Tenacibaculum adriaticum sp. nov., from a bryozoan in the Adriatic Sea

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A rod-shaped, translucent yellow-pigmented, Gram-negative bacterium, strain B390^T, was isolated from the bryozoan *Schizobrachiella sanguinea* collected in the Adriatic Sea, near Rovinj, Croatia. 16S rRNA gene sequence analysis indicated affiliation to the genus *Tenacibaculum*, with sequence similarity levels of 94.8–97.3 % to type strains of species with validly published names. It grew at 5–34 °C, with optimal growth at 18–26 °C, and only in the presence of NaCl or sea salts. In contrast to other type strains of the genus, strain B390^T was able to hydrolyse aesculin. The predominant menaquinone was MK-6 and major fatty acids were iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH and iso-$C_{15:1}$. The DNA G+C content was 31.6 mol%. DNA–DNA hybridization and comparative physiological tests were performed with type strains *Tenacibaculum aestuarii* JCM 13491^T and *Tenacibaculum lutimaris* DSM 16505^T, since they exhibit 16S rRNA gene sequence similarities above 97 %. These data, as well as phylogenetic analyses, suggest that strain B390^T (=DSM 18961^T =JCM 14633^T) should be classified as the type strain of a novel species within the genus *Tenacibaculum*, for which the name *Tenacibaculum adriaticum* sp. nov. is proposed.

The genus *Tenacibaculum* was first proposed by Suzuki *et al.* (2001) as a consequence of the reclassification of [*Flexibacter*] *maritimus* (Wakabayashi *et al.*, 1986) and [*Flexibacter*] *ovolyticus* (Hansen *et al.*, 1992) as *Tenacibaculum maritimum* and *Tenacibaculum ovolyticum*, respectively, and the description of *Tenacibaculum mesophilum* and *Tenacibaculum amylolyticum*. At present, the genus includes five further species: *Tenacibaculum skagerrakense* (Frette *et al.*, 2004), *T. lutimaris* (Yoon *et al.*, 2005), *T. litoreum* (Choi *et al.*, 2006), *T. aestuarii* (Jung *et al.*, 2006) and *T. litopenaei* (Sheu *et al.*, 2007). All of them were isolated from marine environments. Strain B390^T, reported in this study, represents the first taxonomically described *Tenacibaculum* species from the Mediterranean Sea.

Bryozoan specimens were collected in the Adriatic Sea near Rovinj, Croatia, and used as sources for isolation and characterization of associated micro-organisms. Strain B390^T was obtained from a specimen of *Schizobrachiella sanguinea* by the dilution plating technique on tryptic soy broth (TSB; Difco) supplemented with 2.5 % (w/v) NaCl and 1.5 % (w/v) agar. Subcultivation was done on TSB and half-strength marine broth 2216 (MB; Difco) plates at room temperature (approx. 22 °C). Unless otherwise noted, half-strength MB medium was used in the studies. Agar (1.5 % w/v) was added for solidification if needed. Strain B390^T was maintained in MB supplemented with 10 % (v/v) DMSO at −80 °C.

Phenotypic and phylogenetic characterization was done according to the minimal standards for describing new taxa of the family *Flavobacteriaceae* proposed by Bernardet *et al.* (2002). Colony morphology was determined with 5-day cultures on agar plates with a binocular microscope (Wild Heerbrugg). Gram staining was performed with standard stains (carbol–gentian violet solution, Lugol's iodine, 96 % (v/v) ethanol and carbol–fuchsin solution) and by cell lysis using 6 % (w/v) KOH as described by Gregersen (1978). Cell morphology and flagellation were examined by phase-contrast microscopy (Axiophot; Zeiss). The ability to glide was checked with cultures grown under optimal and harsh conditions (regarding temperature, pH and salinity) as described by Bowman (2000) and Bernardet *et al.* (2002). Scanning electron micrographs were taken from cultures grown overnight or for 4 days at room temperature with a Zeiss DSM 940. The presence of flexirubin-type pigments was tested with 20 % KOH (Reichenbach, 1992); Congo red adsorption was determined as described by Bernardet *et al.* (2002).

Growth at various temperatures from 2 to 50 °C was measured with a custom-made temperature-gradient bar over 7 days. Salinity requirement was determined with saltless MB medium [per litre distilled water: 5.0 g Bacto

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B390^T is AM412314.

A 16S rRNA gene sequence-based neighbour-joining tree and detailed Biolog utilization results are available as supplementary material with the online version of this paper.

Table 1. Morphological and physiological characteristics of strain B390^T and the most closely related *Tenacibaculum* species

Taxa: 1, strain B390^T; 2, *T. aestuarii* (data from Jung *et al.*, 2006); 3, *T. litoreum* (Choi *et al.*, 2006); 4, *T. lutimaris* (Yoon *et al.*, 2005); 5, *T. skagerrakense* (Frette *et al.*, 2004); 6, *T. mesophilum* (Suzuki *et al.*, 2001); +, Positive; −, negative; (+), weakly positive; ND, no data available; NG, no growth.

Characteristic	1	2	3	4	5	6
Origin of type strain	Bryozoan, Croatia	Tidal flat, Korea	Tidal flat, Korea	Tidal flat, Korea	Seawater, Skagerrak (Denmark)	Sponge, Japan
Cell size (μm)	0.3 × 1.5–3.5	0.3 × 2.0–3.5	0.3–0.5 × 2–35	0.5 × 2–10	0.5 × 2–15	0.5 × 1.5–10
Filaments (μm)	11–35	ND	ND	ND	ND	ND
Colony morphology						
Shape	Circular, regular edge	Irregular, spreading edge	Irregular, spreading edge	Irregular, spreading edge	Circular, spreading edge	Circular or irregular, spreading edge
Diameter at 5 days (mm)	<5	5–10	5–10	10–20	5–20	30–60
Colour	Translucent yellow	Pale yellow	Pale yellow	Pale yellow, glistening	Bright yellow	Bright yellow
pH range	5–9	5.5–8.5	6–10	5–8	6–8	5.3–9.0
Temperature for growth (°C)						
Range	5–33.5	9–41	5–40	10–39	10–40	15–40
Optimum	18–26	30–37	35–40	30–37	25–37	28–35
Salinity range						
NaCl (% w/v)	1–5	0.5–7	3–5	<8 (2–3)	NG	1–7
Seawater (% v/v)	12.5–175*	ND	25–250*	25–175*	25–150*	10–100
Nitrate reduction	−	−	+	−	+	−
Utilization of:						
DL-Aspartate	+	+†[a]	−	+†[b]	+	+
L-Proline	+	+†[a]	+	+†[b]	+	+
L-Glutamate	+	+†[a]	−	+†[b]	+	+
Citrate	−	−†	−	−†	+	−
L-Leucine	(+)	(+)†[a]	−	(+)†[b]	+	−
D-Glucose	+	−	−	−	+	ND
D-Mannose	+	−	−	−	+	ND
Cellobiose	+	−	ND	−	+	ND
Hydrolysis of:						
Starch	+	−	+	−	+	−
Aesculin	+	−	−	−	ND	−
Chitin	−	−†	ND	−†	−	−
DNA G+C content (mol%)	31.6	33.6	30.0	32.2–32.8	35.2	31.6–32.0

*Percentage calculated using a relation of 100 % seawater=40 g artificial sea salts l^{-1} (Frette *et al.*, 2004).
†Data from this study. Where indicated, a negative result was reported by: *a*, Jung *et al.* (2006); *b*, Yoon *et al.* (2005).

peptone, 1 g yeast extract and 0.1 g ferric citrate] and saltless TSB medium (per litre distilled water: 1.7 g Bacto tryptone, 0.3 g papain-digested soybean meal, 0.25 g glucose and 0.25 g K_2HPO_4). The media were supplemented with NaCl [0–9 % (w/v) in 1 % increments] or artificial sea salts (Tropic Marin; 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70 and 80 g l^{-1}) and incubated for 7 days at room temperature. Growth at different pH values (2.3, 3.5, 5.0, 6.0, 8.5, 9.0 and 9.5) was also investigated.

Type strains of *T. aestuarii* (JCM 13491^T) and *T. lutimaris* (DSM 16505^T), the most closely related species based on 16S rRNA gene sequences, were tested as references in the phenotypic characterization. The API 20E kit (bioMérieux) for identification of Gram-negative rods was performed in duplicate according to the manufacturer's instructions with API NaCl 0.85 % medium and artificial seawater (ASW; Bruns *et al.*, 2001) as suspension fluids. The utilization of different carbon sources was checked with the GN2 MicroPlate (Biolog) following the manufacturer's instructions. Duplicate tests were carried out with bacteria suspended in 1 % (w/v) NaCl solution, and triplicate tests in ASW as suspension medium. The hydrolysis of aesculin was studied as described by Cowan & Steel (1965) and

Table 2. Cellular fatty acid contents (%) of strain B390^T and other *Tenacibaculum* species

Taxa: 1, strain B390^T; 2, *T. aestuarii* (data from Jung *et al.*, 2006); 3, *T. litoreum* (Choi *et al.*, 2006); 4, *T. lutimaris*; 5, *T. skagerrakense*; 6, *T. mesophilum*; 7, *T. maritimum* (data in columns 4–7 from Yoon *et al.*, 2005); 8, *T. litopenaei* (data from Sheu *et al.*, 2007). TBSA, Tuberculostearic acid (10-methyl $C_{18:0}$); ECL, equivalent chain-length; tr, trace (<1 %).

Fatty acid	1	2	3	4	5	6	7	8
Straight-chain								
$C_{15:0}$	2.7	6.1	2.7	8.9	4.9	3.6	2.9	
$C_{16:0}$		0.4	0.9	0.6	0.6	0.7	0.3	1.8
$C_{18:0}$			0.3				1.4	
Branched								
iso-$C_{13:0}$	1.6	1.3	1.4	0.7	0.2	0.8	1.8	tr
iso-$C_{14:0}$	0.4	2.2	0.7	1.7	0.9	0.8	0.8	tr
iso-$C_{15:0}$	25.3	18.9	18.8	17.2	9.5	13.2	16.8	22.0
iso-$C_{15:1}$	13.1	8.7	8.2	5.3	8.2	7.1	7.6	8.7
iso-$C_{16:0}$	0.2	2.0	2.3	3.8	1.3	1.7	0.3	1.8
iso-$C_{16:1}$	0.8	2.3	1.3	1.7	1.7	0.8		1.6
iso-$C_{17:1}\omega 9c$	2.0	0.8	1.6	0.4		0.6		1.6
anteiso-$C_{15:0}$	0.5	1.3	1.8	0.7		1.1	0.8	tr
TBSA	0.4							
Unsaturated								
$C_{15:1}\omega 6c$	5.6	3.0	1.7	4.2		1.6	2.2	1.6
$C_{17:1}\omega 6c$	0.7	1.6	0.9	1.5	1.2	0.9	0.3	1.9
$C_{18:1}\omega 5c$	1.2							
$C_{18:3}\omega 6c$			1.5					
Hydroxylated								
$C_{10:0}$ 3-OH		0.7						
$C_{15:0}$ 2-OH	0.4	0.8	0.7	1.2	2.5	1.1	1.1	tr
$C_{15:0}$ 3-OH	3.2	4.2		3.4	8.6	2.9	3.8	2.7
$C_{16:0}$ 3-OH	0.2	1.0	1.6	1.3	2.1	3.2	1.5	5.4
$C_{17:0}$ 2-OH		0.4	0.9	0.2		0.8		tr
$C_{17:0}$ 3-OH		0.6	0.3	0.9	2.5	0.7	0.6	1.0
iso-$C_{14:0}$ 3-OH	0.3	0.5						
iso-$C_{15:0}$ 3-OH	13.7	6.1	6.6	4.6	7.8	8.0	19.8	4.6
iso-$C_{16:0}$ 3-OH	2.8	12.3	6.8	12.8	12.2	9.0	5.0	3.4
iso-$C_{17:0}$ 3-OH	10.8	9.6	13.6	8.4	11.7	14.9	13.7	12.7
Unknown								
ECL 13.565	0.2		1.3					1.9
ECL 16.582	0.8	1.0	1.3	0.7	0.7	1.0	1.0	tr
Summed features*								
1	1.2							
3	11.8	11.9	19.6	18.1	22.5	24.4	17.9	21.3
4			1.3					

*Summed features contained one or more of the following fatty acids. Summed feature 1, iso-$C_{15:1}$ H, iso-$C_{15:1}$ I and $C_{13:0}$ 3-OH; summed feature 3, $C_{16:1}\omega 7c$ and iso-$C_{15:0}$ 2-OH; summed feature 4, iso-$C_{17:1}$ I and anteiso-$C_{17:1}$ B.

alternatively with half-strength MB plates supplemented with 0.1 % (w/v) aesculin. The tested strains grew considerably better on the MB plates than on the standard plates, and addition of ferric salts for complexation of aesculetin was not necessary since MB contains ferric citrate. L-Tyrosine and starch hydrolysis tests were carried out as described by Cowan & Steel (1965) with the difference that half-strength MB was used as the nutrient source. Casein hydrolysis was determined with plates made as follows: 2.5 g casein was suspended in 1000 ml distilled water, followed by 0.1 g $CaCl_2$ and a few drops of 10 M NaOH. The suspension was left to macerate for 30 min. After addition of 1.5 % (w/v) agar and 18.7 g marine broth 2216, the suspension was brought carefully to the boil, filtered and autoclaved (15 min, 121 °C). In order to test chitin hydrolysis, a bottom-agar plate was covered with a top agar layer containing chitin. The bottom agar consisted of 15 g agar and 20 g artificial sea salts per litre distilled water. The top agar comprised two solutions: 0.8 g chitin was suspended in 100 ml distilled water (solution 1) and 6 g agar was suspended in 300 ml half-strength MB (solution 2). After autoclaving, the two solutions were combined and poured on the bottom-agar plates.

Chemotaxonomic analyses (DNA G+C content, DNA–DNA hybridization, menaquinones, fatty acid methyl esters) were carried out by the Identification Service of the DSMZ (Braunschweig, Germany). DNA was isolated using a French pressure cell (Thermo Spectronic) and purified as described by Cashion *et al.* (1977). The DNA G+C content of strain B390^T was determined according to Mesbah *et al.* (1989). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983). Fatty acid analysis was carried out using the MIDI system (MIDI Inc.).

Genomic DNA for phylogenetic analyses was isolated by consecutive freezing and boiling of picked colonies in DNA-free water (Fluka). 16S rRNA gene amplification was done by PCR with puReTaq Ready-To-Go PCR Beads (Amersham Biosciences) and primers 27f and 1492r (Lane, 1991). PCR product purification and sequencing were done at the Institute for Clinical Molecular Biology of the University Hospital Schleswig-Holstein in Kiel. For each purification reaction, a mixture of 1.5 U exonuclease I (GE Healthcare) and 0.3 U shrimp alkaline phosphatase (SAP; Roche) was added to the PCR product and incubated for 15 min at 37 °C. The enzymes were subsequently inactivated at 72 °C for 15 min. A sequencing reaction was done with the BigDye Terminator v1.1 sequencing kit (Applied Biosystems) and analysed in a 3730xl DNA Analyser (Applied Biosystems) as specified by the manufacturer. The 16S rRNA gene sequence obtained was a continuous stretch of 1441 bp. The sequence was compared in the EMBL nucleotide database using FASTA (Pearson, 1990), available online at the European Bioinformatics Institute homepage. Related sequences from cultivated bacteria were downloaded from the RDP-II Project homepage (Cole *et al.*, 2007) and aligned with the CLUSTAL W program (Thompson *et al.*, 1994). The alignment was checked and, if necessary, corrected manually. Phylogenetic analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004) and the PhyML online web server (Guindon *et al.*, 2005). A distance matrix-based tree using the neighbour-joining

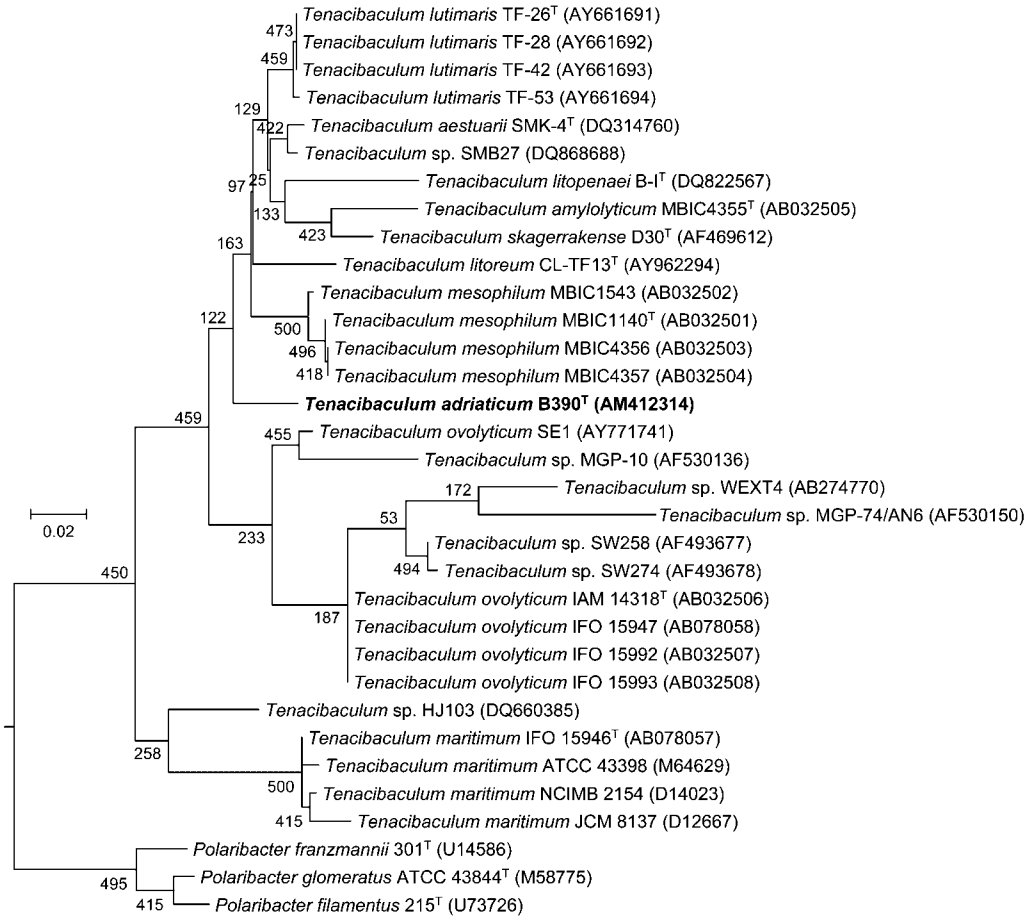

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences available from the databases (accession numbers in parentheses). Bootstrap values based on 500 replications are given at branching points. The tree was calculated using *Flexibacter flexilis* ATCC 23079^T (GenBank accession no. M62794) as an outgroup (not shown). Bar, 0.02 substitutions per nucleotide position.

method (Kimura's two-parameter model) was calculated on the basis of 1000 bootstrap replicates. A maximum-likelihood tree was generated using the general time-reversible (GTR) substitution model with estimated proportion of invariable sites and bootstrap values of 500.

Cells of strain B390^T were rod-shaped, 0.3 μm wide and 1.5–3.5 μm long and Gram-negative. Considerably longer filaments could sometimes be observed, and spherical cells occurred regularly under suboptimal growth conditions such as adverse temperature, salinity or pH. Characteristic morphological, physiological and chemotaxonomic properties are summarized in Table 1 and are included in the species description. The DNA G+C content of strain B390^T was 31.6 mol%, which falls in the range reported for other *Tenacibaculum* species (30–35.2 mol%). Consistent with other *Tenacibaculum* species, as well as with members of the family *Flavobacteriaceae* in general, the major isoprenoid quinone was MK-6 (98.5 %); others were MK-5 (1 %) and MK-7 (0.5 %). The major components of the fatty acid profile were iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, iso-$C_{15:1}$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), iso-$C_{17:0}$ 3-OH and $C_{15:1}\omega6c$, which strengthens the affiliation of strain B390^T to the genus *Tenacibaculum*. Several other fatty acids were detected, but made up less than 5 % of the total mass. A comprehensive list of fatty acids in comparison with other *Tenacibaculum* species is given in Table 2.

A detailed Biolog respiration profile (including profiles of *T. aestuarii* JCM 13491^T and *T. lutimaris* DSM 16505^T and in comparison to published profiles of *T. skagerrakense* D28 and D30^T and *T. litopenaei* B-I^T) is available as Supplementary Table S1 in IJSEM Online. Important differences were that strain B390^T could utilize α-D-glucose and several D-glucose-derived compounds (such as α-cyclodextrin, dextrin, glycogen, cellobiose, gentiobiose, maltose and D-glucose 6-phosphate) as well as succinic acid, succinamic acid and succinic acid monomethyl ester, whereas *T. aestuarii* JCM 13491^T and *T. lutimaris* DSM 16505^T could not. *T. litopenaei* B-I^T and *T. skagerrakense* D28 and D30^T were able to utilize α-D-glucose; the

latter two strains were also positive for utilization of α-cyclodextrin, dextrin, glycogen, cellobiose and maltose, but not succinic acid, succinamic acid or succinic acid monomethyl ester. All strains were respiration-positive for acetic acid, L-glutamic acid and L-proline.

The API 20E test revealed positive responses for gelatinase and cytochrome oxidase. Flexirubin-type pigment was absent and strain B390^T did not adsorb Congo red, indicating the absence of extracellular glycans.

Phylogenetic analysis based on 16S rRNA gene sequences showed that strain B390^T clustered within other *Tenacibaculum* sequences, but formed a distinct branch that was separated from type strains of all hitherto-described *Tenacibaculum* species. This branching was demonstrated by the maximum-likelihood tree (Fig. 1) as well as by the neighbour-joining tree (Supplementary Fig. S1).

Sequence similarities below 97 % were found with the type strains of all other species of *Tenacibaculum* with validly published names except for *T. aestuarii* SMK-4^T and *T. lutimaris* TF26^T, which showed similarities of 97.2 and 97.3 % using FASTA. DNA–DNA hybridization tests were performed. Results of a replicate hybridization test are given in parentheses. The DNA relatedness of strain B390^T was 27.4 (32.9) % to *T. aestuarii* JCM 13491^T and 27.1 (20.5) % to *T. lutimaris* DSM 16505^T. According to the recommendation of Wayne *et al.* (1987), who defined a threshold value over 70 % for DNA–DNA relatedness as a definition of bacterial species, strain B390^T is not a member of *T. aestuarii* or *T. lutimaris*.

In conclusion, the phenotypic and phylogenetic data presented indicate that strain B390^T should be classified within a novel species, for which the name *Tenacibaculum adriaticum* sp. nov. is proposed.

Description of *Tenacibaculum adriaticum* sp. nov.

Tenacibaculum adriaticum (ad.ri.a′ti.cum. L. neut. adj. *adriaticum* of the Adriatic Sea).

Cells are Gram-negative, rod-shaped, 0.3 μm wide and 1.5–3.5 μm long, non-flagellated but motile by means of gliding. Spherical cells (0.5–0.8 μm in diameter) occur in the stationary phase and under suboptimal growth conditions. Longer rods (up to 35 μm in length) are observed regularly. Colonies are circular with a regular edge and translucent yellow; flexirubin-type pigment is absent. Requires NaCl for growth (optimum 1–2 %) and grows in media containing artificial sea salts up to 70 g l^{-1}. Temperature range for growth is 5–34 °C, with an optimum at 18–26 °C. Grows at pH 5–9, with an optimum at pH 7. Aesculin, casein, tyrosine, starch and gelatin are hydrolysed. Oxidase-positive. According to Biolog tests, α-cyclodextrin, dextrin, glycogen, cellobiose, gentiobiose, maltose, D-glucose 6-phosphate, succinic acid, succinamic acid and succinic acid monomethyl ester are utilized, as well as Tween 40, maltose, acetic acid, D- and L-lactic acid, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-ornithine and L-proline as carbon sources. The G+C content of the DNA of the type strain is 31.6 mol%. The major isoprenoid quinone is MK-6 (98.5 %). Major fatty acids are iso-$C_{15:0}$ (25.3 %), iso-$C_{15:0}$ 3-OH (13.7 %) and iso-$C_{15:1}$ (13.1 %).

The type strain is B390^T (=DSM 18961^T =JCM 14633^T), isolated from the bryozoan *Schizobrachiella sanguinea* from the Adriatic Sea.

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