Tenacibaculum litopenaei sp. nov., isolated from a shrimp mariculture pond

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A yellow-coloured, aerobic, Gram-negative, rod-shaped bacterial strain, designated B-I^T, was isolated from the water of a shrimp (*Litopenaeus vannamei*) mariculture pond in Taiwan. No species with a validly published name showed 16S rRNA gene sequence similarity of more than 96.5 % to this novel isolate. The highest sequence similarities displayed by strain B-I^T (93.2–96.1 %) were to members of the genus *Tenacibaculum*. The phenotypic properties of this organism were consistent with its classification in the genus *Tenacibaculum*. The novel isolate could be distinguished from all *Tenacibaculum* species by several phenotypic characteristics. The major fatty acids were iso-C_{15:0} (22 %), summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 21.3 %), iso-C_{17:0} 3-OH (12.7 %) and iso-C_{15:1} (8.7 %). The G + C content of the genomic DNA was 35.2 mol%. Hence, genotypic and phenotypic data demonstrate that strain B-I^T should be classified within a novel species in the genus *Tenacibaculum*, for which the name *Tenacibaculum litopenaei* sp. nov. is proposed. The type strain is B-I^T (=BCRC 17590^T=LMG 23706^T).

The genus *Tenacibaculum* Suzuki *et al.* 2001 is a member of the family *Flavobacteriaceae* (Reichenbach, 1992a, b; Bernardet *et al.*, 1996, 2002). It currently contains eight species isolated from different marine sources. *Tenacibaculum maritimum* and *T. ovolyticum* [previously *Flexibacter maritimus* (Wakabayashi *et al.*, 1986) and *Flexibacter ovolyticus* (Hansen *et al.*, 1992)] were isolated from diseased red sea bream fingerling and halibut egg, respectively. *Tenacibaculum mesophilum* and *T. amyolyticum* were isolated from sponge homogenate and the surface of marine macroalgae, respectively (Suzuki *et al.*, 2001). *Tenacibaculum skagerrakense* was isolated from seawater (Frette *et al.*, 2004), while *Tenacibaculum lutimaris*, *T. litoreum* and *T. aestuarii* were isolated from tidal flat sediment (Yoon *et al.*, 2005; Choi *et al.*, 2006; Jung *et al.*, 2006).

In August 2000, in the course of a study aiming at isolating chitinolytic bacteria, a surface water sample was collected from a shrimp (*Litopenaeus vannamei*) mariculture pond located in Pingtung County, southern Taiwan. The water sample was serially diluted and plated on chitin salt agar plates and incubated at 28 °C. Chitin salt medium contained

(l⁻¹) 5 g colloidal chitin, 30 g NaCl, 1.0 g MgSO₄·7H₂O, 0.2 g K₂HPO₄, 0.5 g CaCl₂·2H₂O and 1.0 ml trace element solution. The pH was adjusted to 7.5. Colloidal chitin was prepared from commercial chitin (Ohka Chemical) as described by Chang *et al.* (2004). The trace element solution contained (l⁻¹) 2.8 g H₃BO₃, 2.0 g MnSO₄·4H₂O, 0.2 g ZnSO₄·7H₂O, 0.08 g CuSO₄·5H₂O, 0.1 g FeCl₃ and 0.14 g Na₂MoO₄·2H₂O (Chang *et al.*, 2004). Agar plates were prepared by adding 1.5 % (w/v) Bacto agar (BD Difco) to the chitin salt medium. A bacterial strain that showed large clear zones around the colonies resulting from chitin degradation was isolated and designated B-I^T. Strain B-I^T was further subcultivated on marine agar 2216 (MA; BD Difco) and the organism was subjected to a polyphasic taxonomic study.

The morphology of bacterial cells was observed during the lag, exponential and stationary phases of growth under a phase-contrast microscope. Gliding motility was tested as described by Bowman (2000) and Bernardet *et al.* (2002). Flagellar staining was performed using the Spot Test flagella stain (BD Difco). The Gram reaction was assessed using the Gram stain set (BD Difco) and the Ryu non-staining KOH method (Powers, 1995). Accumulation of poly-β-hydroxybutyrate granules was observed by light microscopy after staining cells with Sudan black. Cellular pigments were

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B-I^T is DQ822567.

extracted from cultures grown on MA by using an acetone/methanol mixture (7:2, v/v) and absorption spectra were determined with a scanning UV/visible spectrophotometer. The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992a) and Bernardet *et al.* (2002). The pH range for optimum growth was examined in marine broth 2216 (MB) using appropriate biological buffers (pH 3–11) (Chung *et al.*, 1995). The pH was adjusted prior to sterilization; post-sterilization controls revealed that only minor changes in pH had occurred. Requirement for NaCl was determined using nutrient broth containing no NaCl and 0.5 % and 1.0–10.0 % NaCl (w/v, at 1.0 % intervals). The temperature range for growth (4, 10, 15, 20, 25, 30, 35, 39 and 40 °C) was examined in MB adjusted to pH 7. Growth was examined by measuring the turbidity (OD₆₀₀) of cultures grown at various pH, NaCl concentrations and temperatures. Anaerobic cultivation was performed on MA using the Oxoid AnaeroGen system.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Chen *et al.* (2001). Sequence reaction fragments were separated using a DNA sequencer (ABI PRISM 310 instrument; Applied Biosystems) and sequences were assembled by using the Fragment Assembly System program from the Wisconsin package 9.1 (GCG, 1995). The resulting sequence was compared with available 16S rRNA gene sequences from the RDP2 and GenBank databases. Multiple-sequence alignments of strain B-I^T and its closest relatives were performed using the BioEdit software (Hall, 1999). Phylogenetic trees were inferred using the maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. An evolutionary distance matrix was generated for the neighbour-joining algorithm using the Jukes & Cantor (1969) distance model and bootstrap analysis (1000 resamplings).

A comparison of the nearly complete 16S rRNA gene sequence (1471 nt) of strain B-I^T with those of representative members of the genera classified in the family *Flavobacteriaceae* showed that this organism fell within the evolutionary radiation of the genus *Tenacibaculum* (Fig. 1). Strain B-I^T was most closely related (99.5–99.7 %) to '[*Flexibacter*] *echinica*', an unpublished bacterial species isolated from diseased sea urchin (Tajima *et al.*, 1997a, b) and now considered as a member of *Tenacibaculum* (Masuda *et al.*, 2004). Unfortunately, it has not been possible to obtain strains of '[*Flexibacter*] *echinica*' for comparison with strain B-I^T. Among species with validly published names, strain B-I^T was most closely related to *T. mesophilum* MBIC1140^T and *T. lutimaris* CL-TF13^T, sharing a 16S rRNA gene sequence similarity of 96.1 % with each. Strain B-I^T shared lower 16S rRNA gene sequence similarity with the type strains of *T. aestuarii* (95.7 %), *T. amylolyticum* (95.5 %), *T. litoreum* (95.4 %), *T. skagerrakense* (95.0 %), *T. ovolyticum* (93.3 %) and *T. maritimum* (93.2 %). Similarity levels of strain B-I^T to other bacterial species in the family *Flavobacteriaceae* were less than 93 %.

DNA–DNA hybridization experiments were performed in triplicate with photobiotin-labelled probes as described by Ezaki *et al.* (1989). Strain B-I^T showed low DNA–DNA hybridization values with *T. mesophilum* MBIC1140^T (26 ± 4 %) and *T. lutimaris* CL-TF13^T (12 ± 3 %), clearly indicating that it represents a novel species in the genus *Tenacibaculum*.

Fatty acid methyl esters were extracted from cells grown on MA for 48 h at 28 °C and prepared by the standard protocol of the Microbial Identification System (MIDI; Microbial ID). The fatty acid composition of strain B-I^T was dominated by iso-C_{15:0} (22 %), summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 21.3 %), iso-C_{17:0} 3-OH (12.7 %) and iso-C_{15:1} (8.7 %). The detailed fatty acid compositions

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of strain B-I^T, other *Tenacibaculum* species and related taxa. Sequences were retrieved from the EMBL database (accession numbers in parentheses). Numbers at nodes are bootstrap values (%) based on 1000 resampled datasets; only values above 50 % are given. Bar, 2% sequence dissimilarity per nucleotide position. The sequence of *Cytophaga hutchinsonii* ATCC 33406^T was used as an out-group. The maximum-parsimony tree showed a very similar topology (data not shown).

Table 1. Fatty acid compositions of strain B-I^T and type strains of other *Tenacibaculum* species

Strains: 1, B-I^T (*T. litopenaei* sp. nov.); 2, *T. aestuarii* SMK-4^T; 3, *T. litoreum* CL-TF13^T; 4, *T. lutimaris* TF-26^T; 5, *T. maritimum* NCIMB 2154^T; 6, *T. mesophilum* MBIC1140^T; 7, *T. skagerrakense* D30^T. Data are percentages of total fatty acids. —, Not detected; tr, trace (<1%). ECL, Equivalent chain-length. Data were taken from Jung *et al.* (2006), Choi *et al.* (2006), Yoon *et al.* (2005) and this study. No fatty acid data were available for *T. ovolyticum* or *T. amylolyticum*. For fatty acid analysis, all strains studied were grown on MA. The incubation temperature was 28 °C for strain B-I^T and 30 °C for all other strains studied, while the incubation time was 24 h for *T. litoreum* CL-TF13^T and 48 h for all other strains studied.

Fatty acid	1	2	3	4	5	6	7
Straight-chain							
C _{15:0}	—	6.1	2.7	8.9	2.9	3.6	4.9
C _{16:0}	1.8	tr	tr	tr	tr	tr	tr
C _{18:0}	—	—	tr	—	1.4	—	—
Branched							
iso-C _{13:0}	tr	1.3	1.4	tr	1.8	tr	tr
iso-C _{14:0}	tr	2.2	tr	1.7	tr	tr	tr
iso-C _{15:0}	22.0	18.9	18.8	17.2	16.8	13.2	9.5
iso-C _{15:1}	8.7	8.7	8.2	5.3	7.6	7.1	8.2
anteiso-C _{15:0}	tr	2.0	1.8	tr	tr	1.1	—
iso-C _{16:0}	1.8	2.3	2.3	3.8	tr	1.7	1.3
iso-C _{16:1}	1.6	tr	1.3	1.7	—	tr	1.7
iso-C _{17:1ω9c}	1.6	1.3	1.6	tr	—	tr	—
Unsaturated							
C _{15:1ω6c}	1.6	3.0	1.7	4.2	2.2	1.6	—
C _{17:1ω6c}	1.9	1.6	tr	1.5	tr	tr	1.2
C _{18:3ω6c} (6,9,12)	—	—	1.5	—	—	—	—
Hydroxy							
C _{10:0} 3-OH	—	tr	—	—	—	—	—
iso-C _{14:0} 3-OH	—	tr	—	—	—	—	—
C _{15:0} 2-OH	tr	tr	tr	1.2	1.1	1.1	2.5
C _{15:0} 3-OH	2.7	4.2	—	3.4	3.8	2.9	8.6
iso-C _{15:0} 3-OH	4.6	6.1	6.6	4.6	19.8	8.0	7.8
C _{16:0} 3-OH	5.4	1.0	1.6	1.3	1.5	3.2	2.1
iso-C _{16:0} 3-OH	3.4	12.3	6.8	12.8	5.0	9.0	12.2
C _{17:0} 2-OH	tr	tr	tr	tr	—	tr	—
C _{17:0} 3-OH	1.0	tr	tr	tr	tr	tr	2.5
iso-C _{17:0} 3-OH	12.7	9.6	13.6	8.4	13.7	14.9	11.7
Unknown fatty acids							
ECL 13.565	1.9	—	1.3	—	—	—	—
ECL 16.582	tr	1.0	1.3	tr	1.0	1.0	tr
Summed features*							
Summed feature 3	21.3	11.9	19.6	18.1	17.9	24.4	22.5
Summed feature 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 3 contained C_{16:1ω7c} and/or iso-C_{15:0} 2-OH. Summed feature 4 contained iso-C_{17:1} I and/or anteiso-C_{17:1} B.

of strain B-I^T and other *Tenacibaculum* species are shown in Table 1. The fatty acid profile of strain B-I^T was in accordance with those of other *Tenacibaculum* species (Jung *et al.*, 2006; Choi *et al.*, 2006; Yoon *et al.*, 2005), although distinguishable by the absence of C_{15:0} (Table 1).

The DNA G+C content of strain B-I^T was determined in triplicate as described by Mesbah *et al.* (1989). The G+C content of strain B-I^T was 35.2±1.0 mol%.

The commercially available API 20NE (bioMérieux), API ZYM (bioMérieux) and Biolog GN2 (Biolog) microtest systems were used according to the manufacturers' instructions to determine the biochemical properties, enzyme activities and carbohydrate utilization pattern of strain B-I^T. The API ZYM test was read after 4 h incubation at 37 °C, while API 20NE and Biolog GN2 were read after 72 h at 28 °C. The three commercial systems were inoculated with a cell suspension in artificial seawater (NaCl, 24 g; MgCl₂, 5.1 g; Na₂SO₄, 4 g; CaCl₂, 1.1 g; KCl, 0.7 g; NaHCO₃, 0.2 g; KBr, 0.1 g; H₃BO₃, 0.027 g; SrCl₂, 0.024 g; NaF, 0.003 g; distilled water, 1 l; Lyman & Fleming, 1940). Sensitivity of strain B-I^T to different antibiotics was analysed by the diffusion method on MA plates. The following antibiotic discs (Oxoid) were used: ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), rifampicin (5 µg), penicillin G (10 U), streptomycin (10 µg) and tetracycline (30 µg). The effect of antibiotics on cell growth was assessed after 2 days of incubation at 28 °C.

Detailed results of phenotypic study are provided in Table 2 and in the species description. Strain B-I^T could be distinguished from its phylogenetic relatives using a combination of phenotypic properties, especially nitrate reduction, hydrolysis of chitin, starch, gelatin and Tween 80 and utilization of various organic compounds (such as citrate, L-leucine, L-proline, L-glutamate, L-aspartate, D-glucose and sucrose).

Genotypic and phenotypic data support the description of a novel species in the genus *Tenacibaculum* to accommodate strain B-I^T. The name *Tenacibaculum litopenaei* sp. nov. is proposed for this taxon.

Description of *Tenacibaculum litopenaei* sp. nov.

Tenacibaculum litopenaei (li.to.pen.ae'i. N.L. n. *Litopenaeus* the scientific name of a genus of penaeid shrimp; N.L. gen. n. *litopenaei* of *Litopenaeus*, referring to the isolation of the type strain from a shrimp belonging to the genus *Litopenaeus*).

Cells are aerobic, Gram-negative, non-flagellated, non-spore-forming, straight rods, 0.3–0.5 µm in width and 2–10 µm in length and motile by gliding. Degenerative spherical cells are occasionally observed in ageing broth cultures. Poly-β-hydroxybutyrate granules are not accumulated. Forms

Table 2. Phenotypic characteristics that differentiate strain B-I^T from the type strains of other *Tenacibaculum* species

Strains: 1, B-I^T (*T. litopenaei* sp. nov.); 2, *T. amylolyticum* MBIC4355^T; 3, *T. aestuarii* SMK-4^T; 4, *T. litoreum* CL-TF13^T; 5, *T. lutimaris* TF-26^T; 6, *T. maritimum* NCIMB 2154^T; 7, *T. mesophilum* MBIC1140^T; 8, *T. ovolyticum* IAM 14318^T; 9, *T. skagerrakense* D30^T. Data were taken from Jung *et al.* (2006), Choi *et al.* (2006), Yoon *et al.* (2005), Frette *et al.* (2004), Suzuki *et al.* (2001) and this study. Some strains were grown on different media to determine colony and cell morphology; consult original papers for direct comparisons. +, Positive; –, negative; W, weakly positive; NA, no data available; NG, no growth with NaCl only.

Characteristic	1	2	3	4	5	6	7	8	9
Origin	Shrimp culture pond, Taiwan	Macroalgae, Japan	Tidal flat, Korea	Tidal flat, Korea	Tidal flat, Korea	Diseased red sea bream, Japan	Sponge and macroalgae, Japan	Halibut egg, Norway	Pelagic, Denmark
Colony morphology	Circular, convex, spreading edge	Circular, spreading edge	Irregular, spreading edge	Irregular, spreading edge	Irregular, spreading edge	Uneven edge	Irregular, spreading edge	Regular edge	Circular, convex, spreading edge
Colony diameter at 5 days (mm)	5–20	23–27	5–10	5–10	10–20	<5	30–60	NA	5–20
Gliding motility	+	+	+	+	+	+	+	+	–
Colour	Yellow	Yellow	Pale yellow	Pale yellow	Pale yellow	Pale yellow	Yellow	Pale yellow	Bright yellow
Cell size (µm)	0.3–0.5 × 2–10	0.4 × 2–5	0.3 × 2–3.5	0.3–0.5 × 2–35	0.5 × 2–10	0.5 × 2–30	0.5 × 1.5–10	0.5 × 2–20	0.5 × 2–15
Spherical cells in ageing broth culture	Very rare	Very rare	NA	Very rare	Very rare	Frequent	Very rare	NA	Frequent
Tolerance of NaCl (%)	2–10	3 (W)	<7	3–5	<8	NG	1–7	NG	NG
Growth at pH 5	+	–	–	–	+	–	–	–	–
Nitrate reduction	–	W	–	+	–	+	–	+	+
Hydrolysis of:									
Chitin	+	–	NA	NA	NA	–	–	+	–
Starch	+	+	–	+	–	–	–	–	+
Gelatin	+	+	+	+	+	–	+	+	NA
Tween 80	–	+	+	+	–	+	+	+	–
Carbon source utilization									
Citrate	–	–	NA	–	NA	–	–	–	+
L-Leucine	–	–	–	–	–	–	–	–	W
L-Proline	+	+	–	+	–	–	+	–	+
L-Glutamate	+	+	–	–	–	W	+	–	+
L-Aspartate	–	–	–	–	–	–	+	–	+
D-Glucose	+	NA	–	–	–	NA	NA	NA	+
Sucrose	–	–	–	–	–	–	–	–	+
DNA G+C content (mol%)	35.2	30.9	33.6	30	32.6	31.6	31.8	30.3	35.2

yellow-coloured, circular, convex colonies with spreading margins after 48 h of incubation at 28 °C on MA. Flexirubin-type pigments are absent. Carotenoid pigments are produced with absorption peaks at 480, 454 and 425 nm. Grows at 10–39 °C, in the presence of 2–10 % NaCl and at pH 5–10. Optimum growth occurs at 28–37 °C, with 3 % NaCl and at pH 7.0–8.0. Positive for gelatin hydrolysis and for oxidase, alkaline phosphatase, C4 esterase, C8 lipase, C14 lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activities; negative for nitrate reduction, indole production, arginine fermentation, aesculin hydrolysis, catalase, urease, arginine dihydrolase, trypsin, α -chymotrypsin and β -glucuronidase activities (API 20NE and API ZYM). The following carbon sources are utilized (Biolog GN2): α -D-glucose, acetic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, L-glutamic acid, hydroxy-L-proline, L-proline, L-serine, L-threonine and uridine. The following carbon sources are not utilized: α -cyclodextrin, dextrin, glycogen, Tweens 40 and 80, *N*-acetyl-D-galactosamine, adonitol, arabinose, arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, *myo*-inositol, α -D-lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β -D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, methyl pyruvate, monomethyl succinate, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, *p*-hydroxyphenylacetic acid, α -, β - and γ -hydroxybutyric acids, itaconic acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, alaninamide, D- and L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-histidine, glycyl L-aspartic acid, glycyl L-glutamic acid, L-leucine, L-ornithine, L-phenylalanine, L-pyroglutamic acid, D-serine, DL-carnitine, γ -aminobutyric acid, urocanic acid, inosine, thymidine, phenyl ethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL- α -glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. Resistant to ampicillin, gentamicin, kanamycin, penicillin G and streptomycin; sensitive to chloramphenicol, erythromycin, nalidixic acid, novobiocin, rifampicin and tetracycline. The major fatty acids are iso-C_{15:0}, summed feature 3 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH), iso-C_{17:0} 3-OH and iso-C_{15:1}. The G + C content of the DNA is 35.2 mol%.

The type strain, B-I^T (= BCRC 17590^T = LMG 23706^T), was isolated from a water sample collected from a shrimp (*Litopenaeus vannamei*) mariculture pond located in Pingtung County, southern Taiwan.

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