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Labilibacter aurantiacus gen. nov., sp. nov., isolated from sea squirt (*Styela clava*) and reclassification of *Saccharicrinis marinus* as *Labilibacter marinus* comb. nov.

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Abstract

A Gram-stain-negative, facultatively anaerobic, orange-pigmented bacterium, designated HQYD1^T, was isolated from a sea squirt (*Styelaclava*) and characterized using a polyphasic approach. Morphologically, strain HQYD1^T exhibited rods with gliding motility. This novel isolate grew optimally at 28 °C in the presence of 2–3 % (w/v) NaCl. The 16S rRNA gene sequence was most similar to [*Saccharicrinis*] *marinus* Y11^T (96.3 %), followed by *Saccharicinis fermentans* DSM 9555^T (93.8 %). The dominant fatty acids of strain HQYD1^T were identified as $C_{16:0}$, $C_{18:0}$ and iso-$C_{15:0}$. Major polar lipids included an unidentified lipid and a phospholipid. The major respiratory quinone was found to be MK-7, and the genomic DNA G+C content was determined to be 35.1 mol%. Based on evidence from this taxonomic study, a novel genus, *Labilibacter* gen. nov., is proposed in the family *Marinilabiliaceae* with type species *Labilibacter aurantiacus* sp. nov. The type strain of the type species is HQYD1^T (=MCCC 1K02304^T=KCTC 42583^T). As [*Saccharicrinis*] *marinus* Y11^T clustered phylogenetically with strain HQYD1^T, we also propose [*Saccharicrinis*] *marinus* Y11^T be reclassified as *Labilibacter marinus* comb. nov. (type strain Y11^T=CICC 10837^T=KCTC 42400^T).

The family *Marinilabiliaceae* suggested by Ludwig *et al.* [1] comprises 10 genera at the time of writing. Species in the genera *Alkaliflexus*, *Alkalitalea*, *Anaerophaga*, *Mangroviflexus*, *Natronoflexus* and *Thermophagus* are anaerobic, while species in the genera *Geofilum*, *Marinilabilia*, *Carboxylicivirga* and *Saccharicrinis* are capable of fermentative metabolism. Members of the family *Marinilabiliaceae* are Gram-stain-negative rods and contain MK-7 as the respiratory quinone [2–12].

In this study, an orange-pigmented bacterium, HQYD1^T, was isolated from a sea squirt collected from the coast of Weihai, China. Phenotypic and genotypic characteristics, fatty acid and menaquinone compositions and phylogenetic findings support the establishment of a novel genus in the family *Marinilabiliaceae*. Based on our analyses, we also propose the reclassification of (*Saccharicrinis*) *marinus* Y11^T to this novel genus.

Strain HQYD1^T was isolated from a sea squirt (*Styela clava*) collected from the coastal area of Weihai, China (37° 31′ 33″ N 122° 0′ 37″ E). For isolation, sea squirt tissue was homogenized and then serially diluted in sterile water. Samples were then taken from each serial dilution, plated on marine agar 2216 (MA; Difco) and incubated at 28 °C in aerobic conditions for 7 days. Orange-pigmented colonies, designated HQYD1^T, were picked and streaked several times on the same medium. Once isolated, strain HQYD1^T was routinely grown on MA or MB-agarose [marine broth 2216 (MB; Difco) supplemented with 0.05 % agarose] at 28 °C. For long-term preservation, cells were stored at −80 °C in sterile 1 % (w/v) saline medium containing 15 % (v/v) glycerol. [*Saccharicrinis*] *marinus* Y11^T (from our laboratory), *Saccharicrinis carchari* SS12^T (from our lab) and *Saccharicrinis fermentans* DSM 9555^T [Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany] were used as reference strains. Unless stated otherwise, all organisms were grown on MA or MB-agarose under identical conditions for comparative purposes.

Cell size, morphology and the presence of flagella were investigated by light microscopy (Ci-L; Nikon) and transmission electron microscopy (Jem-1200; Jeol), using cells grown on MA at 28 °C for 48, 72, 120 and 168 h. Gliding motility was examined according to the method described by Bowman [13]. Gram-reaction was performed as described by Smibert and Krieg [14]. Growth ranges and

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optima of temperature were indicated by visible colonies on MA and in MB-agarose via turbidity change at 4, 8, 10, 12, 15, 20, 25, 28, 30, 33, 35, 37, 40, 42 and 45 °C. The tolerance range for NaCl was tested in MB-agarose prepared with artificial seawater (per litre: 3.2 g $MgSO_4$, 2.2 g $MgCl_2$, 1.2 g $CaCl_2$, 0.7 g KCl, 0.2 g $NaHCO_3$) containing NaCl at concentrations from 0 to 10 % (w/v, in 1 % intervals). The effect of pH on growth was investigated in MB-agarose. The pH was adjusted by addition of MES (for pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5) and CAPSO (pH 9.0 and 9.5). Bacterial growth was monitored by using a spectrophotometer at 660 nm. Oxidase activity was tested using the oxidase reagent kit (BioMérieux) according to manufacturer's instructions (after incubation for 48 h). Catalase activity in bacterial colonies was detected by exposure to a 3 % H_2O_2 solution. Anaerobic growth was tested for 7 days at 28 °C on MA with or without 0.1 % (w/v) $NaNO_3$ in an anaerobic jar (Whitley Jar Gassing System; Don Whitely Scientific). Degradation of agar, alginate and starch, susceptibility to antibiotics and hydrolysis of Tween 80 were tested as described previously [15]. Nitrate reduction was tested as described by Dong and Cai [16]. Oxidation of carbohydrates, alcohols, organic acids, amino acids and nucleosides as sole carbon sources was evaluated in Biolog GEN III MicroPlates. Other biochemical and physiological characterizations of strain HQYD1^T were performed using API 20E, API ZYM and API 50 CHB fermentation kits (bioMérieux) according to the manufacturer's instructions, except that the suspension was prepared in 3 % (w/v) sterile sea-salt solution (Sigma).

Cellular menaquinones and polar lipids were identified from a freeze-dried sample (200 mg) of cells grown under optimal culture conditions on MB-agarose to late-exponential growth phase. Menaquinones were analysed as described by Minnikin *et al.* [17] using reversed-phase HPLC. For fatty acids analysis, the cell mass of strain HQYD1^T was harvested from MA plates after cultivation for 72 h at 28 °C. Fatty acids were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System [18]. Polar lipid analysis was performed by the Identification Service of the DSMZ. Polar lipids were extracted from 100 mg freeze-dried cell material using a chloroform/methanol/0.3 % (w/v) aqueous NaCl mixture (1 : 2 : 0.8, by vol.), modified from the Bligh and Dyer protocol [19]. Lipids were recovered in the chloroform phase by adjusting the chloroform/methanol/0.3 % (w/v) aqueous NaCl mixture to a ratio of 1 : 1 : 0.9 (by vol.), and were separated by two-dimensional silica gel thin-layer chromatography (Macherey-Nagel Art. No. 818 135) [20].

Colonies of strain HQYD1^T were orange-pigmented, transparent, circular and approximately 0.5 mm in diameter on MA after incubation for 72 h at 28 °C under aerobic

Table 1. Differential characteristics of strain HQYD1^T and with its phylogenetically related neighbours

Strains: 1, HQYD1^T (data from this study); 2, [*Saccharicrinis*] *marinus* Y11^T (this study); 3, *S. fermentans* DSM 9555^T (this study); 4, *S. carchari* SS12^T (this study); 5, *Carboxylicivirga mesophila* MEBiC 07026^T [11]. All strains were Gram-stain-negative, facultatively anaerobic, rod-shaped. and require NaCl for active growth. The major respiratory quinone of all strains was MK-7. +, Positive; −, negative; w, weakly positive; v, variable.

Characteristic	1	2	3	4	5
Colony colour	Orange	Yellow	Bright yellow	Yellow	Yellow
Cell size (μm)	0.3–0.5×1.5–20.0	0.3–0.5×2–17	0.3–0.7×8–50	0.5–0.7×7–14	0.4–0.8×8.2–11.8
Temperature range (optimum) for growth (°C)	4–37 (28)	4–33 (28–30)	18.5–37.5 (30)	10–40 (28–30)	18.2–38.1 (30.3)
Gliding motility	+	+	−	+	−
Catalase	w	−	+	+	+
Oxidase	−	+	−	−	−
Nitrate reduction	−	+	−	+	−
Indole production	−	+	−	+	v
Hydrolysis of:					
Agar	+	+	+	−	−
Gelatin	+	−	−	−	+
Urea	−	−	−	+	+
Enzyme activities					
Esterase (C4)	+	+	−	w	−
Esterase lipase (C8)	−	−	+	+	+
Valine arylamidase	+	−	−	−	−
Major fatty acids	$C_{16:0}$, $C_{18:0}$, iso-$C_{15:0}$	iso-$C_{15:0}$, $C_{16:0}$, anteiso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$	iso-C15 : 0, anteiso-$C_{15:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$
DNA G+C content (mol%)*	35.1	36.1	37.6[a]	40.0[b]	44.0

*Data from: *a*, Yang *et al.* [11]; *b*, Liu *et al.* [12].

conditions. Cells were Gram-negative rods (0.3–0.5 μm×1.5–20.0 μm) with gliding motility and had a single polar flagellum (Fig. S1, available in the online Supplementary Material). Strain HQYD1^T and [*Saccharicrinis*] *marinus* Y11^T were readily distinguished from *S. fermentans* DSM 9555^T and *S. carchari* SS12^T by physiological features, such as growth at 4 °C and the presence of esterase, β-galactosidase and naphthol-AS-BI-phosphohydrolase. Detailed comparison of major features of strain HQYD1^T with its phylogenetically related neighbours are shown in Table 1.

Genomic DNA of the isolate was extracted and purified using a genomic DNA extraction kit (Takara) and the DNA G+C content was determined by HPLC [21]. A restriction cut lambda standard was used as molecular ladder. The 16S rRNA gene was amplified by PCR using two universal primers as described by Liu *et al.* [12]. Sequencing of the 16S rRNA gene and phylogenetic analysis were performed as described by Wang *et al.* [15]. Comparison of this sequence with the 16S rRNA gene sequences of established species was performed using the EzTaxon server version 2.1 [22]. A phylogenetic tree was reconstructed with the neighbour-joining algorithm implemented in the software package MEGA (version 6.0) [23]. Phylogenetic trees were also generated with the maximum-likelihood [24] and maximum-parsimony algorithms [23] and showed the same phylogenetic trends as the neighbour-joining tree.

The 16S rRNA gene sequence of strain HQYD1^T was composed of 1442 bp. Comparison of this sequence with the 16S rRNA gene sequences of established species indicated that the closest relatives of the novel organism were members of the family *Marinilabiliaceae*, order *Bacteroidales*, class *Bacteroidia* in the phylum *Bacteroidetes*. The sequence was 96.3 % identical to *S. marinus* Y11^T, 93.8 % identical to *S. fermentans* DSM9555^T, and 92.2 % identical to *S. carchari* SS12^T. The sequence of [*Saccharicrinis*] *marinus* Y11^T was 93.0 % identical to *S. marinus* Y11^T and 93.4 % identical to *S. fermentans* DSM9555^T. Strain HQYD1^T formed a phylogenetic cluster with strain [*Saccharicrinis*] *marinus* Y11^T at a bootstrap confidence level of 100 % and was distinctly separated from members of genus *Saccharicrinis* at a bootstrap confidence level of 98 % (Fig. 1). This can be estimated and verified by the maximum-likelihood tree and maximum-parsimony tree. Comparative 16S rRNA gene sequence analysis suggests that strain HQYD1^T should be classified as a new genus and species in the family *Marinilabiliaceae* (Fig. 1).

In addition to its unique 16S rRNA gene sequence, there are a number of other features that may be used to clearly distinguish the proposed genus from other members of the *Marinilabiliaceae*. For example, members of the novel genus can grow under 10 °C, and cannot grow at 40 °C, unlike genera *Saccharicrinis*, *Carboxylicivirga*, *Geofilum*, *Marinilabilia*, *Alkaliflexus*, and *Thermophagus*. Additional differential characteristics are shown in Table 2. The DNA G+C content of strain HQYD1^T is 35.1 mol%, which is closer to [*Saccharicrinis*] *marinus* Y11^T (36.1 mol%).

Similar to other species in the family *Marinilabiliaceae*, the predominant respiratory quinone present in strain HQYD1^T was identified as MK-7. Strain HQYD1^T and [*Saccharicrinis*] *marinus* Y11^T have rich linear-saturated fatty acids which were quite different from those of *S. fermentans* DSM 9555^T and *S. carchari* SS12^T (Table S1). Furthermore, *S. fermentans* DSM 9555^T and *S. carchari* SS12^T have rich branched fatty acids (>50 %), which were quite different from those of strain HQYD1^T and [*Saccharicrinis*] *marinus* Y11^T (Table S1). These findings suggest that [*Saccharicrinis*] *marinus* Y11^T is most closely related to strain HQYD1^T, rather than *S. fermentans* DSM 9555^T or *S. carchari* SS12^T. The major polar lipids of strain HQYD1^T included an unidentified lipid and phospholipid. Aminophospholipid was also detected (Fig. S2), which was similar to [*Saccharicrinis*] *marinus* Y11^T [25] but unlike *S. fermentans* DSM 9555^T and *S. carchari* SS12^T [11, 12]. More differential characteristics are shown in Table 2.

Based on the results of this taxonomic study using a polyphasic approach, strain HQYD1^T is considered to represent a novel species of a new genus of the family *Marinilabiliaceae*, for which the name *Labilibacter aurantiacus* gen. nov., sp. nov is proposed. In addition, we propose that [*Saccharicrinis*] *marinus* Y11^T be reclassified as *Labilibacter marinus* comb. nov.

DESCRIPTION OF *LABILIBACTER* GEN. NOV.

Labilibacter (La.bi.li.bac′ter. L. adj. *labilis*, gliding; N.L. masc. n. *bacter*, rod; N.L. masc. n. *Labilibacter*, gliding rod).

Cells are Gram-negative, facultatively anaerobic rods with gliding motility. No growth is observed without NaCl. The main respiratory quinine is MK-7. The predominant cellular fatty acids are $C_{16:0}$, $C_{18:0}$ and iso-$C_{15:0}$. The range of DNA G+C contents is 35–37 mol%. Phylogenetically, the genus is a member of the family *Marinilabiliaceae*, order *Marinilabiliales*, class *Bacteroidia* in the phylum *Bacteroidetes*.

The type species is *Labilibacter aurantiacus*.

DESCRIPTION OF *LABILIBACTER AURANTIACUS* SP. NOV.

Labilibacter aurantiacus (au.ran.ti′a.cus. N.L. masc. adj. *aurantiacus* orange-coloured).

In addition to the description of the genus, the following properties are exhibited. Cells are approximately 0.3–0.5 μm wide and 1.5–20.0 μm long. Colonies on MA are orange-pigmented, circular and about 0.5 mm in diameter after 72 h of growth at 28 °C. Growth was observed at 4–37 °C, pH 6.0–8.5 and in the presence of 1–5 % (w/v) NaCl [with optimum growth at 28 °C, pH 7.0–7.5 and with 2–3 % (w/v) NaCl]. Can hydrolyse agar gelatin and alginate, but not starch, urea or Tween 80. Cells are weakly positive for catalase and negative for oxidase activity and indole and H_2S production. Nitrate is not reduced to nitrite. When assayed

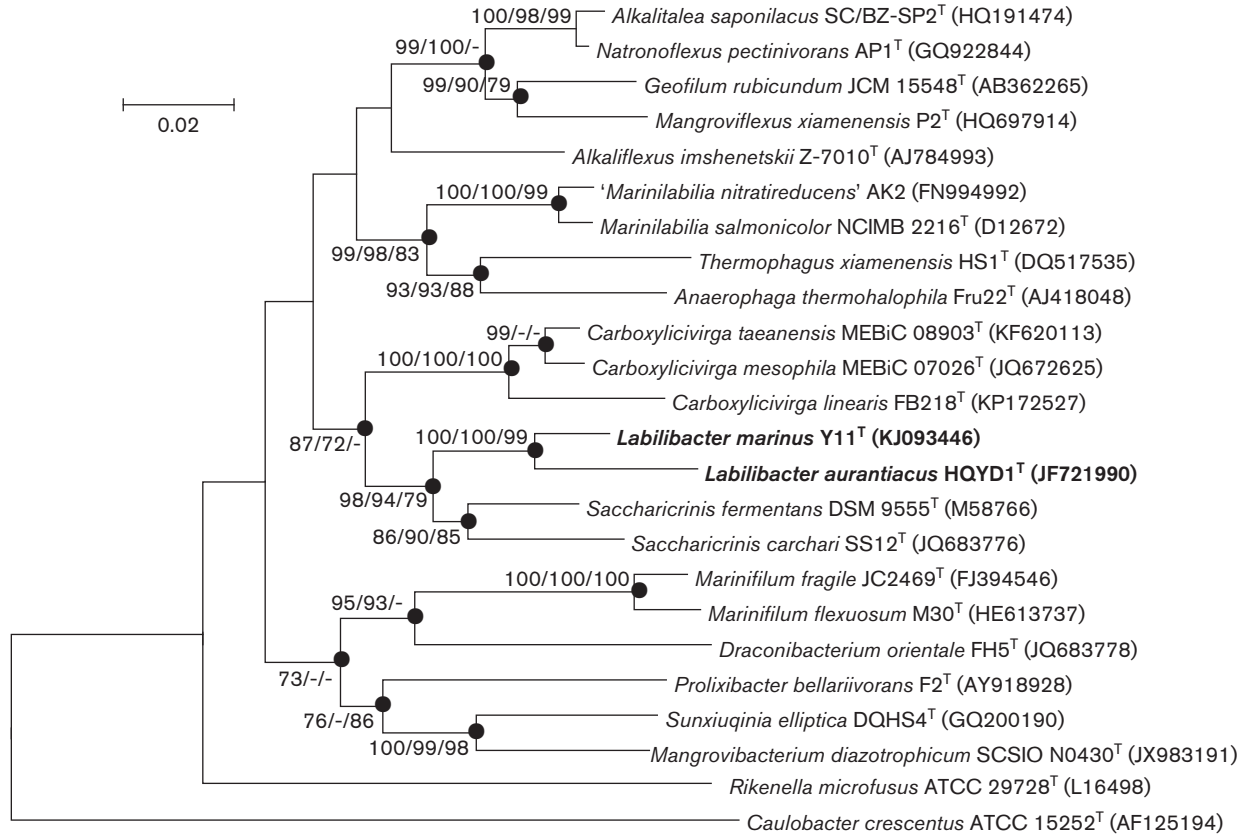

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain HQYD1^T and some other phylogenetically related taxa. Bootstrap values >70 % (based on 1000 replicates) from neighbour-joining, maximum-likelihood and maximum parsimony methods, respectively, are indicated at branch points; values <70 % are indicated by a dash. GenBank accession numbers are shown in parentheses. Bar, 0.02 substitutions per nucleotide position.

with the API ZYM system, alkaline phosphatase, esterase (C4), valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but esterase lipase (C8), lipase (C14), leucine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-

Table 2. Differential characteristics of the proposed novel genus *Labilibacter* sp. nov. and closely related genera in the family *Marinilabiliaceae*

Taxa: 1, *Labilibacter* sp. nov. (data from this study); 2, *Saccharicrinis* (this study, [11, 12]); 3, *Carboxylicivirga* [11, 26]; 4, *Geofilum* [8]; 5, *Marinilabilia* [10]; 6, *Mangroviflexus* [4]; 7, *Alkaliflexus* [2]; 8, *Alkalitalea* [3]; 9, *Anaerophaga* [5]; 10, *Natronoflexus* [6]; 11, *Thermophagus* [7]. +, Positive; –, negative; W, weakly positive; V, variable; ND, not detected; F, Facultatively anaerobic; S, Strictly anaerobic; A, Anaerobic, low O_2 tolerance.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Colony colour	Orange, yellow	Bright yellow, yellow	Yellow	Salmon pink	Yellow, salmon pink	Yellow	Yellow to pink	Salmon pink	Orange-red	Pink	White
O_2 metabolism	F	F	F	F	F	S	A	S	S	S	S
Growth at 10 °C	+	V	V	+	–	–	+	+	–	–	–
Growth at 40 °C	–	+	V	+	+	–	+	+	+	+	+
Gliding motility	+	+	V	+	+	+	+	+	ND	+	+
Catalase	–	+	+	+	+	–	+	–	–	W	+
Oxidase	V	–	–	–	ND	–	–	–	ND	ND	–
Nitrate reduction	V	V	V	+	+	–	–	ND	–	ND	–
DNA G+C content (mol%)	35–37	37.5–40	40–44.5	42.9	37	44.0	44.3	39.5±0.9	41.8	40.6	38.7

glucosaminidase, α-mannosidase and β-fucosidase are absent. Acid is produced from D-ribose, D-xylose, D-galactose, D-glucose, aesculin, cellobiose, lactose, amygdalin, starch, glycogen, D-gentiobiose and 5-keto-potassium gluconate. The following substrates are oxidized: maltose, cellobiose, gentiobiose, α-D-lactose, *N*-acetyl-β-D-mannosamine, D-mannose, D-mannitol, *myo*-inositol, gelatin, L-histidine, D-galacturonic acid, citric acid and α-ketoglutaric acid. The type strain is susceptible to acetylspiramycin, erythromycin, penicillin, cefotaxime, ceftriaxone, clindamycin, lincomycin, chloramphenicol, sulfamethoxy hoxydiazine and rifampicin. The major polar lipids are an unidentified lipid and phospholipid. In addition, phosphatidylethanolamine, an aminophospholipid and three unidentified lipids are present in moderate to minor amounts in the polar lipid profile.

The type strain, HQYD1^T (=MCCC 1K02304^T=KCTC 42583^T), was isolated from a sea squirt (*Styela clava*) collected from the coastal area of Weihai, China. The DNA G+C content of the type strain is 35.1 mol%.

DESCRIPTION OF *LABILIBACTER MARINUS* COMB. NOV.

Labilibacter marinus (ma.ri'nus. L. masc. adj. *marinus* from the sea).

Basonym: *Saccharicrinis marinus* Liu *et al.* 2015, 3429 [25].

The description remains as given by Liu *et al.* [25] with the following emendations. The major cellular fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and $C_{16:0}$. The DNA G+C content is 36.1 mol%.

The type strain, Y11^T (=CICC 10837^T= KCTC 42400^T), was isolated from marine sediment at Weihai in China.

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Conflicts of interest
The authors declare that they have no conflict of interest.

Ethical statement
This article does not contain any studies with animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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