maximum-likelihood (ML; Adachi & Hasegawa, 1996) algorithms. The topology of the trees was calculated by bootstrap resampling analysis (Felsenstein, 1985) of 1000 replicates for NJ and MP and local bootstrap probabilities (Hasegawa & Kishino, 1994) of 100 replicates for ML.

The results of an initial BLAST search showed a cloned sequence from coral (GenBank accession no. EF206921; Koren & Rosenberg, 2006) to be the closest relative of strain FYK2218^T, with a 16S rRNA gene sequence similarity of 93.3 %. Phylogenetic trees inferred from NJ, MP and ML algorithms based on 1262 and 667 bp, respectively, of the 16S rRNA gene sequence are shown in Fig. 1 and Supplementary Fig. S2. Strain FYK2218^T forms a distinct clade in the radiation of subdivision 8 of the 'Acidobacteria' (Fig. 1 and Supplementary Fig. S2), supported by high bootstrap values. The most closely related species to strain FYK2218^T were *Holophaga foetida* (83.6 % identity to the type strain) and *Geothrix fermentans* (83.6 % identity). The results of the phylogenetic analysis are supported by the phenotypic differences of strain FYK2218^T from *Holophaga foetida* and other phylogenetic neighbours.

Fig. 1. Neighbour-joining tree of the phylum 'Acidobacteria' based on 16S rRNA gene sequences using 1262 bp. Cultivated strains are shown in bold. Branch points supported by NJ, ML and MP analysis are indicated by solid circles. Bootstrap values above 50 % are shown (above the nodes, NJ; below, ML).

Table 1. Differential characteristics of FYK2218^T and members of related genera in subdivision 8 of the phylum 'Acidobacteria'

Strains: 1, strain FYK2218^T; 2, *Geothrix fermentans* H-5^T (data from Coates *et al.*, 1999); 3, *Holophaga foetida* TMBS4^T (Liesack *et al.*, 1994). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3
Pigmentation	Yellow	White	Translucent–beige
Motility	+	–	–
Optimum temperature for growth (°C)	30	35	35
Optimum pH	7.0–8.0	ND	6.8–7.5
Aerobic growth	+	–	–
Anaerobic growth	–	+	+
Use as electron donor and carbon source of:			
Glucose	+	–	ND
Acetate	–	+	ND
Propionate	–	+	ND
Succinate	–	+	–
Pyruvate	–	ND	+
Fructose	–	ND	–
Citrate	–	+	ND
Lactate	–	+	–
DNA G + C content (mol%)	56.7	ND	62.5

(Table 1). Based on these results, we conclude that strain FYK2218^T represents a new genus and species in subdivision 8 of 'Acidobacteria', for which the name *Acanthopleuribacter pedis* gen. nov., sp. nov. is proposed.

In phylogenetic trees obtained with the NJ, MP and ML algorithms, the cluster of subdivision 8 in the 'Acidobacteria' including FYK2218^T was independent and was supported by high bootstrap values (Fig. 1). Furthermore, levels of 16S rRNA gene sequence similarity between FYK2218^T and other members of subdivision 8 of the 'Acidobacteria' were very low (82.7–83.6%). It is therefore also proposed that subdivision 8 of the phylum 'Acidobacteria' should be classified not in the class *Acidobacteria* and order *Acidobacteriales* (Cavalier-Smith, 2002) but as a new class, *Holophagae* classis nov., with two new orders, *Acanthopleuribacterales* ord. nov. and *Holophagales* ord. nov. Order *Acanthopleuribacterales* contains the new family *Acanthopleuribacteraceae* fam. nov., with *Acanthopleuribacter* as its type genus. The genus *Holophaga* is placed in a new family *Holophagaceae* fam. nov. in the order *Holophagales*.

Description of *Acanthopleuribacter* gen. nov.

Acanthopleuribacter (A.can'tho.pleu.ri.bac'ter. N.L. n. *Acanthopleura* a zoological genus name of shellfish; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Acanthopleuribacter* a rod from *Acanthopleura*, referring to the isolation of the first strain from the chiton *Acanthopleura japonica*).

Cells are Gram-negative, motile, strictly aerobic rods. Predominant fatty acids are iso-C_{15:0}, iso-C_{17:0} and C_{16:0}, while the major hydroxy fatty acids are iso-C_{13:0} 3-OH and iso-C_{17:0} 3-OH. MK-6 and MK-7 are the menaquinones. The G+C content of the genomic DNA of the type strain of the type species is 56.7 mol%. The type species is *Acanthopleuribacter pedis*.

Description of *Acanthopleuribacter pedis* sp. nov.

Acanthopleuribacter pedis (pe'dis. L. gen. n. *pedis* of the foot, referring to the isolation of the type strain from the foot of a chiton).

In addition to the characteristics listed for the genus, the following are observed. Cells are 0.7–1.0 × 2.4–4.7 µm and motile by peritrichous flagella. Colonies on marine agar are circular, smooth and yellow in colour. Grows on marine agar at 15–30 °C (optimally at 30 °C). Grows at pH 5.0–9.0 (optimally at pH 7.0–8.0). Growth is observed on R2A agar supplemented with 50–150% ASW (optimally with 70–120% ASW). Catalase and oxidase activities are present. Nitrate and nitrite are not reduced, acid is not produced from glucose and indole is not produced from tryptophan. β-Glucosidase and protease activities are present. Arginine dihydrolase and urease activities are not detected. Growth by fermenting glucose under anaerobic conditions is not observed. The following substrates are utilized for growth: α-D-glucose, L-alanine, hydroxy-L-proline, L-serine,

L-threonine, inosine, uridine and thymidine. Activities in the API ZYM strip are detected for alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase. Tests for esterase C4, ester lipase C8, cystine arylamidase and β-glucosidase are weakly positive. Activities of lipase C4, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase and α-fucosidase are not observed. The quinone system of the type strain consists of MK-6 (57.8%) and MK-7 (42.2%).

The type strain, FYK2218^T (=NBRC 101209^T =KCTC 12899^T), was isolated from a chiton, *Acanthopleura japonica*, from the Boso peninsula, Japan.

Description of *Acanthopleuribacteraceae* fam. nov.

Acanthopleuribacteraceae (A.can'tho.pleu.ri.bac'te.ra'ce.ae. N.L. masc. n. *Acanthopleuribacter* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Acanthopleuribacteraceae* the family of the genus *Acanthopleuribacter*).

The description is the same as that for the genus *Acanthopleuribacter*. The family contains the type genus *Acanthopleuribacter*.

Description of *Acanthopleuribacterales* ord. nov.

Acanthopleuribacterales (A.can'tho.pleu.ri.bac'te.ra'les. N.L. masc. n. *Acanthopleuribacter* type genus of the order; -ales ending to denote an order; N.L. fem. pl. n. *Acanthopleuribacterales* the order of genus *Acanthopleuribacter*).

The description is the same as that for the genus *Acanthopleuribacter*. The order contains the family *Acanthopleuribacteraceae*. The type genus is *Acanthopleuribacter*.

Description of *Holophagaceae* fam. nov.

Holophagaceae (Ho.lo.pha.ga'ce.ae. N.L. fem. n. *Holophaga* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Holophagaceae* the family of the genus *Holophaga*).

Defined by phylogenetic analyses based on 16S rRNA gene sequences. The family contains the genera *Holophaga* (Liesack *et al.*, 1994) and *Geothrix* (Coates *et al.*, 1999). The type genus is *Holophaga*.

Description of *Holophagales* ord. nov.

Holophagales (Ho.lo.pha.ga'les. N.L. fem. n. *Holophaga* type genus of the order; -ales ending to denote an order; N.L. fem. pl. n. *Holophagales* the order of genus *Holophaga*).

The description is the same as for the family *Holophagaceae*. The order contains the family *Holophagaceae*. The type genus is *Holophaga*.

Description of *Holophagae* classis nov.

Holophagae (Ho.lo.pha'gae. N.L. fem. pl. n. *Holophagales* type order of the class; -ae ending to denote a class; N.L. fem. pl. n. *Holophagae* the class of the order *Holophagales*).

Equivalent to subdivision 8 (Hugenholtz *et al.*, 1998) of the phylum '*Acidobacteria*' and is defined by phylogenetic analyses based on 16S rRNA gene sequences obtained from three cultured representatives and a wide range of uncultured bacteria retrieved mainly from marine and soil habitats. Gram-negative. The class comprises the orders *Holophagales* and *Acanthopleuribacterales*. The type order is *Holophagales*.

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