Marinifilum fragile gen. nov., sp. nov., isolated from tidal flat sediment

Hyunsoo Na, Seil Kim, Eun Young Moon and Jongsik Chun

Correspondence

Jongsik Chun

jchun@snu.ac.kr

School of Biological Sciences, Seoul National University, Shillim-dong, Kwanak-gu, Seoul 151-742, Republic of Korea

A facultatively anaerobic, moderately halophilic, Gram-negative, filamentous bacterium, designated JC2469^T, was isolated from tidal flat sediment in Korea. Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate belonged to the phylum *Bacteroidetes* and its closest taxonomic relative was *Cytophaga fermentans* NCIMB 2218^T (89.6% sequence similarity). Cells appeared as filaments during exponential growth, but became fragmented to rods at stationary phase. Spherical cells were also observed in aged cultures. Strain JC2469^T contained iso-C_{15:0} (56.2%) and MK-7 as the predominant fatty acid and respiratory quinone, respectively. On the basis of evidence from this polyphasic study, the isolate showed substantial differences from other genera. The phylogenetic and physiological data of the present study strongly suggest that the isolate represents a novel genus and species, for which the name *Marinifilum fragile* gen. nov., sp. nov. is proposed. The type strain of *Marinifilum fragile* is JC2469^T (=IMSNU 14138^T =KCTC 22488^T =JCM 15579^T).

Tidal flats have been shown to harbour highly diverse strains of bacteria (Kim *et al.*, 2004, 2005). During the study on culturable bacteria from a tidal flat in Korea, a novel bacterial strain, designated JC2469^T, was isolated from a tidal flat sediment sample from Yeocha, Ganghwa Island, South Korea (37° 37' N 126° 22' E). Following inoculation, enrichment was carried out in modified DSMZ medium 198 supplemented with 3.7 mM sodium acetate, incubated anaerobically at 30 °C. A pure culture of strain JC2469^T was obtained via serial dilution and plating on marine agar 2216 (MA; Difco) plates. As the isolate also showed growth under aerobic conditions, it was routinely cultivated aerobically on MA at 30 °C. Cultures were stored as glycerol suspensions (20%, v/v) at –85 °C.

DNA extraction, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chun & Goodfellow, 1995). The identification of phylogenetic neighbours was initially carried out using BLAST (Altschul *et al.*, 1997) and FASTA (Pearson & Lipman, 1988) against the database of prokaryotic type strains with validly published names using the EzTaxon server (Chun *et al.*, 2007). A preliminary similarity search indicated that our isolate belonged to the phylum *Bacteroidetes*. Alignment and phylogenetic analyses were carried out using jPHYDIT (Jeon *et al.*, 2005) and PAUP version 4.0 (Swofford, 1998) as described previously (Kim, *et al.*, 2005; Yi & Chun, 2006a). Phylogenetic trees were constructed according to the Fitch–Margoliash (Fitch & Margoliash,

1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. Pairwise evolutionary distances were computed according to the model of Jukes & Cantor (1969). Branch stability was evaluated by 1000 neighbour-joining replications (Felsenstein, 1985).

The results of a comprehensive phylogenetic analysis are shown in Fig. 1. On the basis of 16S rRNA gene sequence similarities, the closest relative was *Cytophaga fermentans* NCIMB 2218^T (89.6%), followed by *Alkaliflexus imshenetskii* Z-7010^T (87.9%), *Prolixibacter bellariivorans* F2^T (86.3%), *Paludibacter propionigenes* WB4^T (86.0%), *Zhouia amylolytica* HN-171^T (85.6%), *Aequorivita antarctica* SW49^T (84.9%) and *Actibacter sediminis* JC2129^T (84.4%). Strain JC2469^T formed a monophyletic clade with *C. fermentans* NCIMB 2218^T, though it was weakly supported, with a bootstrap value of 46%.

The Gram reaction was checked by standard Gram staining (Difco) and KOH tests (Powers, 1995). Cell morphology was examined by phase-contrast (Axioskop 40; Zeiss), scanning electron (JSM-5410LV; JEOL) and energy-filtering transmission electron (Libra 120; Zeiss) microscopes using 2-, 7- and 12-day-old cultures grown on MA at 30 °C. Motility was examined on semi-solid MA medium after incubation at 30 °C for 24 h. Gliding motility was checked by direct microscopic examination of the edges of colonies during exponential growth on MA, as described previously (Yi & Chun, 2006b). Heat resistance was examined by heating 2- or 3-week-old spherical cell-containing cultures at 80 °C for 10 min. Desiccation

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JC2469^T is FJ394546.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain JC2469^T among related species of the phylum *Bacteroidetes*. Bootstrap values (>50%) are shown at branch nodes. Solid circles indicate that the corresponding nodes are also recovered in Fitch–Margoliash, maximum-likelihood and maximum-parsimony trees. The sequence of *Halicomonobacter hydrossis* DSM 1100^T (GenBank accession no. AJ784892) was used as the outgroup (not shown). Bar, 0.05 substitutions per nucleotide position.

resistance was assessed as described by Wise *et al.* (2001). Growth under different conditions with a temperature-gradient incubator (TVS126MA; Advantec) was monitored by measuring the optical density at 600 nm. Growth was evaluated at various temperatures (10, 16, 20, 24, 27, 30, 34, 37, 41, 45, 49 and 55 °C) in marine broth. Growth at various pH values (pH 4, 5, 6, 7, 8, 9 and 10) was evaluated in marine broth adjusted with 6 M HCl or 10 M KOH. Salt requirement and tolerance were determined in ZoBell medium (Yi & Chun, 2006b) with artificial sea salts (Sigma) or NaCl (0, 1, 3, 5, 7 and 9%, w/v).

Cells of strain JC2469^T were Gram-negative, non-motile and non-gliding. Endospore formation was not observed throughout the entire phase of growth. Cells were filamentous during exponential growth, and then fragmented into rods (Fig. 2a, b). They later became spherical during the stationary phase. A similar morphological transition has been reported for *Alkaliflexus imshenetskii*, one of the isolate's phylogenetic neighbours (Zhilina *et al.*, 2004). The filaments and rods were generally 0.5 µm wide and variable in length. Transmission electron microscopy showed a 0.3–3.8 µm long flagella-like structure at the end of filaments (Fig. 2c), despite the fact that we were not able to find motile cells under light microscopy and the cells gave a negative result for the motility test based on semi-solid media. Ultrathin sections often exhibited bud-like cells at the end of the filaments (Fig. 2f). Our isolate was sensitive to heat and did not survive 1 week of desiccation. Colonies on MA plates appeared circular, in some cases with erose edges, and became ivory to brownish ivory coloured as they grew. Strain JC2469^T grew at 20–37.4 °C

(optimum 33 °C), at pH 6–8 (optimum pH 7) and at 1–7% (w/v) artificial sea salt concentration (optimum 3%). No growth was observed in the absence of sea salts or with NaCl alone.

Catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and Kovács' reagent (Kovács, 1956). Other enzyme activities were tested at 37 °C using API ZYM, substrate utilization and biochemical tests were carried out at 30 °C with API 50CH B/E and nitrate reduction, indole production and H₂S production tests were performed using API 20NE and API 20E (all from bioMérieux). Strain JC2469^T showed no reaction with the sea-salt-free API 50CH B/E medium. Therefore, the medium was amended with filter-sterilized artificial sea salts at a final concentration of 3% for strain JC2469^T and *C. fermentans* KCTC 2901^T. The API tests were performed for strain JC2469^T, *C. fermentans* KCTC 2901^T and *Paludibacter propionigenes* JCM 13257^T. Our isolate showed negative reactions for both catalase and oxidase. The results of biochemical and physiological tests are given in Table 1 and in the genus and species descriptions.

To determine the end products of glucose fermentation, the basal medium (Kim *et al.*, 2007) was slightly modified to contain the following (l⁻¹ distilled water): 1 g NH₄Cl, 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 30 g sea salts, 3.6 g glucose, 0.5 g cysteine hydrochloride, 0.3 g Na₂S·9H₂O, 1 mg resazurin (Sigma), 1 ml trace mineral element solution (DSMZ medium 318) and 1 ml vitamin solution (DSMZ medium 141). After 3 weeks of incubation at 30 °C, the fermentation end products were analysed with GC (ACME 6000GC; Young Lin) and HPLC (HP1100; Hewlett

Fig. 2. Electron micrographs of strain JC2469^T: scanning electron micrographs of cells in the exponential (a) and stationary (b) phases; transmission electron micrograph (c); ultrathin sections revealing the membrane structure of a filament (d) and a spherical cell (e); bud-like structures are often observed at one end of the filament (f). Bars, 5 μm (a, b), 0.5 μm (c) and 100 nm (d–f).

Packard) as described previously (Kim *et al.*, 2007). The G+C content was determined with HPLC of the deoxyribonucleosides as described by Mesbah & Whitman (1989), using a reversed-phase column (Supelcosil LC-18-S; Supelco). Isoprenoid quinones were extracted from 2-day-old cells grown on MA at 30 °C, according to the method of Minnikin *et al.* (1984), and analysed by reversed-phase TLC and HPLC as described by Collins (1985). Cellular fatty acids of the test strains were analysed as methyl esters by GC according to the instructions of the Microbial Identification System (MIDI). Fatty acid methyl esters were prepared from biomass grown on MA at 30 °C for 2 days.

Strain JC2469^T produced acetate and propionate as major fermentation products from glucose. The G+C content of strain JC2469^T was 45 mol%. The major respiratory quinone of JC2469^T was MK-7, as also reported for *C. fermentans* (Nakagawa & Yamasato, 1993), but different from those of *Paludibacter propionigenes* (MK-8; Ueki *et al.*, 2006) and *Z. amylolytica* (MK-6; Liu *et al.*, 2006). The major cellular fatty acids were iso-C_{15:0} (56.2 %), iso-

C_{15:0} 3-OH (9.1 %), iso-C_{17:0} 3-OH (7.0 %), iso-C_{13:0} (4.4 %) and anteiso-C_{15:0} (3.5 %). A comparison of the cellular fatty acids of strain JC2469^T and its phylogenetic neighbours is shown in Table 2.

Strain JC2469^T shows very low 16S rRNA gene sequence similarity (<90 %) to its closest neighbours in the phylum *Bacteroidetes*. Its dramatic morphological transformation during growth is a unique characteristic that can be used to differentiate it from its closest neighbours. Additionally, several other physiological and biochemical characteristics differentiate strain JC2469^T from related species (Table 1). On the basis of evidence presented here, it is proposed that the tidal flat isolate be classified in a novel genus in the phylum *Bacteroidetes* as *Marinifilum fragile* gen. nov., sp. nov.

Description of *Marinifilum* gen. nov.

Marinifilum (Ma.ri.ni.fi'lum. L. adj. *marinus* belonging to the sea; L. neut. n. *filum* a thread; N.L. neut. n. *Marinifilum* a thread belonging to or living in the sea).

Table 1. Comparison of major features of strain JC2469^T with its phylogenetic neighbours

Strains: 1, *Marinifilum fragile* gen. nov., sp. nov. JC2469^T (data from this study); 2, *Cytophaga fermentans* KCTC 2901^T (unless indicated, data from Reichenbach, 1989); 3, *Alkaliflexus imshenetskii* DSM 15055^T (Zhilina *et al.*, 2004); 4, *Prolixibacter bellariivorans* JCM 13498^T (Holmes *et al.*, 2007); 5, *Paludibacter propionigenes* JCM 13257^T (Ueki *et al.*, 2006); 6, *Zhouia amylolytica* JCM 14016^T (Liu *et al.*, 2006); 7, *Aequorivita antarctica* DSM 14231^T (Bowman & Nichols, 2002); 8, *Actibacter sediminis* JCM 14002^T (Kim *et al.*, 2008). +, Positive; –, negative; w, weak; ND, not detected/not reported.

Characteristic	1	2	3	4	5	6	7	8
Source of isolation	Marine environment	Marine environment	Soda lake	Marine environment	Rice field	Marine environment	Marine environment	Marine environment
Optimal temperature (°C)	33.8	30	35	22	30	30	20	37
Catalase	–	+	+	–	–	+	+	+
Utilization of:								
Arabinose	–	+*	+	–	+*	–	–	–
Cellobiose	–	+*	+	+	+*	+	–	ND
Fructose	–	+*	–	–	+*	+	–	ND
Galactose	+	–*	+	+	+*	+	–	ND
Gelatin	+	ND	–	+	–*	+	+	+
Glycerol	+	–*	–	–	–*	–	–	–
Glycogen	–	+*	+	ND	–*	+	ND	ND
Lactose	+	+*	–	+	–*	+	–	ND
Maltose	+	+*	+	+	+*	+	–	–
Mannose	–	+*	+	+	+*	w	–	–
Trehalose	–	–*	+	+	–*	+	–	ND
Turanose	–	–*	ND	+	+*	+	ND	–
Xylose	–	+*	+	+	+*	ND	ND	–
Fermentation end products from glucose†	A, P	A, P, S	ND	–	A, P, S	–	–	–
DNA G + C content (mol%)	45	39	44.3	44.9	39.3	34.5	38–39	43–45

*Data from this study.

†A, Acetate; P, propionate; S, succinate.

Gram-negative, oxidase-positive and catalase-negative. Cells are filamentous. Facultatively anaerobic, chemo-organotrophic and moderately halophilic. Ferment sugars. Cellular fatty acids are dominated by saturated iso-branched fatty acids. Possesses menaquinone MK-7 as the major respiratory quinone. Acetate and propionate are produced as major fermentation products from glucose. According to the 16S rRNA gene sequence of the type strain of the type species, the genus belongs to the phylum *Bacteroidetes*. The only known strain was isolated from tidal flat sediment. The DNA G + C content of the type strain of the type species is 45 mol%. The type species is *Marinifilum fragile*.

Description of *Marinifilum fragile* sp. nov.

Marinifilum fragile (fra'gi.le. L. neut. adj. *fragile* fragile).

Displays the following properties in addition to those given in the genus description. Cells are initially filamentous,

becoming spherical in aged cultures. Filaments are generally 0.5 µm wide and variable in length, with a 0.3–3.8 µm long flagella-like structure at the end. Heat sensitive. Colonies on MA are circular, in some cases with erose edges, and ivory or brownish ivory coloured. Growth occurs at pH 6–8 (optimum pH 7) and 20–37.4 °C (optimum 33 °C). Requires artificial 1–7% (w/v) sea salts (optimum 3%). Does not grow with 0–9% (w/v) NaCl alone. Utilizes glycerol, galactose, glucose, *N*-acetylglucosamine, maltose, lactose, melibiose and starch, but not turanose, D-xylose, fructose, gentiobiose, glycogen, mannose or mannitol. Produces β-galactosidase, gelatinase, alkaline phosphatase, trypsin, acid phosphatase and *N*-acetyl-β-glucosaminidase. Nitrate is reduced to nitrite. Indole is produced, but H₂S is not. The major fatty acid is iso-C_{15:0}.

The type strain is JC2469^T (=IMSNU 14138^T =KCTC 22488^T =JCM 15579^T), isolated from tidal flat sediment of Yeocha, Ganghwa Island, South Korea.

Table 2. Cellular fatty acid composition of strain JC2469^T and its phylogenetic neighbours

Strains: 1, *Marinifilum fragile* gen. nov., sp. nov. JC2469^T (data from this study); 2, *Cytophaga fermentans* KCTC 2901^T (this study); 3, *Alkaliflexus imshenetskii* DSM 15055^T (Zhilina *et al.*, 2004); 4, *Prolixibacter bellariivorans* JCM 13498^T (Holmes *et al.*, 2007); 5, *Paludibacter propionigenes* JCM 13257^T (Ueki *et al.*, 2006); 6, *Zhouia amylytica* JCM 14016^T (Liu *et al.*, 2006); 7, *Aequorivita antarctica* DSM 14231^T (Bowman & Nichols, 2002); 8, *Actibacter sediminis* JCM 14002^T (Kim *et al.*, 2008). Only fatty acids representing at least 1 % of the total fatty acids for at least one of the strains are shown. —, Not detected/not reported.

Fatty acid (%)	1	2	3	4	5	6	7	8
C _{13:0}	—	—	1.1	0.1	—	—	—	—
iso-C _{13:0}	4.4	—	1.4	0.1	0.3	—	—	6.4
C _{14:0}	0.2	9.0	—	0.2	2.8	2.5	—	—
iso-C _{14:0}	0.5	—	1.1	2.5	0.3	—	0.2	—
C _{15:0}	0.1	0.5	39.0	20.7	19.0	9.4	0.6	—
anteiso-C _{15:0}	3.5	—	11.6	23.3	30.8	1.1	15.7	3.7
iso-C _{15:0}	56.2	—	29.4	13.7	1.1	14.9	7.6	19.8
iso-C _{15:0} 3-OH	9.1	—	—	—	1.1	3.9	5.4	—
C _{15:0} 3-OH	—	—	2.4	0.5	2.6	—	—	2.1
C _{16:0}	0.3	18.0	1.0	5.6	4.9	4.9	1.9	—
anteiso-C _{17:0} 3-OH	—	—	—	—	17.9	—	2.9	—
iso-C _{17:0} 3-OH	7.0	—	—	—	6.2	—	2.0	13.7
C _{18:1} ω7c	—	12.5	—	—	—	—	—	—
Summed feature 3*	0.3	35.3	—	—	—	—	—	4.8

*Summed features are groups of two or three fatty acids that cannot be separated by GC with the MIDI system. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

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