Maritimimonas rapanae gen. nov., sp. nov., isolated from gut microflora of the veined rapa whelk, *Rapana venosa*

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A yellow-pigmented, Gram-negative, aerobic bacterial strain comprising rod-shaped cells devoid of flagellar and gliding motility, designated strain A31^T, was isolated from a veined rapa whelk (*Rapana venosa*) collected from the South Sea, Republic of Korea. Results from 16S rRNA gene sequence analysis indicated that the isolate belonged to the family *Flavobacteriaceae*; the highest level of nucleotide sequence similarity (92.6 %) was observed with *Tenacibaculum aestuarii* KCTC 12569^T. The predominant cellular fatty acids were iso-C_{15:1} G (24.2 %), iso-C_{15:0} (20.1 %) and iso-C_{17:0} 3-OH (11.2 %). Flexirubin-type pigments were absent. The major isoprenoid quinone was MK-6. The DNA G + C content was 31.7 mol%. Data from a polyphasic taxonomic study suggested that the isolate represents a novel species in a new genus of the family *Flavobacteriaceae*, for which the name *Maritimimonas rapanae* gen. nov., sp. nov. is proposed. The type strain of *Maritimimonas rapanae* is A31^T (=KCTC 22186^T =JCM 15075^T).

The family *Flavobacteriaceae* (Bernardet *et al.*, 2002; Reichenbach, 1989) is one of the largest phylogenetic groups within the phylum *Bacteroidetes* (Garrity & Holt, 2001). The family currently comprises more than 45 genera with validly published names, most of them originating from diverse environments including freshwater, brackish and marine waters, soil and epibenthic fauna. Members of the family are known to be proficient in degrading various biopolymers such as cellulose, chitin and pectin (Kirchman, 2002). Recently, phylogenetic analyses have revealed that many marine species in the family clustered together into a well-defined 'marine clade' (Bowman & Nichols, 2005). These marine flavobacteria are known to play a role in the utilization of high-molecular-mass dissolved organic matter (Kirchman, 2002). In the course of our study on gut microflora of the veined rapa whelk (*Rapana venosa*), a yellow-pigmented bacterial strain, designated A31^T, was isolated and subjected to taxonomic investigation. In this study, the description of a novel *Flavobacterium*-like bacterium that showed relatively low levels of 16S rRNA gene sequence similarity to members of the family *Flavobacteriaceae* with validly published names is reported.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A31^T is EU290161.

A Fitch–Margoliash tree based on 16S rRNA gene sequences and an SEM of cells of strain A31^T are available as supplementary material with the online version of this paper.

Strain A31^T was isolated using the standard dilution plating technique from a veined rapa whelk sample collected from the South Sea (Yeoja Bay; 34° 44' N 127° 44' E) off the coast of the Republic of Korea during July 2008. Isolation was achieved using marine agar 2216 (MA; Difco) (Yang *et al.*, 2007) at 25 °C for 7 days. The isolate was cultured routinely on MA and maintained at –80 °C as a suspension in marine broth 2216 (MB; Difco) containing glycerol (20 %, w/v).

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chun & Goodfellow, 1995). The resultant sequence of strain A31^T (1416 nt) was aligned manually against sequences obtained from GenBank. Phylogenetic trees were inferred from the regions available for all sequences (positions 22–1450; *Escherichia coli* numbering system) using the neighbour-joining (Saitou & Nei, 1987) and Fitch–Margoliash (Fitch & Margoliash, 1967) methods. Evolutionary distance matrices were generated according to Jukes & Cantor (1969). The resultant neighbour-joining tree topology was evaluated by means of bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out using the program jPHYDIT (available at <http://plaza.snu.ac.kr/~jchun/jphydit/>) and PAUP 4.0 (Swofford, 1998) as described previously (Chun *et al.*, 2000).

Preliminary sequence comparison with 16S rRNA gene sequences held in GenBank indicated that our isolate showed low levels of similarity to strains of known species of the family *Flavobacteriaceae*. Strain A31^T was most closely related to members of the genera *Tenacibaculum* (90.1–92.6% 16S rRNA gene sequence similarity), *Polaribacter* (89.8–91.4%), *Lutibacter* (90.9%), *Actibacter* (91.2%), *Lutimonas* (90.3%) and *Aestuariicola* (90.2%). No strains of other species with validly published names exceeded 90.5% 16S rRNA gene sequence similarity with strain A31^T. To elucidate the phylogenetic relationship between the novel isolate and other species of the family *Flavobacteriaceae*, phylogenetic trees were constructed by using two different tree-making algorithms. The neighbour-joining tree (Fig. 1) showed that strain A31^T formed a very robust clade with strains of *Tenacibaculum*, *Polaribacter*, *Lutibacter*, *Lutimonas*, *Actibacter* and *Aestuariicola* species, but could not be linked to any of the known genera in the family *Flavobacteriaceae*. The tree based on the Fitch–Margoliash method showed a similar topology (see Supplementary Fig. S1, available in IJSEM Online). The tree suggested that strain A31^T formed a monophyletic clade with those genera with 100% bootstrap support. Thus, strain A31^T should be recognized as a member of a new genus.

Cells grown for 7 days at 30 °C on MA were observed with phase-contrast (TMS-F; Nikon) and scanning electron (S-4800; Hitachi) microscopes. Gliding motility was investigated by examining the edge of a hanging drop of fresh MB

culture, as recommended by Bernardet *et al.* (2002). The requirement for sea salts (Sigma) for growth was determined at various concentrations (final concentrations of 0–12% in increments of 1%) on nutrient agar (NA; Difco), trypticase soy agar (TSA; Difco), R2A agar (Difco) and ZoBell's agar (ZoBell, 1941; 5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate and 15 g Bacto agar in 1 l distilled water). All media used in the physiological tests were supplemented with 4% sea salts unless MA or MB was used. The pH range for growth was determined in ZoBell's liquid medium. The medium was adjusted to pH 3–11 (in increments of 1 pH unit) by the addition of HCl/NaOH and sterilized by filtration. Growth temperatures (4–50 °C) and growth in an anaerobic chamber (CO₂/H₂/N₂, 10:10:80; Sheldon Manufacturing) were determined on MA after incubation for up to 1 week. The nature of growth on the surface, subsurface and bottom of tubes was determined in MB tubes cultivated for up to 14 days at 30 °C. Catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and Kovács' reagent (Kovács, 1956), respectively. Acid production from glucose was tested as described by Yamaguchi & Yokoe (2000). Nitrate reduction was tested in MB containing 0.2% KNO₃ (Skerman, 1967). H₂S production was determined on Kligler iron agar (Difco). Hydrolysis of CM-cellulose (1%, w/v), casein [2% (w/v) skimmed milk], chitin from crab shell (0.5%, w/v; Sigma), dextran (1%, w/v), egg yolk (10%, w/v), elastin (0.5%, w/v), starch (0.2%, w/v) and Tween 80 (1%, w/v) was tested as described by Smibert & Krieg (1994) using MA as the basal medium.

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain A31^T and members of the family *Flavobacteriaceae*. Numbers at nodes are levels of bootstrap support (%) based on neighbour-joining analyses of 1000 resampled datasets; only values greater than 50% are shown. The sequence of *Bacteroides fragilis* ATCC 25285^T (GenBank accession no. X83935) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.

Hydrolysis of L-tyrosine (0.5 %, w/v) and xylan (1 %, w/v) was tested using MA as the basal medium (Barrow & Feltham, 1993). Cellulase activity was tested by observing the disintegration of filter paper in an MB culture (Smibert & Krieg, 1994; Bernardet *et al.*, 2002). DNase activity was determined with DNase test agar (Difco). Other biochemical tests and enzyme activity determinations were performed using the API 20E and API ZYM kits (bioMérieux) and GN2 MicroPlates (Biolog) prepared according to the manufacturers' instructions except that bacterial strains were suspended in distilled water supplemented with 4 % sea salts. Antibiotic resistance was determined by the disc-diffusion method using commercial antibiotic-impregnated discs (BBL Becton Dickinson); after 5 days of incubation at 25 °C on MA, results were interpreted according to the guidelines set forth by the

CLSI (2003). The presence of flexirubin-type pigments was examined as described by Bernardet *et al.* (2002).

Colonies of strain A31^T on MA were yellow-pigmented, convex and did not adhere to the agar. Flexirubin-type pigments were not detected. Physiological, morphological and biochemical characteristics are described in Table 1 and in the species description. A number of phenotypic characteristics clearly distinguished strain A31^T from members of other genera in the family *Flavobacteriaceae*. Strain A31^T was oxidase-positive, whereas strains of *Lutibacter* and *Lutimonas* are negative. Strain A31^T was unable to hydrolyse aesculin or starch, unlike most members of related genera. Significant differences between strain A31^T and members of other genera were found in the utilization of carbon sources.

Table 1. Differential phenotypic characteristics of strain A31^T and members of related genera of the family *Flavobacteriaceae*

Taxa: 1, strain A31^T; 2, *Lutibacter litoralis* CL-TF09^T (data from Choi & Cho, 2006); 3, *Tenacibaculum* ($n=14$; Choi *et al.*, 2006; Frette *et al.*, 2004; Heindl *et al.*, 2008; Jung *et al.*, 2006; Piñeiro-Vidal *et al.*, 2008a, b; Sheu *et al.*, 2007; Wang *et al.*, 2008); 4, *Polaribacter* ($n=6$; Gosink *et al.*, 1998; Nedashkovskaya *et al.*, 2005; Yoon *et al.*, 2006); 5, *Actibacter sediminis* JC2129^T (Kim *et al.*, 2008); 6, *Lutimonas vermicola* IMCC1616^T (Yang *et al.*, 2007); 7, *Aestuariaicola saemankumensis* SMK-142^T (Yoon *et al.*, 2008). +, Positive; –, negative; v, variable; NA, data not available.

Characteristic	1	2	3	4	5	6	7
Cell morphology*	R	R	R	R/F	R	R	R/O/C
Gliding motility	—	—	v	—	—	+	—
Colony colour†	Y	Y	Y/PY	OR/SP	Y	Y	Y
Sea salts requirement	+	+	v	v	—	NA	+
Growth at:							
pH 6	+	—	v	NA	+	+	+
25 °C	+	+	+	—	+	+	+
37 °C	+	—	v	—	+	+	—
Oxidase	+	—	+	v	+	—	+
Nitrate reduction	—	—	v	v‡	—	—	—
Hydrolysis of:							
Aesculin	—	+	—§ ^a	v	+	+	—
Gelatin	+	+	v	v	+	+	—
Starch	—	+	v	+	+	+	+
Utilization of:							
Sucrose	—	—	v	v	+	+	—
L-Glutamate	+	—	v	—§ ^b	+	+	—
L-Leucine	—	+	v	—§ ^b	—	—	—
L-Proline	+	—	v	—§ ^b	—	+	—
Citrate	—	+	v	—§ ^b	+	+	—
Pyruvate	—	+	—§ ^c	—§ ^b	+	—	—
Succinate	—	+	v¶	—§ ^b	—	+	—
DNA G + C content (mol%)	31.7	33.9	29.8–35.2	30.0–33.2	43–45	40.1	37.2

*C, Cocci; F, filaments; O, ovals; R, rods.

†OR, Orange; PY, pale yellow; SP, salmon pink; Y, yellow.

‡All species except *P. glomeratus* are negative.

§Data not available for: a, *T. gallaicum* or *T. soleae*; b, *P. putkevichii*; c, *T. aiptasiae*, *T. adriaticum*, *T. gallaicum* or *T. soleae*.

||All type strains for which data are available are negative apart from that of *T. skagerrakense*, which is weakly positive. Data not available for *T. gallaicum* or *T. soleae*.

¶All type strains for which data are available are negative apart from that of *T. adriaticum*, which is positive. Data not available for *T. aestuarii*, *T. aiptasiae*, *T. gallaicum*, *T. lutimaris* or *T. soleae*.

Table 2. Cellular fatty acid compositions (%) of strain A31^T and members of related genera in the family *Flavobacteriaceae*

Taxa: 1, strain A31^T; 2, *Lutibacter litoralis* CL-TF09^T (data from Choi & Cho, 2006); 3, *Tenacibaculum* ($n=10$; Choi *et al.*, 2006; Frette *et al.*, 2004; Hansen *et al.*, 1992; Jung *et al.*, 2006; Piñeiro-Vidal *et al.*, 2008b; Sheu *et al.*, 2007; Wakabayashi *et al.*, 1986; Yoon *et al.*, 2005); 4, *Polaribacter* ($n=6$; Gosink *et al.*, 1998; Nedashkovskaya *et al.*, 2005; Yoon *et al.*, 2006); 5, *Actibacter sediminis* JC2129^T (Kim *et al.*, 2008); 6, *Lutimonas vermicola* IMCC1616^T (Yang *et al.*, 2007); 7, *Aestuariicola saemankumensis* SMK-142^T (Yoon *et al.*, 2008). Fatty acid data are not available for *T. amylolyticum*, *T. discolor*, *T. gallaicum* or *T. ovolyticum*. Values are percentages of total fatty acids. tr, Trace amount (<1 %); ECL, equivalent chain-length. All strains studied except *Polaribacter* strains were grown on MA.

Fatty acid	1	2	3	4	5	6	7
Straight-chain saturated							
C _{13:0}					6.4		
C _{15:0}	3.3	1.7	2.7–8.9 ^{*bc}	2.3–8.1 ^{†hi}	19.8	3.8	4.0
C _{16:0}	3.8		tr–1.8 ^{*a}		1.2		
C _{18:0}	3.1	1.3	tr–1.4 ^{†de}				
C _{15:0} 2-OH	tr	2.1	tr–2.5		2.2		1.2
C _{15:0} 3-OH	tr	1.5	2.7–8.6 ^{*d}	12.0–38.0	2.1		
C _{16:0} 3-OH	2.2		tr–5.4				
C _{17:0} 2-OH	tr	3.6	tr ^{*abefg}		1.6		tr
C _{17:0} 3-OH	tr		tr–2.5 ^{*ag}		2.9		
Branched							
iso-C _{13:0}	tr	tr	tr–1.8 ^{*b}	2.0–16.4		1.0	6.5
iso-C _{14:0}	tr	2.2	tr–2.4 ^{*b}				tr
iso-C _{15:0}	20.1	16.7	9.5–25.3	9.0–22.0		16.5	21.3
iso-C _{15:1}			5.3–13.1	6.0–15.4 ^{*h}		6.7	
iso-C _{16:0}	2.5	tr	tr–3.8			2.5	1.4
iso-C _{16:1}			tr–2.4 ^{*e}				
iso-C _{15:0} 3-OH	7.8	17.4	4.6–19.8			3.2	7.4
iso-C _{16:0} 3-OH	4.5	13.4	2.8–12.8	5.0 ^{†i}	1.2	3.1	3.3
iso-C _{17:0} 3-OH	11.2	3.9	2.9–14.9	8.4 ^{†i}	13.7	8.8	12.5
iso-C _{15:1} F							1.2
iso-C _{15:1} G	24.2	4.2			14		13.6
iso-C _{16:1} H		1.3					1.2
iso-C _{17:1} ω9c		tr	tr–2.0 ^{*efg}		3.7	8.2	3.7
anteiso-C _{13:0}		1.4					tr
anteiso-C _{15:0}	1.3	15.1	tr–1.8 ^{*bf}	4.0–6.0 ^{*hi}	3.7	10.9	1.9
anteiso-C _{15:1}						1.0	
anteiso-C _{15:1} A	tr	1.6			1.2		tr
Unsaturated							
C _{15:1} ω6c		1.5	1.6–12.2 ^{*f}	3.0–9.0	5.2	1.4	1.9
C _{17:1} ω6c	tr	1.0	tr–2.0		2.3		2.6
C _{17:1} ω8c					1.7		
C _{18:1} ω5c			1.2 ^{†a}				
C _{18:3} ω6c			1.5 ^{†d}				
Unknown							
ECL 11.543	tr	2.3					tr
ECL 13.565	tr		tr–1.9 ^{†acd}				1.9
ECL 16.582			tr–1.3				
Summed features[‡]							
1		1.1	1.2 ^{†a}				
3	7.5	1.0	11.0–24.4	6.6 ^{†i}	4.8	7.1	7.6
4			1.3 ^{†d}				1.3

*Not detected in the following species: a, *T. adriaticum*; b, *T. aptasiae*; c, *T. litopenaei*; d, *T. litoreum*; e, *T. maritimum*; f, *T. skagerrakense*; g, *T. soleae*; h, *P. butkevichii*; i, *P. dokdonensis*.

†Detected in the following species: a, *T. adriaticum*; c, *T. litopenaei*; d, *T. litoreum*; e, *T. maritimum*; g, *T. soleae*; h, *P. butkevichii*; i, *P. dokdonensis*.

‡Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 1 contained iso-C_{15:1} and/or C_{13:0} 3-OH; summed feature 3 contained C_{16:1} ω7c and/or iso-C_{15:0} 2-OH; summed feature 4 contained iso-C_{17:1} I and/or anteiso-C_{17:1} B.

Cells of strain A31^T grown on MA for 2 days at 30 °C were prepared (in duplicate) and analysed for fatty acids by GLC according to the instructions of the Microbial Identification System (MIDI, 1999). For G+C content calculations, DNA samples were prepared in triplicate and G+C content was determined by the thermal denaturation method of Marmur & Doty (1962). Isoprenoid quinones were isolated by using the method of Minnikin *et al.* (1984) and were analysed by HPLC (Varian) as described by Collins (1985).

The fatty acid profiles of strain A31^T and related members of the family *Flavobacteriaceae* are shown in Table 2. The fatty acid profile of strain A31^T (>1.0 % of total fatty acids) included the branched fatty acids iso-C_{15:0} (20.1 %), iso-C_{16:0} (2.5 %) and anteiso-C_{15:0} (1.3 %), the hydroxy fatty acids iso-C_{17:0} 3-OH (11.2 %), iso-C_{15:0} 3-OH (7.8 %), iso-C_{16:0} 3-OH (4.5 %) and C_{16:0} 3-OH (2.2 %), the straight-chain fatty acids C_{16:0} (3.8 %), C_{15:0} (3.3 %) and C_{18:0} (3.1 %), the unsaturated fatty acid iso-C_{15:1} G (24.2 %) and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 7.5 %). Significant differences were found in the levels of iso-C_{15:1} G, anteiso-C_{15:0} and summed feature 3 in strain A31^T and members of related genera. The DNA G+C content of strain A31^T was 31.7 ± 0.4 mol%, a value within the range reported for members of the family *Flavobacteriaceae*. The predominant isoprenoid quinone of strain A31^T was MK-6, and small amounts of MK-7 and MK-8 were also found (MK-6/MK-7/MK-8 ratio 91:5:4), as observed in all members of the family *Flavobacteriaceae*.

On the basis of the data from this polyphasic study, strain A31^T represents a novel species in a new genus in the family *Flavobacteriaceae*, for which the name *Maritimimonas rapanae* gen. nov., sp. nov. is proposed.

Description of *Maritimimonas* gen. nov.

Maritimimonas (Ma.ri'ti.mi.mo'nas. L. adj. *maritimus* of the sea, marine; L. fem. n. *monas* a unit, a monad; N.L. fem. n. *Maritimimonas* a monad from the sea, pertaining to the habitat of the animal that harboured the type species, a marine rock).

Cells are rod-shaped, non-flagellated, yellow-pigmented and non-gliding. Gram-negative. Aerobic, chemoheterotrophic and mesophilic. Oxidase- and catalase-positive. Spores are not formed. Flexirubin-type pigments are absent. The major isoprenoid quinone is MK-6. The predominant cellular fatty acids are iso-C_{15:0}, iso-C_{15:1} G and iso-C_{17:0} 3-OH. Based on 16S rRNA gene sequence analysis, the genus is a member of the family *Flavobacteriaceae*. The type species is *Maritimimonas rapanae*.

Description of *Maritimimonas rapanae* sp. nov.

Maritimimonas rapanae (ra.pa'na.e. N.L. gen. n. *rapanae* of *Rapana*, the generic name of the mollusc from which the type strain was isolated).

Cells are 0.3–0.4 µm wide and 0.8–1.2 µm long and sometimes show filamentous growth (Supplementary

Fig. S2). Colonies on MA are circular, smooth, convex, yellow-pigmented and glistening with entire margins, do not adhere to the agar and are approximately 2.0 mm in diameter after 7 days at 30 °C (pH 7). Growth occurs at 10–37 °C (optimum, 30 °C) and pH 5–9 (optimum, pH 7). Growth occurs in media (ZoBell's, TSA, R2A and NA) containing 3–6 % sea salts (optimum, 4–5 %), but not in media supplemented with NaCl (1–12 %) alone. Cells grow in the bottom of tubes as viscous sediments. Nitrate is not reduced to nitrite. Indole and H₂S are not produced. Gelatin is degraded, but aesculin, DNA, agar, casein, CM-cellulose, crystalline cellulose (filter paper), chitin, starch and Tween 80 are not. In API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, leucine arylamidase and valine arylamidase activities are present, cystine arylamidase and trypsin activities are weakly positive and α-glucosidase, lipase (C14), α-galactosidase, β-galactosidase, β-glucosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-fucosidase and α-mannosidase activities are absent. Acid is produced from glucose. In GN2 MicroPlate (Biolog) tests, D-glucose, L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-glutamic acid, hydroxy-L-proline, L-ornithine, L-serine and L-threonine are utilized. Sensitive to (µg per disc, unless indicated) chloramphenicol (30), erythromycin (15), tetracycline (30) and vancomycin (30); resistant to amikacin (30), ampicillin (10), gentamicin (10), kanamycin (30), nalidixic acid (30), penicillin (10 IU), polymyxin B (300 IU) and streptomycin (10). The predominant cellular fatty acids are iso-C_{15:1} G, iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{15:0} 3-OH and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c). The DNA G+C content of the type strain is 31.7 mol%.

The type strain is A31^T (=KCTC 22186^T =JCM 15075^T), isolated from a sample from a veined rapa whelk (*Rapana venosa*) collected from the South Sea, Republic of Korea.

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