Draconibacterium orientale gen. nov., sp. nov., isolated from two distinct marine environments, and proposal of *Draconibacteriaceae* fam. nov.

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The taxonomic characteristics of two bacterial strains, $FH5^{T}$ and SS4, isolated from enrichment cultures obtained from two distinct marine environments, were determined. These bacteria were Gram-stain-negative, facultatively anaerobic rods. Growth occurred at 20–40 °C (optimum, 28–32 °C), pH 5.5–9.0 (optimum, pH 7.0–7.5) and in the presence of 1–7% NaCl (optimum, 2–4%). The major cellular fatty acids were anteiso- $C_{15:0}$ and iso- $C_{15:0}$. Menaquinone 7 (MK-7) was the sole respiratory quinone. The major polar lipids were phosphatidylethanolamine, an unkown phospholipid and an unknown lipid. The DNA G+C contents of strains FH5^T and SS4 were both determined to be 42.0 mol%. The results of DNA–DNA hybridization studies indicated that the FH5^T and SS4 genomes share greater than 95% relatedness. The strains formed a distinct phyletic line within the class *Bacteroidia*, with less than 89.4% sequence similarity to their closest relatives with validly published names. On the basis of physiological and biochemical characteristics, 16S rRNA gene sequences and chemical properties, a novel genus and species, *Draconibacterium orientale* gen. nov., sp. nov., within the class *Bacteroidia*, are proposed, with strain FH5^T (=DSM 25947^T=CICC 10585^T) as the type strain. In addition, a new family, *Draconibacteriaceae* fam. nov., is proposed to accommodate *Draconibacterium* gen. nov.

The class Bacteroidia was proposed by Krieg (2011) to accommodate Gram-stain-negative, non-spore-forming rods and coccobacilli that were non-motile or capable of gliding motility. Although most of the members identified in this class are anaerobic, several aerobic and facultatively anaerobic strains have been reported recently. Sunxiuginia faeciviva and Sunxiuginia elliptica were isolated from deep sub-seafloor sediment and sediment in a sea cucumber farm, respectively (Qu et al., 2011; Takai et al., 2013), Prolixibacter bellariivorans was found in a marine-sediment fuel cell (Holmes et al., 2007), and Marinifilum fragile and Marinifilum flexuosum were recovered from tidal flat sediment and surface seawater, respectively (Na et al., 2009; Ruvira et al. 2013). Recently, a novel family, 'Prolixibacteraceae' fam. nov., was proposed to accommodate the genera Sunxiuqinia, Prolixibacter and a newly described genus 'Mangrovibacterium' (Huang et al. 2013). In this study, we report the characterization of two

deep-branching strains isolated from a marine sediment sample and a shark gill sample. The novel isolates represented a deep-rooting lineage belonging to the class *Bacteroidia* which, at present, constitutes a single order, the *Bacteroidales*. The novel isolate showed closest 16S rRNA gene sequence similarity with members of the established genera *Marinifilum* and *Prolixibacter*. On the basis of the distinct genotypic and phenotypic properties when compared with members of the most closely related taxon, the novel strain is proposed as representing a novel genus and species for which the name *Draconibacterium orientale* gen. nov., sp. nov. is proposed.

Strains FH5^T and SS4 were isolated from a marine sediment sample from the coast of Weihai, China (122° 03′ 44.01″ E 37° 32′ 01.93″ N), and a dead shark (*Cetorhinus maximus*) that caught by fishermen from the Yellow Sea, China, respectively, using an enrichment culture technique. The medium consisted of the following ingredients in 1000 ml seawater: 0.1 % NH₄Cl, 0.2 % CH₃COONa, 0.02 % MgSO₄. 7H₂O, 0.02 % yeast extract, 0.02 % peptone. The pH of the medium was adjusted to pH 7.5 and then autoclaved. A 10 % (w/v) NaHCO₃ solution was filtered and a 2 % (w/v)

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of *Draconibacterium orientale* FH5^{T} and SS4 are JQ683778 and KF041475, respectively.

A supplementary figure is available with the online version of this paper.

 $\rm KH_2PO_4$ solution was autoclaved. Each of the two solutions was added to the autoclaved media (10 ml per litre). Incubation was performed at 25 °C for 21 days with a 500 ml sealed glass bottle (filled with medium and 10 g sample was added). The bottle was shaken twice a day and kept sealed during the incubation. The anaerobic enrichment cultures were diluted with 9 ml sterile seawater and spread onto 2216 marine agar (MA; Hopebio). The plates were incubated aerobically at 28 °C for 5 days. Strains FH5^T and SS4 were obtained in pure culture after three successive transfers to fresh MA plates and stored at -80 °C in 20 % (v/v) glycerol.

Routine tests, including Gram staining and agarase, amylase, urease, catalase, gelatinase and oxidase activities, were carried out as described by Smibert & Krieg (1994). Cell morphology and flagella were observed by transmission electron microscopy (Jem-1200; Jeol). Colony morphology was observed on MA after 4 days of incubation at 28 °C. Antibiotic sensitivity was assessed as described by the Clinical and Laboratory Standards Institute (CLSI, 2012): a cell suspension (McFarland standard 0.5) was swabbed over the surface of Iso-Sensitest agar (Oxoid) plates to create a uniform lawn before aseptic placement of antibiotic discs onto the agar surface. Inoculated plates were incubated at 28 °C for up to 7 days. The effects of different temperatures on growth were assessed on MA plates with incubation at temperatures ranging from 4 to 42 °C. Plates were incubated for 4 weeks and growth was indicated by visible colonies. The pH range for growth was determined by adding no buffer (pH 5.0), MES (pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5) or CAPSO (pH 9.0 and 9.5) to 2216 marine broth (MB; Hopebio) at concentrations of 20 mM. The pH of the medium was adjusted by addition of 1M HCl or NaOH before autoclaving. The effects of different NaCl concentrations on growth were assessed by using 2216 MA made with artificial seawater (consisting of 0.6 % MgCl, 0.32 % Na₂SO₄, 0.18 % CaCl₂, 0.06 % KCl, 0.02 % Na₂CO₃, traces of Na₂SiO₃ and NaF) and NaCl concentrations ranging from 0-15% (w/v).

Gliding motility was observed by oil-immersion phasecontrast microscopy (AX70; Olympus) according to the method described by Bowman (2000). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on 2216 MA with or without 0.25 % (w/v) NaNO₃ for 7 days at 28 °C. Physiological and biochemical characteristics were determined with API 20E and API ZYM kits (bioMérieux) according to the manufacturer's instructions, except that the suspension medium was replaced by 3 % (w/v) NaCl solution. Tests of acid production from carbohydrates were performed by using the API 50CHB fermentation kit (bioMérieux) according to the manufacturer's instructions. The salinity of the 50CHB medium was adjusted by addition of autoclaved 30% (w/v) NaCl solution before inoculation. The API 50CHB strips were read after 7 days of incubation at 28 °C. The isolates were further tested for their ability to utilize different carbon sources using GEN III MicroPlates (Biolog) according to the manufacturer's instructions. Data were

analysed using the software package provided by Biolog. All of the tests were performed in duplicate.

Cellular fatty acids were determined on a 4-day-old culture (end of the exponential growth phase) grown on 2216 MA plates after incubation at 28 °C. The fatty acids were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system (Sasser, 1990). Respiratory quinones were extracted and purified according to the protocol of Collins (1985) and were analysed by HPLC (Kroppenstedt, 1982). Polar lipid analysis was carried out by the Identification Service, Leibniz Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

DNA was extracted and purified using a bacteria genomic DNA Mini kit (TaKaRa Bio) following the manufacturer's protocol. The gene encoding 16S rRNA was amplified by PCR with two universal primers, 27f and 1492r (Jordan et al., 2007). The purified PCR product was ligated to the vector pMD 18-T (TaKaRa Bio) and cloned according to the manufacturer's instructions. Sequencing reactions were carried out using an ABI BigDye 3.1 Sequencing kit (Applied BioSystems) and an automated DNA sequencer (model ABI3730; Applied BioSystems). The nearly complete 16S rRNA gene sequence of strains FH5^T and SS4 was submitted to GenBank and EMBL to search for similar sequences using the BLAST algorithm. 16S rRNA gene sequences of several closely related species were first aligned by using CLUSTAL X (version 1.81) (Thompson et al. 1997), and then the alignments were corrected manually. A phylogeny of the taxa was inferred by using the neighbour-joining method implemented in the computer program MEGA version 5.2.1 (Tamura et al., 2011), and statistical reliability was assessed from 1000 bootstrap replicates.

For DNA-DNA hybridization, genomic DNA from all strains was extracted from cultured cells using the Qiagen DNAeasy Midi kit (Qiagen) and subsequently quantified using the Qaunt-iT PicoGreen dsDNA assay kit (Invitrogen). The DNA G+C content (mol%) of the study strains was determined using the method of Gonzalez & Saiz-Jimenez (2002). The type strains Marinifilum fragile NRRL B-59702^T, Wandonia haliotis NRRL B-59700^T, Escherichia coli NRRL B-3054, Deinococcus radiodurans NRRL B-2906^T, Streptosporangium roseum NRRL 2505^T, Bacillus subtilis subsp. subtilis NRRL NRS-1315^T, Streptomyces coelicolor NRRL B-2812^T and Clostridium aminophilum NRRL B-23502^T were used as reference taxa to generate a calibrated regression equation from which G+C mol% could be determined. Subsequent DNA-DNA hybridization experiments were conducted using the fluorometric method of Gonzalez & Saiz-Jimenez (2005). Prior to analysis, isolated DNA was renatured at the optimum temperature for renaturation (T_{or}) as described by De Ley et al. (1970). Renaturation conditions consisted of a denaturation step of 99 °C for 10 min, followed by an annealing period of 8 h at T_{or}, which was approximated according to the method of De Ley et al. (1970) using the equation $T_{\rm or}$ =0.51(%G+C) + 47.0. This was followed by progressive 60 min steps, each at 10 °C below the previous one, until room temperature was reached. Renatured DNA was diluted to 10 ng ul⁻¹ in 0.1 × SSC, pH 8.0, containing 1 × SYBR Green (Molecular Probes). Fluorescence measurements were recorded with a Rotor-gene Q (Qiagen) instrument. The thermal profile consisted of a 15 min hold at 25 °C followed by a 25–99 °C ramp in 0.2 °C steps with a 5 s hold. The mean of three or more replicates was used to determine the $T_{\rm m}$ (melt temperature). $T_{\rm m}$ values of DNA from homologous and hybrid DNA solutions were calculated as the temperatures corresponding to a 50% decrease in fluorescence. Percentage similarity was estimated based on the $\Delta T_{\rm m}$ values (Gonzalez & Saiz-Jimenez, 2005).

After 3 weeks of enrichment culture at 25 °C, strains FH5^T and SS4 were isolated. The two isolates exhibited almost identical characteristics and both produced colonies on 2216 MA that were circular with entire edge, light pink to tawny in colour and nearly opaque. Microscopic examination suggested that the cells of the two strains were straight to slightly curved rods, and flagella were not observed. Cells were non-motile and non-endospore-forming. Both strains contained MK-7 (100%) as the sole menaquinone. The major polar lipids of strain FH5^T were phosphatidylethanolamine, an unkown phospholipid and an unknown lipid. A two-dimensional TLC plate image of lipids of strain $FH5^{T}$ is available as Fig. S1 in the online Supplementary Material. The predominant cellular fatty acids of strain FH5^T were anteiso- $C_{15:0}$ (25.6%), iso- $C_{15:0}$ (17.1%), $C_{17:0}$ 2-OH (9.7%) and iso- $C_{17:0}$ 3-OH (6.3%). The complete morphological, physiological and biochemical characteristics of strain FH5^T are given in the species description. The characteristics that differentiate strain FH5^T from related genera of the class *Bacteroidia* are shown in Table 1.

The DNA G+C contents of strains $FH5^{T}$ and SS4 were both determined to be 42.0%. The results of DNA-DNA hybridization studies indicated that the FH5^T and SS4 genomes are highly similar with a $\Delta T_{\rm m}$ <1 °C, which corresponds to an overall relatedness greater than 95% based on the method of Gonzalez & Saiz-Jimenez (2005). $\Delta T_{\rm m}$ values between homologous and hybrid DNA of 5 °C or lower are considered as corresponding to the same microbial species (Rosselló-Mora & Amann, 2001; Wavne et al., 1987), indicating that strains FH5^T and SS4 are thus conspecific. We compared these strains to the most closely related strains that we identified, W. haliotis and Marinifilum fragile, using the EzTaxon-e 16S rRNA gene sequence database (Kim et al. 2012), and which we could obtain from public culture collections. The results of these analyses suggested that FH5^T and SS4 represent a distinct species from Marinifilum fragile with $\Delta T_{\rm m}$ values of 7.4 and 8.2 °C, respectively, corresponding to an overall genome relatedness of $\sim 60-65$ %. FH5^T and SS4 were also found to represent a distinct species from W. haliotis with $\Delta T_{\rm m}$ values of 7.4 and 7.6 °C, respectively, suggesting an overall genome relatedness of ~60-65 %.

The 16S rRNA gene analysis placed strain FH5^T within the class Bacteroidia. Similarity values calculated for isolate FH5^T indicated a remote relationship (less than 90%) similarity) with members of the class Bacteroidia. 16S rRNA gene sequence similarity calculations indicated that strain FH5^T showed the greatest degree of similarity to Marinifilum fragile JC2469^T (GenBank accession no. FJ394546; 89.4%); other closely related strains produced similar values, including *P. bellariivorans* F2^T (AY918928; 89.0%) and Sunxiuginia elliptica DOHS-4^T (GO200190; 88.4%). Phylogenetic trees obtained by using the neighbour-joining method (Fig. 1) revealed clear affiliation of the novel isolates to the class Bacteroidia, being positioned on a separate branch within this class. This toplogy was also supported by the minimum-evolution and maximumlikelihood algorithms (data not shown). The Draconibacterium clade was also shown to be a well separated lineage with less than 90 % 16S rRNA gene sequence similarity to the other recognized genera within the class Bacteroidia. We therefore propose to create a new genus, namely Draconibacterium gen. nov. Strain FH5^T represents the type strain of the type species, Draconibacterium orientale sp. nov.

The proposed genus Draconibacterium can be readily distinguished from the genus Marinifilum because members of the latter can hydrolyse starch, cannot grow at pH 5.5 and have negative catalase activity; it can be distinguished from the genus Prolixibacter because members of the latter can grow at 4 °C, hydrolyse starch and have MK7(H6) as one of the respiratory quinones. The proposed genus Draconibacterium can be distinguished from the genus Sunxiuginia by the ability to hydrolyse gelatin, and can be distinguished clearly from the genus 'Mangrovibacterium' because members of the latter contain the respiratory quinones MK-7(H2), MK-7(H6) and MK-9 in addition to MK7. Strains FH5^T and SS4 can also be distinguished from strains belonging to the family Marinilabiliaceae by their lack of gliding motility, and from strains of the families Bacteroidaceae, Rikenellaceae, Prevotellaceae and Porphyromonadaceae by their marine origin and ability to grow aerobically. Based on the distinct phylogenetic position of the Draconibacterium gen. nov. lineage in the class Bacteroidia and the large degree of divergence revealed through 16S rRNA gene sequence comparisons (strain FH5^T formed a deep branch in class *Bacteroidia*, clearly distinguished from other described families of the class), a novel family, Draconibacteriaceae fam. nov. is proposed.

Description of Draconibacterium gen. nov.

Draconibacterium (Dra.co.ni.bac.te'ri.um. L. masc. n. draco -onis snake; L. n. bacterium staff, rod and, in biology, a bacterium; N.L. neut. n. Draconibacterium the dragon bacterium).

Cells are Gram-stain-negative, straight to slightly curved rods, facultatively anaerobic, non-motile or non-gliding, and non-endospore-forming. Chemo-organotrophic. Oxidase- and

Table 1. Differential characteristics of strain FH5^T and related genera of the class *Bacteroidia*

Genera: 1, *Draconibacterium* gen. nov. (strain FH5^T); 2, *Marinifilum* (data from Na *et al.*, 2009; Ruvira *et al.*, 2013); 3, *Prolixibacter* (Holmes *et al.*, 2007; Huang *et al.*, 2013); 4, *Sunxiuqinia* (Qu *et al.*, 2011; Takai *et al.*, 2013); 5, '*Mangrovibacterium*' (Huang *et al.*, 2013); 6, *Marinilabilia* (Krieg, 2011; Shalley *et al.*, 2013); 7, *Bacteroides* (Lan *et al.*, 2006; Robert *et al.*, 2007; Hayashi *et al.*, 2007; Chassard *et al.*, 2008; Ueki *et al.*, 2008, 2011; Nishiyama *et al.*, 2009; Watanabe *et al.*, 2010; Kim *et al.*, 2010; Kitahara *et al.*, 2011; Sakamoto & Ohkuma, 2013). +, Positive; -, negative; v, variable; ND, no data available; A, aerobic; F, facultatively anaerobic; An, strictly anaerobic.

Characteristic	1	2	3	4	5	6	7
Pigment	Light pink to tawny	Ivory or brownish ivory/unpigmented	White	White to orange–red	Orange–red	Pink to salmon/ reddish-orange	White to grey*
O ₂ metabolism	F	F	F	A/F	F	A/F	An
Motility	-	_	_	V	_	Gliding	_
Catalase	+	_	_	V	+	+	—†
Growth at:							
4 °C	_	_	+	V	_	_	ND
pH 5.5	+	-	+	+	_	—	ND
Hydrolysis of:							
Starch	_	+	+	_	_	V	$+\dagger$
Gelatin	+	+	+	_	+	V	—†
Utilization of:							
Lactose	_	+	ND	_	ND	ND	V
Arabinose	_	_	+	_	ND	ND	+
Respiratory quinones	МК-7	MK-7	MK-7, MK-7(H6)	MK-7	MK-7, MK-7(H2), MK- 7(H6), MK-9	МК-7	MK8, MK-9, MK-10, MK-11, MK12
DNA G+C content (mol%)	42.0	36	44.9	41.8-43.5	43.28	37–41	36.4–49

*Bacteroides faecis was pale yellow.

†>85 % Negative/positive.



Fig. 1. Neighbour-joining tree reconstructed with 16S rRNA gene sequences, showing the phylogenetic position of strain FH5^T within the class *Bacteroidia*. The tree was reconstructed using the neighbour-joining algorithm. The strains characterized in this study are shown in bold type. Numbers at nodes represent bootstrap values; only values greater than 50% are shown. GenBank accession numbers are given in parentheses. Bar, 0.05 substitutions per nucleotide position.

catalase-positive. NaCl is required for growth. The principal menaquinone is MK-7. The predominant cellular fatty acids are anteiso- $C_{15:0}$ and iso- $C_{15:0}$. The major polar lipids are phosphatidylethanolamine, an unkown phospholipid and an unknown lipid.

The type species is Draconibacterium orientale.

Description of *Draconibacterium orientale* sp. nov.

Draconibacterium orientale (o.ri.en.ta'le. L. neut. adj. orientale of or belonging to the east, oriental).

Displays the following properties in addition to those given in the genus description. Cells are 1.3–1.8 μ m in length and 0.3–0.5 μ m in width. Colonies on marine agar 2216 are light pink to tawny, smooth, circular, convex and 1.0– 1.5 mm in diameter after 4 days of incubation at 28 °C. Growth occurs at 20–40 °C and pH 5.5–9.0 and in the presence of 1–7 % (w/v) NaCl. Optimal growth is observed at 28–32 °C, pH 7.0–7.5 and with 2–4 % (w/v) NaCl. No growth is observed without NaCl. Fermentation of glucose occurs under anaerobic conditions with acid production, but no gas. Tween 80 is hydrolysed, but CM-cellulose, starch, sodium alginate and agar are not. In API 20E tests, negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, H₂S production and Voges-Proskauer reaction, but positive for gelatinase, tryptophan deaminase, indole production and Simmons' citrate utilization. Acid is produced from D-arabinose, Larabinose, D-fructose, D-galactose, D-mannose, trehalose, N-acetylglucosamine, cellobiose, starch, glycogen, melibiose, D-xylose, aesculin ferric citrate, inulin, sucrose, turanose, raffinose, melezitose, gentiobiose, potassium 5-keto-D-gluconate, methyl α-D-glucoside and methyl α-D-mannoside in API 50CHB strips. In API ZYM kits, activities of alkaline phosphatase, esterase lipase (C8), trypsin, α -galactosidase, β -galactosidase, naphthol-AS-BIphosphohydrolase, α -glucosidase, β -glucosidase, β -fucosidase and N-acetyl- β -glucosaminidase are positive and activities of leucine arylamidase and acid phosphatase are weakly positive. Negative for activities of esterase (C4), lipase (C14), valine arylamidase, cystine arylamidase, chymotrypsin, β -glucuronidase and α -mannosidase. The

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following results for carbon source assimilation are positive in Biolog GEN III MicroPlates: trehalose, cellobiose, sucrose, turanose, raffinose, D-fructose, L-fucose, L-rhamnose, Dsorbitol, L-lactic acid, α -ketobutyric acid, *N*-acetylneuraminic acid, gelatin, L-serine, propionic acid and acetic acid. The sole menaquinone is MK-7. The predominant cellular fatty acids are anteiso-C_{15:0}, iso-C_{15:0}, C_{17:0} 2-OH and iso-C_{17:0} 3-OH. In addition to the major polar lipids in the genus description, aminolipid and one unknown lipid are present in moderate and minor amounts.

The type strain, FH5^{T} (=DSM 25947^T=CICC 10585^T), was isolated from a sediment sample collected from the coastal area of Weihai, China. The genomic DNA G + C content of the type strain is 42.0 mol%. The type strain is resistant to nalidixic acid (30 µg), cotrimoxazole (25 µg), gentamicin (10 µg), kanamycin (30 µg), tobramycin (10 µg), neomycin (30 µg), polymyxin B (30 µg), amikacin (30 µg) and oxacillin (1 µg), but sensitive to nitrofurantoin (30 µg), acetylspiramycin (30 µg), midecamycin (30 µg), vancomycin (30 µg), chloramphenicol (30 µg), rocephin (30 µg), furazolidone (300 µg), novobiocin (30 µg), cefalexin (30 µg) and rifampicin (5 µg) as determined by antibiotic discs.

Description of Draconibacteriaceae fam. nov.

Draconibacteriaceae (Dra.co.ni.bac.te.ri.a.ce'ae. N.L. neut. n. *Draconibacterium* type genus of the family; suff. *-aceae* ending to denote family; N.L. pl. n. *Draconibacteriaceae* the *Draconibacterium* family).

Cells are gram-stain-negative, straight or curved rods, mesophilic, non-motile and non-endospore-forming. Chemoorganotrophic. The major respiratory quinone is MK-7. Phosphatidylethanolamine and at least one lipid are present as major polar lipids. Segregation of these organisms into a new family is justified by their distinct phyletic lineage based on 16S rRNA gene sequences and chemotaxonomic analyses for menaquinone. At present the family only contains the type genus *Draconibacterium*.

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