Tenacibaculum jejuense sp. nov., isolated from coastal seawater

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A Gram-staining-negative, strictly aerobic and rod-shaped bacterium, designated strain CNURIC013^T, was isolated from seawater collected on the coast of Jeju Island, South Korea. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain CNURIC013^T belonged to the genus *Tenacibaculum*, within the family *Flavobacteriaceae*. Sequence similarities between the novel strain and the type strains of recognized species of the genus *Tenacibaculum* were 93.6–96.0 %, the highest value being with *Tenacibaculum litopenaei* B-1^T (96 %). The DNA G + C content of the novel strain was 34.5 mol% and the major respiratory quinone was menaquinone-6. The major fatty acids were summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 26.0 %), iso-C_{15:0} (24.4 %), iso-C_{15:1} G (18.5 %) and iso-C_{17:0} 3-OH (8.1 %). The polar lipids consisted of phosphatidylethanolamine, one unknown aminophospholipid and nine unknown polar lipids. On the basis of the phenotypic, phylogenetic and genotypic data, strain CNURIC013^T represents a novel species within the genus *Tenacibaculum*, for which the name *Tenacibaculum jejuense* sp. nov. is proposed. The type strain is CNURIC013^T (=KCTC 22618^T=JCM 15975^T).

The genus *Tenacibaculum* was first proposed by Suzuki *et al.* (2001), who described *Tenacibaculum mesophilum* and *Tenacibaculum amylolyticum* and reclassified *Flexibacter maritimus* (Wakabayashi *et al.*, 1986) and *Flexibacter ovolyticus* (Hansen *et al.*, 1992) as *Tenacibaculum maritimum* and *Tenacibaculum ovolyticum*, respectively. The genus *Tenacibaculum* belongs to the family *Flavobacteriaceae* and currently comprises 15 recognized species isolated from different marine habitats. *T. maritimum*, *T. ovolyticum*, *Tenacibaculum discolor*, *Tenacibaculum aiptasiae* and *Tenacibaculum soleae* were isolated from diseased marine animals (Wakabayashi *et al.*, 1986; Hansen *et al.*, 1992; Wang *et al.*, 2008; Piñeiro-Vidal *et al.*, 2008a, b). Although *T. mesophilum*, *Tenacibaculum adriaticum* and *Tenacibaculum crassostreae* were also isolated from marine animals (Suzuki *et al.*, 2001; Heindl *et al.*, 2008; Lee *et al.*, 2009), *T. amylolyticum* was isolated from marine macroalgae (Suzuki

et al., 2001), *Tenacibaculum lutimaris*, *Tenacibaculum litoreum* and *Tenacibaculum aestuarii* were isolated from tidal-flat sediments (Yoon *et al.*, 2005; Choi *et al.*, 2006; Jung *et al.*, 2006) and *Tenacibaculum skagerrakense*, *Tenacibaculum litopenaei* and *Tenacibaculum gallaicum* were isolated from seawater (Frette *et al.*, 2004; Sheu *et al.*, 2007; Piñeiro-Vidal *et al.*, 2008a). In this study, a *Tenacibaculum*-like bacterial strain, CNURIC013^T, was isolated from a seawater sample collected from the coast of Jeju Island, South Korea, before its taxonomy was investigated using a polyphasic approach.

Strain CNURIC013^T was isolated, using a standard dilution-plating technique, on marine agar 2216 (MA; Difco), after incubation at 25 °C for 5 days. Subcultivation was routinely performed on MA at 30 °C for 3 days under aerobic conditions and the strain was preserved at –80 °C in marine broth (MB, Difco) supplemented with 20 % (v/v) glycerol. *T. amylolyticum* JCM 21776^T, *T. litopenaei* B-1^T and *T. skagerrakense* D30^T were used as reference strains in the phenotypic tests and fatty acid analysis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CNURIC013^T is FJ490366.

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

Genomic DNA of strain CNURIC013^T was extracted and purified, using a Wizard genomic extraction kit (Promega), so that the 16S rRNA gene sequence could be amplified using bacterial universal primers, 27F and 1492R (Weisburg *et al.*, 1991). The PCR product was purified with a QIAquick PCR purification kit (Qiagen) and the purified 16S rRNA gene of CNURIC013^T was then sequenced in an automated sequencer (ABI 3730XL; Applied Biosystems) at Solgent Co. Ltd. The resulting 16S rRNA gene sequence (1392 bp) was compared with the 16S rRNA gene sequences available in GenBank, using BLAST (<http://www.ncbi.nlm.nih.gov/blast/>) to determine the novel strain's approximate phylogenetic affiliation and CLUSTAL_X (Thompson *et al.*, 1997) to align the sequences of the novel strain and the closely related recognized species. Sequence similarity values were computed using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Gaps at the 5' and 3' ends and ambiguous bases were removed from the alignments using BioEdit (Hall, 1999). Phylogenetic trees based on comparison of 1376 bases were constructed by using the neighbour-joining and maximum-parsimony algorithms in the MEGA 4.0 program (Tamura *et al.*, 2007), with bootstrap values based on 1000 replications (Felsenstein, 1985). Evolutionary distance matrices were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1983). Phylogenetic analysis based on the nearly complete 16S rRNA gene sequences showed that strain CNURIC013^T was closely related to the genus *Tenacibaculum* in the family *Flavobacteriaceae* and formed a distinct phyletic line with the type strain of *T. litopenaei*, an observation supported by a

high corresponding bootstrap value (92 %) in the neighbour-joining tree (Fig. 1). A tree based on maximum-parsimony showed essentially the same topology (data not shown). The level of 16S rRNA gene sequence similarity between strain CNURIC013^T and *T. litopenaei* B-I^T (96.0 %) was greater than that between strain CNURIC013^T and any other recognized species of the genus *Tenacibaculum* (93.6–95.7 %).

The following investigations were performed on strain CNURIC013^T and three reference strains: *T. amylyticum* JCM 21776^T, *T. litopenaei* B-I^T and *T. skagerrakense* D30^T. Cells were grown aerobically on MA at 30 °C for 2 days before colony morphology, size and colour were observed. Cell morphology and size were examined by light (FDX-35; Nikon) and transmission electron (JEM-1010; JEOL) microscopy, using cells from exponentially growing cultures. The optimal temperature and temperature range for growth were investigated on MA at 4, 10, 15, 20, 25, 30, 35, 37 and 42 °C. NaCl requirement and tolerance were determined in the presence of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 % (w/v) NaCl in synthetic ZoBell agar (ZoBell, 1941) made by dissolving 5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate and 15 g Bacto agar in 1 litre of modified artificial seawater (5.94 g MgSO₄·7H₂O, 4.53 g MgCl₂·6H₂O, 0.64 g KCl and 1.3 g CaCl₂ 1⁻¹). Tolerance to sea salts (Sigma) was tested in synthetic ZoBell agar containing 0–10 % sea salts (w/v, at 1 % intervals). The pH range for growth was determined in MB at pH 4.0–11.0 (at intervals of 0.5 pH unit). The pH of the sterilized medium being adjusted with 1 M NaOH or 1 M HCl. The Gram reaction was determined by using a

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain CNURIC013^T and type strains of species of the genus *Tenacibaculum*. The numbers at branching points are bootstrap percentage values based on 1000 replications. Only values >50 % are shown. *Aestuariaicola saemankumensis* SMK-142^T was used as the outgroup. Bar, 0.01 substitution per nucleotide position.

commercial Gram staining kit (bioMérieux) according to the manufacturer's instructions. Gliding motility was investigated using phase-contrast light microscopy and the hanging drop method (Bernardet *et al.*, 2002). Anaerobic growth was tested on MA incubated for up to 15 days in the AnaeroPack-Anaero system (Mitsubishi Gas Chemical). Catalase and oxidase activities were assessed using standard methods (Tindall *et al.*, 2007). The degradation of agar, DNA, starch, carboxymethylcellulose and xylan were assessed according to the methods of Smibert & Krieg (1994). Casein hydrolysis was assessed by the method of Lányi (1987) and hydrolysis of chitin and Tweens 20, 40, 60 and 80 was assessed as described by Baumann & Baumann (1981). To detect flexirubin-type pigments, cell mass of each of the four strains was subjected to the KOH test, as described by Bernardet *et al.* (2002). Antibiotic sensitivity of strain CNURIC013^T was assessed, after 2 days of incubation at 30 °C on MA, by the disc diffusion method, using commercial discs (BBL Becton Dickson) impregnated with ampicillin (10 µg), carbenicillin (100 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), lincomycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), novobiocin (5 µg), oleandomycin (15 µg), penicillin (10 U), polymyxin B (300 µg), streptomycin (10 µg) or tetracycline (30 µg). The results were interpreted according to the guidelines set forth by CLSI (2003). Further phenotypic and enzymatic characterizations of strain CNURIC013^T and the three reference strains were conducted using the API 20E, API 20NE and API 50CH test kits (bioMérieux) at 30 °C for 3 days and the API ZYM test kit (bioMérieux) at 30 °C for 8 h. The utilization of different carbon sources was tested with the GN2 MicroPlate (Biolog) according to the manufacturer's instructions. All commercial kits were inoculated with bacterial suspensions in 3% (w/v) sea salts.

Colonies of strain CNURIC013^T grown on MA for 3 days at 30 °C were circular, convex, smooth, entire, pale yellow in colour and 1–5 mm in diameter. Cells of strain CNURIC013^T are Gram-staining-negative, non-motile, and rod-shaped (0.3–0.6 µm in width and 2.5–3.0 µm in length). The strain was able to grow at a temperature range of 15–35 °C (optimum, between 25 and 30 °C); no growth occurred at 10 °C or 37 °C. The pH range for growth was pH 6.0–9.0 (optimum, between pH 7.0 and 8.0) and the strain was able to grow with 1.0–4.0% NaCl (optimum, 3%). Flexirubin-type pigments were absent. The strain was oxidase-positive and catalase-positive but negative for nitrate reduction. Aesculin, agar, casein, carboxymethylcellulose, DNA, gelatin, starch, xylan, Tweens 20, 40, 60 and 80 were hydrolysed but chitin was not. Additional characteristics of the novel strain are given in the species description, and Table 1 provides a comparison of the characteristics of CNURIC013^T and the three reference strains.

For cellular fatty acid analysis, cells of strain CNURIC013^T and the three reference strains were grown aerobically on MA at 30 °C for 3 days. Fatty acid methyl esters were

obtained by saponification, methylation and extraction, following the standard protocol in version 4.5 of the Sherlock Microbial Identification System (MIDI, 2002). Fatty acids were analysed by GC (6890N; Agilent) and identified by using the TSBA50 database of the Microbial Identification System (Sasser, 1990). The major respiratory quinones were analysed by HPLC (ZQ 4000; Waters) with a reversed-phase column (300 × 3.9 mm, Nova-Pak 130 C18; Waters), as described by Komagata & Suzuki (1987). Polar lipids were extracted using 6.5 × 6.5 cm TLC silica gel 60 F₂₅₄ plates (Merck) according to the procedures described by Minnikin *et al.* (1984). Individual phospholipids were identified by two-dimensional TLC, after spraying with the appropriate detection reagents, through co-migration with authentic standards (Sigma) (Komagata & Suzuki, 1987). The G + C content of the DNA of strain CNURIC013^T was determined using the thermal denaturation method (Marmur & Doty, 1962), an Ultrospec 2100 spectrophotometer (Pharmacia Biotech) and, as a control, DNA from *Escherichia coli* K-12.

The major components of the cellular fatty acid profile of the novel strain were summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 26.0%), iso-C_{15:0} (24.4%), iso-C_{15:1} G (18.5%) and iso-C_{17:0} 3-OH (8.1%). This fatty acid profile was similar to those of the three *Tenacibaculum* type strains used for reference, although there were differences, in content and proportion, for some fatty acids (Table 2). The main polar lipids in strain CNURIC013^T were phosphatidylethanolamine, one unknown aminophospholipid and nine unknown polar lipids. Although the polar lipid profile of CNURIC013^T was similar to those of other species of the genus *Tenacibaculum*, some unknown polar lipids of strain CNURIC013^T were not detected in the reference strains (see Supplementary Fig. S1, in IJSEM Online). The G + C content of the DNA of strain CNURIC013^T was 34.5 mol%, which is within the range of DNA G + C contents of the other type strains of species of the genus *Tenacibaculum* (30–35.2 mol%). The major respiratory lipoquinone of strain CNURIC013^T, was menaquinone-6 (MK-6), which is in line with other members of the genus *Tenacibaculum*.

In conclusion, on the basis of the phenotypic characterization and the phylogenetic analysis, strain CNURIC013^T represents a novel species of the genus *Tenacibaculum*, for which the name *Tenacibaculum jejuense* sp. nov. is proposed.

Description of *Tenacibaculum jejuense* sp. nov.

Tenacibaculum jejuense (je.ju.en'se. N.L. neut. adj. *jejuense* of or pertaining to Jeju Island in the Republic of Korea, where the type strain was isolated).

Cells are Gram-staining-negative rods measuring 0.3–0.6 µm in diameter and 2.5–3.0 µm in length, strictly aerobic and non-motile after 3 days incubation. Colonies on marine agar are circular, convex, entire, smooth, pale yellow-coloured and 1.0–5.0 mm in diameter. Growth occurs at 15–35 °C (optimum between 25 and 30 °C), at

Table 1. Phenotypic characteristics that differentiate strain CNURIC013^T from the type strains of other species of the genus *Tenacibaculum*

Strains: 1, CNURIC013^T; 2, *T. amylolyticum* JCM 21776^T; 3, *T. litopenaei* B-1^T; 4, *T. skagerrakense* D30^T. +, Positive; –, negative; w, weakly positive. All data from this study except the DNA G+C contents of *T. amylolyticum* JCM 16310^T, *T. litopenaei* B-1^T and *T. skagerrakense* D30^T, which are from Suzuki *et al.* (2001), Sheu *et al.* (2007) and Frette *et al.* (2004), respectively.

Characteristic	1	2	3	4
Colony morphology	Circular, regular edge	Circular, spreading edge	Circular, convex, spreading edge	Circular, convex, spreading edge
Cell size (µm)	0.3–0.6 × 2–3	0.5 × 2–5	0.3–0.5 × 2–10	0.5 × 2–15
Spherical cells in ageing broth	Very rare	Very rare	Very rare	Frequent
Gliding motility	+	+	+	–
Salinity tolerance:				
NaCl (%)	1–4	2–3	2–9	1–3
Seawater (%)*	50–125	50–100	50–125	25–150
pH range	6.0–9.0	5.5–8.5	5.0–10.0	6.0–9.0
Temperature for growth:				
Range	15–35	20–35	10–37	10–40
Optimum	25–30	30	25–30	25–30
Nitrate reduction	–	–	–	+
Hydrolysis of:				
Aesculin	+	+	–	+
Agar	+	–	–	–
Chitin	–	–	+	–
Carboxymethylcellulose	+	–	–	–
Xylan	+	–	–	–
Tween 80	+	+	–	–
Utilization of:				
Citrate	–	–	–	+
Succinate	–	–	+	–
L-Alanine	+	–	–	+
L-Aspartate	+	+	–	+
L-Leucine	–	–	–	+
L-Ornithine	+	+	–	+
L-Serine	+	–	+	+
Sucrose	+	–	–	+
D-Galactose	+	–	–	+
D-Mannose	+	+	–	+
Cellobiose	+	w	–	+
API ZYM:				
Lipase (C14)	–	+	+	–
Trypsin	+	–	–	+
α-Chymotrypsin	+	w	–	+
β-Glucosidase	–	+	–	+
DNA G+C content (mol%)	34.5	30.9	35.2	35.2

*Percentage calculated assuming that a solution containing 40 g artificial sea salts l^{–1} is equivalent to 100% seawater (Frette *et al.*, 2004).

pH 6.0–9.0 (optimum, between pH 7.0 and 8.0), and with 1.0–4.0 % (w/v) NaCl (optimum, 3.0 %). Growth occurs in sea salts concentrations of 2–5 % (w/v). Catalase- and oxidase-positive. Flexirubin-type pigments are absent. Aesculin, agar, casein, starch, carboxymethylcellulose, DNA, xylan, Tweens 20, 40, 60 and 80 are hydrolysed, but chitin is not. In API 20E tests, gives a negative result for the production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H₂S and tryptophan deaminase

and for the utilization of citrate. Negative result in the Voges–Proskauer reaction. In API 20NE tests, nitrate is not reduced to nitrite, and there is no indole production, urease activity or glucose fermentation. Does not produce acid from carbohydrate. In tests based on the API 50CH kit, acid is only produced aerobically from aesculin-ferric citrate and 5-ketogluconate. The API ZYM kit reveals the presence of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine

Table 2. Fatty acid composition (%) of strain CNURIC013^T and type strains of related species of the genus *Tenacibaculum*

Strains: 1, CNURIC013^T; 2, *T. amylolyticum* JCM 21776^T; 3, *T. litopenaei* B-1^T; 4, *T. skagerrakense* D30^T. Fatty acids amounting to <1.0 % in all strains are not listed. All data are from this study. tr, Trace (<1.0 %); –, not detected.

Fatty acid	1	2	3	4
Straight-chain				
C _{14:0}	1.2	tr	1.2	tr
C _{15:0}	4.3	5.4	6.4	3.7
C _{16:0}	–	2.0	4.4	–
Branched				
iso-C _{13:0}	1.0	tr	1.5	–
iso-C _{14:0}	–	–	–	1.8
iso-C _{15:0}	24.4	17.9	32.4	29.6
iso-C _{15:1} G	18.5	14.6	11.7	15.2
iso-C _{16:0}	–	2.2	1.7	tr
iso-C _{16:1} G	–	2.8	–	–
iso-C _{17:0}	–	–	tr	–
anteiso-C _{15:0}	–	–	1.1	1.2
iso-C _{17:1} ω9c	1.6	1.7	3.0	2.4
Unsaturated				
C _{15:1} ω6c	4.8	–	1.5	–
C _{17:1} ω6c	–	1.0	tr	tr
Hydroxy				
C _{15:0} 2-OH	–	–	–	1.4
C _{15:0} 3-OH	–	–	–	3.2
C _{16:0} 3-OH	1.8	3.3	2.0	–
iso-C _{15:0} 3-OH	5.4	4.6	2.1	5.5
iso-C _{16:0} 3-OH	2.1	4.3	1.3	2.3
iso-C _{17:0} 3-OH	8.1	7.9	4.7	8.2
Summed features*				
3	26.0	31.7	14.4	24.0
4	5.3	2.9	3.7	–
Unknown fatty acids				
Equivalent chain length 11.543	–	–	1.8	–
Equivalent chain length 13.563	–	1.6	7.3	1.1

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

arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities, but lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. The following substrates can each be utilized as sole carbon source (in GN2 Microplates): α-cyclodextrin, dextrin, glycogen, Tween 40, cellobiose, D-galactose, α-D-glucose, maltose, D-mannose, acetic acid, α-ketobutyric acid, DL-lactic acid, propionic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-ornithine, L-proline, L-serine, inosine, α-D-glucose 1-phosphate and D-glucose 6-phosphate. No other

carbon sources in the GN2 Microplate are utilized. Susceptible to ampicillin (10 µg), carbenicillin (100 µg), chloramphenicol (30 µg), erythromycin (15 µg), lincomycin (15 µg), novobiocin (5 µg), oleandomycin (15 µg), penicillin (10 U) and tetracycline (30 µg), but resistant to gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (30 µg), polymyxin B (300 µg) and streptomycin (10 µg). The major cellular fatty acids are summed feature 3 (comprising C_{16:1} ω7c and/or iso-C_{15:0} 2-OH), iso-C_{15:0}, iso-C_{15:1} G and iso-C_{17:0} 3-OH. The polar lipids consist of phosphatidylethanolamine, one unknown aminophospholipid and nine unknown polar lipids.

The type strain, CNURIC013^T (=KCTC 22618^T=JCM 15975^T), was isolated from seawater in Jeju Island, Korea. The DNA G+C content of the type strain is 34.5 mol% and the major respiratory quinone is menaquinone-6.

Acknowledgements

We thank Professor Dr Wen-Ming Chen (Laboratory of Microbiology, Department of Seafood Science, National Kaohsiung Marine University) and Professor Dr Niels Kroer (Department of Environmental Chemistry and Microbiology, National Environmental Research Institute) for providing *T. litopenaei* B-1^T and *T. skagerrakense* D30^T. This study was supported by Jeju National University Biotechnology Regional Innovation Center and the 21C Frontier Microbial Genomics and Applications Centre Program, Ministry of Education, Science and Technology, Korea.

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