Tenacibaculum crassostreae sp. nov., isolated from the Pacific oyster, *Crassostrea gigas*

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A rod-shaped, yellow-pigmented, aerobic, Gram-negative bacterium, designated strain JO-1^T, was isolated from an apparently healthy Pacific oyster, *Crassostrea gigas*, collected at Wan Island, Korea. It grew at 15–37 °C (optimum 30 °C) only in the presence of sea salts. Strain JO-1^T hydrolysed casein, Tween 80 and starch. The major fatty acids were iso-C_{15:0} (23.8%), summed feature 3 (comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 14.5%) and iso-C_{15:1} G (14.1%). Analysis of the 16S rRNA gene sequence indicated that strain JO-1^T was a member of the genus *Tenacibaculum* in the family *Flavobacteriaceae*, with sequence similarity of 94.6–97.8% to the type strains of recognized members of the genus. The G + C content of the genomic DNA was 31.4 mol%. DNA–DNA relatedness levels between strain JO-1^T and the five closest relatives, *Tenacibaculum litoreum* KCCM 42115^T, *T. lutimaris* KCTC 12302^T, *T. aestuarii* KCTC 12569^T, *T. mesophilum* DSM 13764^T and *T. adriaticum* JCM 14633^T, were less than 28%. Phylogenetic analyses and differences in physiological and biochemical characteristics suggested that strain JO-1^T (=KCTC 22329^T =JCM 15428^T) should be classified as the type strain of a novel species within the genus *Tenacibaculum*, for which the name *Tenacibaculum crassostreae* sp. nov. is proposed.

The genus *Tenacibaculum*, proposed by Suzuki *et al.* (2001) to reclassify two former species of *Flexibacter*, belongs to the family *Flavobacteriaceae*. At the time of writing, the genus contains 14 species with validly published names, including the recently described species *Tenacibaculum discolor* and *T. gallaicum* (Piñeiro-Vidal *et al.*, 2008a), *T. adriaticum* (Heindl *et al.*, 2008), *T. aiptasiae* (Wang *et al.*, 2008) and *T. soleae* (Piñeiro-Vidal *et al.*, 2008b). *Tenacibaculum* strains are widespread in the marine environment and have been isolated from marine animals (Wakabayashi *et al.*, 1986; Hansen *et al.*, 1992; Suzuki *et al.*, 2001; Piñeiro-Vidal *et al.*, 2008a, b; Heindl *et al.*, 2008; Wang *et al.*, 2008), macroalgae (Suzuki *et al.*, 2001), tidal flats (Yoon *et al.*, 2005; Choi *et al.*, 2006; Jung *et al.*, 2006) and seawater (Frette *et al.*, 2004; Sheu *et al.*, 2007).

Tenacibaculum maritimum, *T. gallaicum*, *T. discolor* and *T. ovolyticum* are pathogenic for many farmed marine fish (Piñeiro-Vidal *et al.*, 2008c). In particular, *T. maritimum* is the causative agent of the well-known bacterial disease tenacibaculosis, which causes severe necrotic and ulcerative lesions on the body surface of a variety of valuable marine fish worldwide (Avendaño-Herrera *et al.*, 2006). *T. soleae* and *T. aiptasiae* were isolated from a diseased sole and sea anemone, respectively. In this study, a bacterium, designated strain JO-1^T, was isolated from an apparently healthy Pacific oyster, *Crassostrea gigas*, and its taxonomic position was investigated using a polyphasic approach.

Strain JO-1^T was isolated from a homogenized oyster sample collected from Wan Island (34° 20' N 126° 44' E), Korea. The sample was serially diluted with sterile artificial seawater (Lyman & Fleming, 1940), spread on marine agar 2216 (MA; Difco) and incubated at 30 °C for 3 days. A colony was selected and subcultured on MA for further characterization. The isolate was preserved in marine broth 2216 (MB) supplemented with 20% (v/v) glycerol at –80 °C.

The GenBank accession number for the 16S rRNA gene sequence of strain JO-1^T is EU428783.

A scanning electron micrograph of cells of strain JO-1^T is available as a supplementary figure with the online version of this paper.

Table 1. Differential phenotypic characteristics of strain JO-1^T and the type strains of other *Tenacibaculum* species

Strains: 1, *Tenacibaculum crassostreae* sp. nov. JO-1^T; 2, *T. adriaticum* B390^T; 3, *T. discolor* LL04 11.1.1^T; 4, *T. gallaicum* A37.1^T; 5, *T. litopenaei* B-I^T; 6, *T. amylolyticum* MBIC4355^T; 7, *T. aestuarii* SMK-4^T; 8, *T. litoreum* CL-TF13^T; 9, *T. lutimaris* TF-26^T; 10, *T. maritimum* NCIMB 2154^T; 11, *T. mesophilum* MBIC1140^T; 12, *T. ovolyticum* IAM 14318^T; 13, *T. skagerrakensense* D30^T; 14, *T. soleae* LL04 12.1.7^T; 15, *T. aiptasiae* a4^T. Data from Suzuki *et al.* (2001), Frette *et al.* (2004), Yoon *et al.* (2005), Choi *et al.* (2006), Jung *et al.* (2006), Sheu *et al.* (2007), Heindl *et al.* (2008), Piñeiro-Vidal *et al.* (2008a, b) and Wang *et al.* (2008). +, Positive; −, negative; ND, no data available; NG, no growth; W, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Gliding motility	+	ND	+	+	+	+ ^{a*}	+	+	+ ^a	+ ^a	+ ^a	+ ^a	− ^a	+	+
Pigmentation†	TY	TY	BY	BY	Y	Y	PY	PY	PY	PY	Y	PY	BY	Y	PY
Occurrence of spherical cells in ageing broth‡	R	ST	R	R	VR	VR ^a	ND	VR	VR	F ^a	VR ^a	NO	F	F	VR
Ranges for growth															
NaCl (%)	NG	1–5	NG	NG	2–10	3 (W)	<7	3–5	<8 ^a	NG	1–7	NG	NG	NG	1–10
Seawater (%)§	75–175	12.5–175	30–100	30–100	ND	50–100	ND	25–250	25–175 ^a	30–100	10–100	70–100	25–150	55–100	ND
pH	6.0–7.0	5.0–9.0	6.0–8.0	6.0–8.0	5.0–10.0	5.3–8.3	5.5–8.5	6.0–10.0	7.0–8.0	5.9–8.6	5.3–9.0	5.9–9.0	6.0–9.0	6.0–8.0	7.0–9.0
Temperature (°C)	15–37	5–33.5	14–38	14–38	10–39	20–35	9–41	5–40	10–39	15–34	15–40	4–25	10–40	14–30	8–40
Temperature optimum (°C)	30	18–26	25–30	25–30	28–37	27–30	30–37	35–40	30–37	30	28–35	ND	25–37	22–25	30–35
Nitrate reduction	−	−	+	+	−	W	−	+	−	+	−	+	+	ND	−
Hydrolysis of:															
Chitin	−	−	ND	ND	+	−	ND	ND	ND	−	−	+	− ^b	ND	−
Starch	+	+	−	−	+	+	−	+	−	−	−	−	+	−	+
Gelatin	+	+	+	+	+	+	+	+	+	−	+	+	ND	+	+
Tween 80	+	−	−	−	−	+ ^a	+	+	−	+ ^a	+ ^a	+ ^a	− ^a	−	+
API ZYM tests															
Lipase (C14)	−	ND	+	+	+	ND	−	−	− ^a	W ^a	ND	ND	ND	+	+
Trypsin	+	ND	+	+	−	ND	−	+	+	W ^a	ND	ND	ND	−	+
Naphthol-AS-BI-phosphohydrolase	+	ND	+	+	+	ND	+	+	+ ^a	W ^a	ND	ND	ND	−	+
Utilization of:															
Citrate	−	−	ND	ND	−	− ^a	ND	−	ND	− ^a	− ^a	− ^a	+ ^a	ND	−
L-Leucine	−	W	ND	ND	−	− ^a	−	−	−	− ^a	− ^a	− ^a	W	ND	−
L-Proline	+	+	+	+	+	+	−	+	−	−	+	−	+ ^a	−	−
L-Glutamate	+	+	+	+	+	+	−	−	−	W ^a	+	−	+ ^a	−	+
DL-Aspartate	+	+	ND	ND	−	−	−	−	−	−	+	−	+	ND	+
α-D-Glucose	−	+	−	−	+	ND	−	−	− ^a	ND	ND	ND	+ ^a	−	−
Sucrose	−	ND	−	−	−	−	−	−	−	−	−	−	+	−	−
DNA G + C content (mol%)	31.4	31.6	32.1	32.7	35.2	30.9	33.6	30	32.3–32.8	31.3–32.5	31.6–32.0	30.3–32.0	35.2	29.8	35.0

*Data from: *a*, Choi *et al.* (2006); *b*, Sheu *et al.* (2007).

†BY, Bright yellow; PY, pale yellow; TY, translucent yellow; Y, yellow.

‡F, Frequent; R, rare; VR, very rare; ST, in stationary phase; NO, not observed.

§Calculated on the basis of 100 % seawater being equivalent to 40 g artificial sea salts l^{−1} (Frette *et al.*, 2004).

||Ranges of values represent data for four (*T. lutimaris* and *T. mesophilum*), three (*T. ovolyticum*) or two (*T. maritimum*) strains, including the respective type strains.

The minimal standards for describing new taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002) were used to describe strain JO-1^T. The optimal temperature and temperature range for growth was tested on MA at 4, 10, 15, 20, 25, 30, 35, 37, 40, 42, 45 and 50 °C. Tolerance to sea salts was determined on ZoBell agar [containing (l⁻¹ distilled water): 5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate, 15 g Bacto agar (ZoBell, 1941)] with 0–10 % (w/v) sea salts (Sigma) at 1.0 % intervals. Growth with NaCl as the sole salt was tested on ZoBell agar supplemented with 3 % NaCl. The pH range for growth was assessed on ZoBell agar containing 2 % sea salts and adjusted to pH 3.0–11.0 (at 1.0 pH unit intervals) with HCl or NaOH before sterilization. Only minor changes in pH were noticed after sterilization. Cell morphology was examined by scanning electron microscopy (S-4800; Hitachi) using cells grown overnight at 30 °C in MB. Gliding motility was tested by the hanging-drop method (Suzuki *et al.*, 2001). Anaerobic growth on MA was tested using the AnaeroPack-Anaero (Mitsubishi Gas Chemical) for up to 10 days. Catalase and oxidase activities were determined using standard methods (Tindall *et al.*, 2007). Hydrolysis of casein, chitin, starch and Tween 80 was examined using MA supplemented with 1 % substrate as described by Hansen & Sørheim (1991). Antibiotic resistance was determined with the disc diffusion method using commercial antibiotic-impregnated discs (BBL; Becton Dickson). The results were interpreted according to the guidelines described by the CLSI (2003). The presence of flexirubin-type pigments was examined as described by Bernardet *et al.* (2002). Other biochemical characteristics of strain JO-1^T were determined using API 20E, API 20NE and API ZYM test kits (bioMérieux) and the utilization of different carbon sources was tested with the GN2 MicroPlate system (Biolog). All kits were used according to the manufacturers' instructions and the bacterial suspensions were prepared in artificial seawater or 2 % sea salts. Detailed results of morphological, physiological and

biochemical tests are given in the species description and in Table 1.

Bacterial genomic DNA was extracted using a commercial kit (Bioneer). The 16S rRNA gene sequence was amplified by using the universal bacterial primers 27f and 1525r (Lane, 1991) and the purified PCR product was sequenced. The almost-complete gene sequence from strain JO-1^T (1394 bp) was aligned with the CLUSTAL W program (Thompson *et al.*, 1994) against sequences obtained from the GenBank database. Phylogenetic trees were inferred from the regions available for all sequences using the Fitch–Margoliash (Fitch & Margoliash, 1967) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices were generated according to Jukes & Cantor (1969). The tree topologies obtained were evaluated by bootstrap analyses (Felsenstein, 1981) of the neighbour-joining method based on 1000 resamplings. The alignment and phylogenetic analysis were carried out using the jPHYDIT program (available at <http://chunlab.snu.ac.kr/jphydit/>) and PAUP 4.0 (Swofford, 1998) as described by Chun *et al.* (2000).

Initial similarity analyses of 16S rRNA gene sequences indicated that strain JO-1^T was closely related to members of the genus *Tenacibaculum* as follows: *T. litoreum* CL-TF13^T (97.8 %), *T. discolor* LL04 11.1.1^T (97.7 %), *T. adriaticum* B390^T (97.6 %), *T. lutimaris* TF-26^T (97.1 %), *T. aiptasiae* a4^T (96.9 %), *T. soleae* LL04 12.1.7^T (96.9 %), *T. aestuarii* SMK-4^T (96.8 %), *T. mesophilum* MBIC1140^T (96.7 %), *T. gallaicum* A37.1^T (96.6 %), *T. litopenaei* B-1^T (95.7 %), *T. skagerrakense* D30^T (95.6 %), *T. amyolyticum* MBIC4355^T (95.2 %), *T. ovolyticum* IAM 14318^T (94.7 %) and *T. maritimum* IFO 15946^T (94.6 %). Strain JO-1^T occupied a distinct position in the neighbour-joining tree (Fig. 1). The tree based on the Fitch–Margoliash method showed essentially the same topology (not shown).

The DNA G+C content was 31.4 mol% as determined using the thermal denaturation method (Marmur & Doty,

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain JO-1^T, other members of the genus *Tenacibaculum* and related genera of the family *Flavobacteriaceae*. Percentages at nodes are levels of bootstrap support (>50 %) based on neighbour-joining analyses of 1000 resampled datasets. The sequence of *Cellulophaga lytica* ATCC 23178^T (GenBank accession no. M62796; not shown) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

1962). For fatty acid analysis, cells grown on MA at 28 °C for 48 h were prepared and analysed by GLC according to the instructions of the Microbial Identification System (MIDI Inc., 1999). The predominant cellular fatty acids of strain JO-1^T were iso-C_{15:0} (23.7%), summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 14.5%) and iso-C_{15:1} G (14.1%). The detailed fatty acid profile of strain JO-1^T is compared with those of members of the genus *Tenacibaculum* in Table 2.

DNA–DNA hybridization was performed by the membrane filter technique using the DIG High Prime DNA Labelling and Detection Starter kit II (Roche Molecular Biochemicals) according to the method described in detail by Lee *et al.* (2003), with the modification that the hybridization temperature was 45 °C. Strain JO-1^T showed relatively low DNA–DNA relatedness to *T. adriaticum* JCM 14633^T (27.7%), *T. litoreum* KCCM 42115^T (27.7%), *T. aestuarii* KCTC 12569^T (26.6%), *T. lutimaris* KCTC

Table 2. Cellular fatty acid compositions of strain JO-1^T and the type strains of other *Tenacibaculum* species

Strains: 1, *T. crassostreae* sp. nov. JO-1^T; 2, *T. adriaticum* B390^T; 3, *T. litopenaei* B-I^T; 4, *T. aestuarii* SMK-4^T; 5, *T. litoreum* KCCM 42115^T; 6, *T. lutimaris* TF-26^T; 7, *T. maritimum* JCM 8137^T; 8, *T. mesophilum* MBIC1140^T; 9, *T. skagerrakense* DSM 14836^T; 10, *T. soleae* LL04 12.1.7^T; 11, *T. aptasiae* a4^T. Data from Yoon *et al.* (2005), Choi *et al.* (2006), Jung *et al.* (2006), Sheu *et al.* (2007), Heindl *et al.* (2008), Piñeiro-Vidal *et al.* (2008c) and Wang *et al.* (2008). No data available for *T. amylolyticum*, *T. discolor*, *T. gallaicum* or *T. ovolyticum*. Values are expressed as percentages of total fatty acids, with those amounting to less than 1% in all strains not listed. Some strains were grown under different culture conditions. –, Not detected; tr, trace (<1%); ECL, equivalent chain-length.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
Straight-chain											
C _{15:0}	2.6	2.7	—	6.1	2.7	8.9	2.9	3.6	4.9	4.8	—
C _{16:0}	5.4	—	1.8	tr	tr	tr	tr	tr	tr	tr	1.1
C _{18:0}	3.8	—	—	—	tr	—	1.4	—	—	—	—
Branched											
iso-C _{13:0}	tr	1.6	tr	1.3	1.4	tr	1.8	tr	tr	tr	—
iso-C _{14:0}	1.2	tr	tr	2.2	tr	1.7	tr	tr	tr	2.4	—
iso-C _{15:0}	23.8	25.3	22.0	18.9	18.8	17.2	16.8	13.2	tr	23.1	12.9
anteiso-C _{15:0}	1.7	tr	tr	1.3	1.8	tr	tr	1.1	—	tr	—
iso-C _{15:1} G	14.1	—	—	—	—	—	—	—	—	—	—
iso-C _{15:1}	—	13.1	8.7	8.7	8.2	5.3	7.6	7.1	8.2	5.7	9.5
iso-C _{16:0}	2.2	tr	1.8	2.0	2.3	3.8	tr	1.7	1.3	1.7	2.8
iso-C _{16:1}	—	tr	1.6	2.3	1.3	1.7	—	tr	1.7	2.4	2.3
iso-C _{17:1} ω9c	tr	2.0	1.6	tr	1.6	tr	—	tr	—	—	1.0
Unsaturated											
C _{15:1} ω6c	tr	5.6	1.6	3.0	1.7	4.2	2.2	1.6	—	12.2	3.3
C _{17:1} ω6c	tr	tr	1.9	1.6	tr	1.5	tr	tr	1.2	1.7	2.0
C _{18:1} ω5c	tr	1.2	—	—	—	—	—	—	—	—	—
C _{18:3} ω6c	—	—	—	—	1.5	—	—	—	—	—	—
Hydroxy											
C _{15:0} 2-OH	tr	tr	tr	tr	tr	1.2	1.1	1.1	2.5	tr	1.9
C _{15:0} 3-OH	tr	3.2	2.7	4.2	—	3.4	3.8	2.9	8.6	3.2	4.4
C _{16:0} 3-OH	1.6	tr	5.4	1.0	1.6	1.3	1.5	3.2	2.1	2.2	3.8
C _{17:0} 3-OH	tr	—	1.0	tr	tr	tr	tr	tr	2.5	—	1.8
iso-C _{15:0} 3-OH	5.4	13.7	4.6	6.1	6.6	4.6	19.8	8.0	7.8	10.6	6.9
iso-C _{16:0} 3-OH	6.6	2.8	3.4	12.3	6.8	12.8	5.0	9.0	12.2	8.4	10.2
iso-C _{17:0} 3-OH	6.7	10.8	12.7	9.6	13.6	8.4	13.7	14.9	11.7	2.9	9.9
Unknown											
ECL 13.565	1.3	tr	1.9	—	1.3	—	—	—	—	tr	—
ECL 16.582	tr	tr	tr	1.0	1.3	tr	1.0	1.0	tr	tr	1.0
Summed features*											
1	—	1.2	—	—	—	—	—	—	—	—	—
3	14.5	11.8	21.3	11.9	19.6	18.1	17.9	24.4	22.5	11.0	19.6
4	—	—	—	—	1.3	—	—	—	—	—	—

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 1 contained one or more of iso-C_{15:1} H, iso-C_{15:1} I and 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.

12302^T (21.0%) and *T. mesophilum* DSM 13764^T (19.9%).

On the basis of the phenotypic and molecular genetic data, it is suggested that strain JO-1^T represents a novel species of the genus *Tenacibaculum*, for which the name *Tenacibaculum crassostreae* sp. nov. is proposed.

Description of *Tenacibaculum crassostreae* sp. nov.

Tenacibaculum crassostreae (cra.sso.stre'ae. N.L. gen. n. *crassostreae* pertaining to *Crassostrea*, the scientific genus name of the Pacific oyster, *C. gigas*, from which the type strain was isolated).

Cells are Gram-negative, aerobic, straight rods, motile by gliding, approximately 0.3 µm in diameter and 1.5–5.0 µm long. On MA, colonies are translucent yellow, circular, low-convex, with regular edges. After 5 days on MA at 30 °C, colonies are approximately 5 mm in diameter. No growth occurs with NaCl only. Growth occurs with 3–7% (w/v) sea salts. Growth occurs at 15–37 °C (optimum 30 °C) but not at 40 °C. The pH range for growth is pH 6.0–7.0. Flexirubin-type pigments are absent. Positive for catalase and oxidase. Casein, starch and Tween 80 are hydrolysed, but chitin is not hydrolysed. According to API ZYM tests, activities for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, whereas activities for alkaline phosphatase, lipase (C14), cystine arylamidase, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl β -glucosaminidase, α -mannosidase and α -fucosidase are absent. API 20E and API 20NE kits yield a positive reaction for gelatinase and negative reactions for nitrate reduction, indole and H₂S production, glucose fermentation, aesculin hydrolysis and urease and arginine dihydrolase. The following substrates are utilized with the Biolog GN2 MicroPlate system: dextrin, L-fucose, myo-inositol, mannitol, turanose, monomethyl succinate, D-glucosaminic acid, α -ketobutyric acid, DL-lactic acid, succinic acid, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, hydroxy L-proline, L-ornithine, L-proline and thymidine. The remaining substrates in the GN2 MicroPlate are not utilized. The type strain is sensitive to (µg per disc unless otherwise stated) ampicillin (10), chloramphenicol (30), erythromycin (15), penicillin (10 IU), nalidixic acid (30), tetracycline (30) and vancomycin (30), but resistant to amikacin (30), gentamicin (10), kanamycin (30), polymyxin B (300 IU) and streptomycin (10). The major fatty acids (>14%) are iso-C_{15:0}, iso-C_{15:1} G 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 31.4 mol%.

The type strain is JO-1^T (=KCTC 22329^T =JCM 15428^T), isolated from a Pacific oyster, *Crassostrea gigas*, collected from Wan Island, Jeonnam province, Korea.

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