

## ***Vitellibacter nionensis* sp. nov., isolated from shallow water hydrothermal vent of Espalamaca, Azores.**

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The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of the strain VBW088<sup>T</sup> is KC534174.

### **Abstract**

A novel, Gram-negative, non-motile, rod-shaped yellow pigmented bacterium, designated VBW088<sup>T</sup> was isolated from shallow water hydrothermal vent of Espalamaca, Azores. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain VBW088<sup>T</sup> was clustered with three type strains of *Vitellibacter* species and exhibited sequence similarity of 97.3 % with *Vitellibacter soesokkakensis* RSSK-12<sup>T</sup>. However, strain VBW088<sup>T</sup> and *V. soesokkakensis* RSSK-12<sup>T</sup> showed a low DNA-DNA relatedness (12.7±3.5 %). Strain VBW088<sup>T</sup> was positive for catalase and oxidase. Growth occurred at 10–37 °C with optimum at 30 °C, at pH 6.0–8.0 (optimum 6.0) and with up to 5 % of NaCl concentration (w/v) with optimum at 1–2 % NaCl. The major fatty acids (>10 %) were iso-C<sub>15:0</sub> (33.5 %) and iso-C<sub>17:0</sub> 3-OH (32.0 %). The polar lipids detected in strain VBW088<sup>T</sup> consisted of phosphatidylethanolamine (PE), one unidentified aminolipid and three unidentified phospholipids. The DNA G+C content of strain VBW088<sup>T</sup> was 36.7 mol%. On the basis of phylogenetic inference, DNA-DNA relatedness, chemotaxonomic analysis and physiological data, the isolate represents a novel species of the genus *Vitellibacter*, for which the name *Vitellibacter nionensis* sp. nov., is proposed, with type strain VBW088<sup>T</sup> (=KCTC 32420<sup>T</sup> = MCC 2354<sup>T</sup>).

**Keywords** Hydrothermal vent; *Vitellibacter nionensis*; Polyphasic taxonomy; Novel species

The genus *Vitellibacter*, a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*, was first established by Nedashkovskaya *et al.* (2003). At the time of writing the genus *Vitellibacter* comprised of only three validly described species, *Vitellibacter vladivostokensis* (Nedashkovskaya *et al.*, 2003), *Vitellibacter aestuarii* (Kim *et al.*, 2010) and *Vitellibacter soesokkakensis* (Park *et al.*, 2014). During our investigation on culturable bacterial diversity from shallow water hydrothermal vent of Espalamaca (Azores, Portugal), we have revealed some novel bacterial taxa belong to the phylum *Bacteroidetes*. Strain VBW088<sup>T</sup> was isolated and characterized in this study. The main aim of the present investigation was to determine the precise taxonomic place for strain VBW088<sup>T</sup> using a polyphasic approach.

A shallow water hydrothermal vent in Espalamaca (38°33'N; 28°39'W) which is located in the Faial Island, Azores, North Atlantic Ocean, was identified in 2010. Gaseous discharges from the vents are mainly composed of CO<sub>2</sub>, low concentration of methane, temperature 35 °C and pH 5.7 (Colaço, personal communication). Bottom water sample from the venting area was collected in September 2010 by scuba diving under Indo-Portugal bilateral program. For isolation of heterotrophic bacteria, serial dilutions of the samples were spread plated on seawater nutrient agar (SWNA: peptone 5.0 g L<sup>-1</sup>, beef extract 1.5 g L<sup>-1</sup>, yeast extract 1.5 g L<sup>-1</sup>, 1.5 % agar prepared in 50 % seawater). The plates were incubated for 2 days at 30 ± 1 °C. Morphologically different colonies were picked and repeatedly quadrant streaked on the same medium to get pure cultures. Subsequently, the growth of strain VBW088<sup>T</sup> was checked on tryptic soy agar (TSA) prepared in 50 % seawater, marine agar 2216 (MA: M384, Himedia) and nutrient agar prepared with artificial seawater (ASWNA). Strain VBW088<sup>T</sup> was maintained at 4 °C on SWNA and as 15 % glycerol suspensions at -80 °C for long-term storage.

Genomic DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. For 16S rRNA genes amplification, eubacterial primers 27F and 1492R were used (Lane, 1991). The PCR amplified 16S rRNA gene product was purified with PCR cleanup kit (Sigma) according to the manufacturer's instructions. The purified 16S rRNA gene was sequenced on automated DNA sequencer 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) with the bacterial primers 27F, 518F, 1053F and 1492R. The acquired near-full-length sequences were subjected to a BLASTn on the National Center for Biotechnology Information (NCBI) and EzTaxon-e (Kim *et al.*, 2012) server to identify the sequences with the highest similarity. Multiple and pairwise sequence alignment were performed using Clustal W sequence alignments program (Thompson *et al.*, 1994). Neighbour-joining (Saitou & Nei, 1987), maximum-

parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods were used to reconstruct phylogenetic trees using MEGA5 software program (Tamura *et al.*, 2011). The topology of the phylogenetic trees was evaluated by bootstrap analysis method of Felsenstein (1985) with 1,000 replications. The genomic DNA isolation and determination of G+C content by reverse-phase HPLC of nucleosides were done according to the protocol described by Mesbah *et al.* (1989). DNA-DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes in microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values are quoted as DNA-DNA relatedness values.

Gram staining was carried out by using the standard Gram reaction (K001, Himedia) and by using the KOH method (Cerny, 1978). Colony morphology was observed using a colony counter and motility by hanging drop method. Cell morphology was examined using light microscopy (Nikon TS100) and scanning electron microscopy (Hitachi TM3000). Growth under anaerobic condition was determined on MA after incubation for 10 days in an anaerobic system with an AnaeroPack (HiMedia, Mumbai). For growth at different pH, the nutrient broth was buffered with citrate (pH 4-5) phosphate (pH 6-8) and carbonate (pH 9-10) buffer systems. Growth was tested on nutrient broth at 4, 10, 20, 25, 30, 37, 40, 45 and 50 °C. In above experiments, cell densities for growth were measured at 600 nm using a spectrophotometer (Cary 300) after 2 days of incubation. Growth in the presence of 0–15 % NaCl (w/v, at 1.0 % intervals) was investigated in nutrient agar prepared in distilled water. Requirement of Mg<sup>2+</sup> was investigated on nutrient agar prepared in distilled water with 4.53 g/L MgCl<sub>2</sub>.6H<sub>2</sub>O without NaCl. Acid production from carbohydrates was determined using API 50CH kit (bioMérieux) according to the manufacturer's instructions using inoculation medium API CHB/E amended with marine cations supplement (MCS, Farmer & Hickman-Brenner, 2006). The methyl red and Voges-Proskauer tests, production of indole and H<sub>2</sub>S, sodium malonate utilisation and nitrate reduction were assessed using Hi25<sup>TM</sup> Enterobacteriaceae identification kit (Himedia).

Catalase activity was tested using 3 % (v/v) H<sub>2</sub>O<sub>2</sub> drop method (Smibert & Krieg, 1994) and oxidase activity was determined using the oxidase discs (DD018, Himedia). Carboxymethyl cellulose (CMC), xylan and starch hydrolysis were determined as per the methods of Khandeparker *et al.* (2011). Hydrolysis of urea and DNA was determined on Urea agar base (M112, Himedia) and DNase test agar (M1041, Himedia) respectively. Hydrolysis of casein (1 %), gelatin (1 %), agar (2 %), Tweens 20 and 80 (1 %), tributyrin (1 %), xanthine (0.4 %) and alginate (1 %) (Smibert & Krieg,

1994) were tested on SWNA medium. Other enzyme activities were assayed using API ZYM strips (bioMérieux) according to the manufacturer's protocol. Antibiotic susceptibility tests were performed in SWNA medium using antibiotic discs (Himedia) containing the following concentrations ( $\mu\text{g}/\text{disc}$ ): amikacin (30), amoxicillin (30), ampicillin (25), bacitracin (8 IU), cefadroxyl (30), ceftazidime (30), chloramphenicol (25), chlortetracycline (30), ciprofloxacin (30), kanamycin (30), lincomycin (10), neomycin (30), norfloxacin (10), novobiocin (30), penicillin G (2 IU), polymyxin B (50 IU), rifampicin (15), streptomycin (300), tetracycline (10), tobramycin (10) and vancomycin (5). The results were observed after 2 days incubation at 30 °C.

Strain VBW088<sup>T</sup> and *V. soesokkakensis* RSSK-12<sup>T</sup> were cultivated on MA at 30 °C for 3 days and harvested for Fatty acid methyl ester (FAME). Fatty acids were saponified, methylated and extracted using the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.2B). The fatty acids were analysed by GC (Hewlett Packard 6890) and identified by using the TSBA6 database of the Microbial Identification System (Sasser, 1990). Cell biomass of strain VBW088<sup>T</sup> for the analysis of isoprenoid quinones and polar lipids was obtained from cultures grown for 2 days at 30 °C in seawater LB broth (M1245, Himedia). Isoprenoid quinones were analyzed by using high performance liquid chromatography (HPLC) and polar lipids were extracted according to the method of Minnikin *et al.* (1984). Briefly, Polar lipids were extracted from freeze-dried cells and separated by two dimensional silica gel thin layer chromatography (Merck). The first direction was developed in chloroform/methanol/water (65:25:3.8, by vol.) and the second in chloroform/methanol/acetic acid/water (40:7.5:6:1.8, by vol.). Total lipid material and specific functional groups were identified using molybdophosphoric acid (total lipids), molybdenum blue spray reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff ( $\alpha$ -glycols), and  $\alpha$ -naphthol reagent (sugars). In addition, three days old cells grown on SWNA medium were suspended in methanol and pigments were extracted. UV-Vis scan was recorded from 200 to 900 nm in methanol using UV-Vis spectrophotometer (Cary 300). The KOH test (Reichenbach, 1992) was carried out to assess the flexirubin type pigments following the minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). The results are presented in a standardized format as described by Kämpfer *et al.* (2003).

The colonies of strain VBW088<sup>T</sup> were small, yellow and circular on MA and SWNA. The cells were Gram-negative and non-spore forming short-rods that are 0.3–0.4  $\mu\text{m}$  wide and 0.8–1.0  $\mu\text{m}$  long (Supplementary Fig. 1). Growth occurs between 10 and 37 °C (optimum 30 °C) at pH 6–8 (optimum pH 6.0) with NaCl concentration of 1–5 % NaCl (optimum 1–2 %). The results obtained from this

study concluded that strain VBW088<sup>T</sup> showed characteristics that were consistent with those of the genus *Vitellibacter*, for instance being Gram-negative, rod-shaped, non-motile, yellow-orange pigmented and oxidase positive (Nedashkovskaya *et al.*, 2003; Kim *et al.*, 2010). Differences noticed on morphological and physiological characteristics of strain VBW088<sup>T</sup> with the closest type strains of *Vitellibacter* are given in Table 1.

Nearly full length 16S rRNA gene sequence of strain VBW088<sup>T</sup> (GenBank accession number KC534174) was compared with the sequences from the GenBank database (<http://www.ncbi.nlm.nih.gov>). 16S rRNA gene sequence similarity values between strain VBW088<sup>T</sup> and other validly named species of the genus *Vitellibacter* in EzTaxon-e database are *V. soesokkakensis* RSSK-12<sup>T</sup> (97.3 %), *V. vladivostokensis* KMM 3516<sup>T</sup> (96.2 %) and *V. aestuarii* JC2436<sup>T</sup> (95.1 %). On the other hand, the species of the genus *Aequorivita* also very close to the genus *Vitellibacter* and had similarity values between 94.6 – 96.5 %. However, both neighbour-joining and maximum-parsimony phylogenetic trees confirmed that strain VBW088<sup>T</sup> clustered within the clade comprising *Vitellibacter* species (Fig. 1 and Supplementary Fig. 2). In addition, DNA-DNA hybridization between strain VBW088<sup>T</sup> and *V. soesokkakensis* RSSK-12<sup>T</sup> showed a low DNA-DNA relatedness (12.7±3.5 %).

Strain VBW088<sup>T</sup> was sensitive to the following antibiotics: amoxicillin, ampicillin, cefadroxyl, ceftazidime, chloramphenicol, chlortetracycline, ciprofloxacin, lincomycin, norfloxacin, novobiocin, penicillin G, rifampicin, streptomycin, tetracycline and vancomycin; resistant to the following antibiotics: amikacin, bacitracin, kanamycin, neomycin, polymyxin B and tobramycin.

The whole cell fatty acid profile of strain VBW088<sup>T</sup> could not provide a precise identification in MIDI database, which concluded that this strain belonged to a novel species. The predominant fatty acids of strain VBW088<sup>T</sup> are iso-C<sub>15:0</sub> (33.5 %) and iso-C<sub>17:0</sub> 3-OH (32.0 %). The fatty acids profile of strain VBW088<sup>T</sup> and the type strains of *Vitellibacter* are almost similar although differences in the percentage (Table 2). Even though strain VBW088<sup>T</sup> and *V. soesokkakensis* RSSK-12<sup>T</sup> were only analysed from this study, fatty acids profile of other two type strains *V. vladivostokensis* KMM 3516<sup>T</sup> and *V. aestuarii* JC2436<sup>T</sup> were also compared since same media and growth conditions had been used in Park *et al.* (2014) for the fatty acid analysis. The polar lipid profile consisted of phosphatidylethanolamine as major component followed by three unidentified phospholipids and one unidentified aminolipid (Supplementary Fig. 3). The major isoprenoid quinone was menaquinone (MK-6) that is consistent with other *Vitellibacter* species. The G+C content of strain VBW088<sup>T</sup> was

36.7 mol% which is very close to *V. soesokkakensis* RSSK-12<sup>T</sup> but however, other two type strains were reported to have more than 40 mol% (Nedashkovskaya *et al.*, 2003; Kim *et al.*, 2010).

The methanol extract of the yellow pigmented strain VBW088<sup>T</sup> has absorption peak at 450 nm (Supplementary Fig. 4). Some of the yellow pigmented *Flavobacterium* and *Zeaxanthinibacter* are known to produce carotenoids with absorption maxima of around 450 nm (Asker *et al.*, 2007). When the extracts were tested for the presence of flexirubin pigments by exposing them to KOH, resulted no change of colour which indicated that the pigment is not belong to flexirubin (Reichenbach *et al.*, 1980). Interestingly, two of the *Vitellibacter* species were able to produce flexirubin pigments (Nedashkovskaya *et al.*, 2003; Kim *et al.*, 2010).

Based on low level of DNA-DNA relatedness (12.7±3.5 %), chemotaxonomic data, phenotypic, and phylogenetic inference presented above, it can be concluded that strain VBW088<sup>T</sup> represents a novel species of the genus *Vitellibacter*, for which the name *Vitellibacter nionensis* sp. nov. is proposed.

#### **Description of *Vitellibacter nionensis* sp. nov.**

*Vitellibacter nionensis* (ni.o.nen'sis. N.L. masc. adj. *nionensis*, arbitrary name from the acronym of National Institute of Oceanography, NIO, where taxonomic studies were performed).

Cells are aerobic, Gram-negative, non-spore forming, non-motile and short-rods (0.3–0.4 × 0.8–1.0 µm). Colonies on MA are circular, raised, smooth, yellow in colour after incubation for 48 h at 30 °C. Optimal growth occurs at 30 °C; growth occurs between 10 and 37 °C. Optimal pH for growth is 6.0; growth occurs at pH 6.0 to 8.0. Optimal growth occurs in the presence of 1 – 2 % (w/v) NaCl; growth occurs in the presence of 5.0 % (w/v) NaCl, but not in the absence of NaCl or above 5.0 % (w/v) NaCl. Mg<sup>2+</sup> not required for growth. Catalase and oxidase are positive. Anaerobic growth does not occur on MA. Carotenoid pigment is produced but not flexirubin. Nitrate is not reduced to nitrite. Citrate utilization, H<sub>2</sub>S and indole production, and the ONPG, methyl red and Voges Proskauer tests are negative. Gelatin, casein, Tween 20 and Tween 80 are hydrolysed, but starch, DNA, CMC, xylan, urea, tributyrin, xanthine and alginate are not hydrolysed. Sodium malonate is utilised as a sole carbon source. Detectable levels of acid are not produced from carbohydrates except potassium 5-keto gluconate. In assays with the API ZYM strips, alkaline phosphatase, esterase (C 4), esterase lipase (C 8), leucine arylamidase, valine arylamidase, cystine arylamidase (weak), acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present, but lipase (C 14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are absent. The predominant

menaquinone is MK-6. The major fatty acids are iso-C<sub>15:0</sub> and iso-C<sub>17:0</sub> 3-OH. The major polar lipids are phosphatidyl ethanolamine, one unidentified aminolipid and three unidentified phospholipids. The DNA G+C content of the type strain is 36.7 mol%.

The type strain is VBW088<sup>T</sup> (= KCTC 32420<sup>T</sup> = MCC 2354<sup>T</sup>), isolated from shallow water hydrothermal vent of Espalamaca, Azores (North Atlantic Ocean), Portugal. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain VBW088<sup>T</sup> is KC534174.

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**Table 1.** Differential characteristic between strain VBW088<sup>T</sup> and type strains of *Vitellibacter* species

Characteristic	1	2	3	4
Pigmentation	Y	Y	Y-O	Y-O
Cell size ( $\geq 5 \mu\text{m}$ )	-	+	+	+
Gliding motility	-	+	-	-
Growth at 40°C	-	-	+	+
Growth at 4°C	-	-	+	-
Catalase	+	+	+	-
NaCl range for growth (%) <sup>*</sup>	1-5	0-12	1-6	2-6
Hydrolysis of: <sup>†</sup>				
DNA	-	ND	+	+
Tween 80 <sup>*</sup>	+	+	-	-
Acid production from <sup>†</sup>				
Potassium 5-keto gluconate	+	ND	-	-
D-galactose <sup>*</sup>	-	+	-	-
API ZYM <sup>*</sup>				
Lipase (C14)	-	-	-	W
Cystine arylamidase	W	+	-	+
Trypsin	-	+	+	-
$\alpha$ -chymotrypsin	-	-	+	-
DNA G+C content	36.7	38.9	41.3	48.7

Strains: 1, VBW088<sup>T</sup> (present study); 2, *V. soesokkakensis* RSSK-12<sup>T</sup> (Park *et al.*, 2014); 3, *V. vladivostokensis* KMM 3516<sup>T</sup> (Nedashkovskaya *et al.*, 2003); 4, *V. aestuarii* JC2436<sup>T</sup> (Kim *et al.*, 2010). <sup>\*</sup>Data for column 1, 2 and 3 from this study; <sup>†</sup>Data for column 1 and 3 from present study. Y, yellow; O, orange; Y-O, yellow to orange; +, positive reaction; -, negative reaction; W, weakly positive; ND, not determined. All strains were positive for oxidase; hydrolysis of casein, gelatin and Tween 20; alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. All strains were negative for anaerobic growth; Gram-staining; nitrate reduction; hydrolysis of starch;  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase.

**Table 2.** Fatty acid profile of the strain VBW088<sup>T</sup> and type strains of *Vitellibacter* species

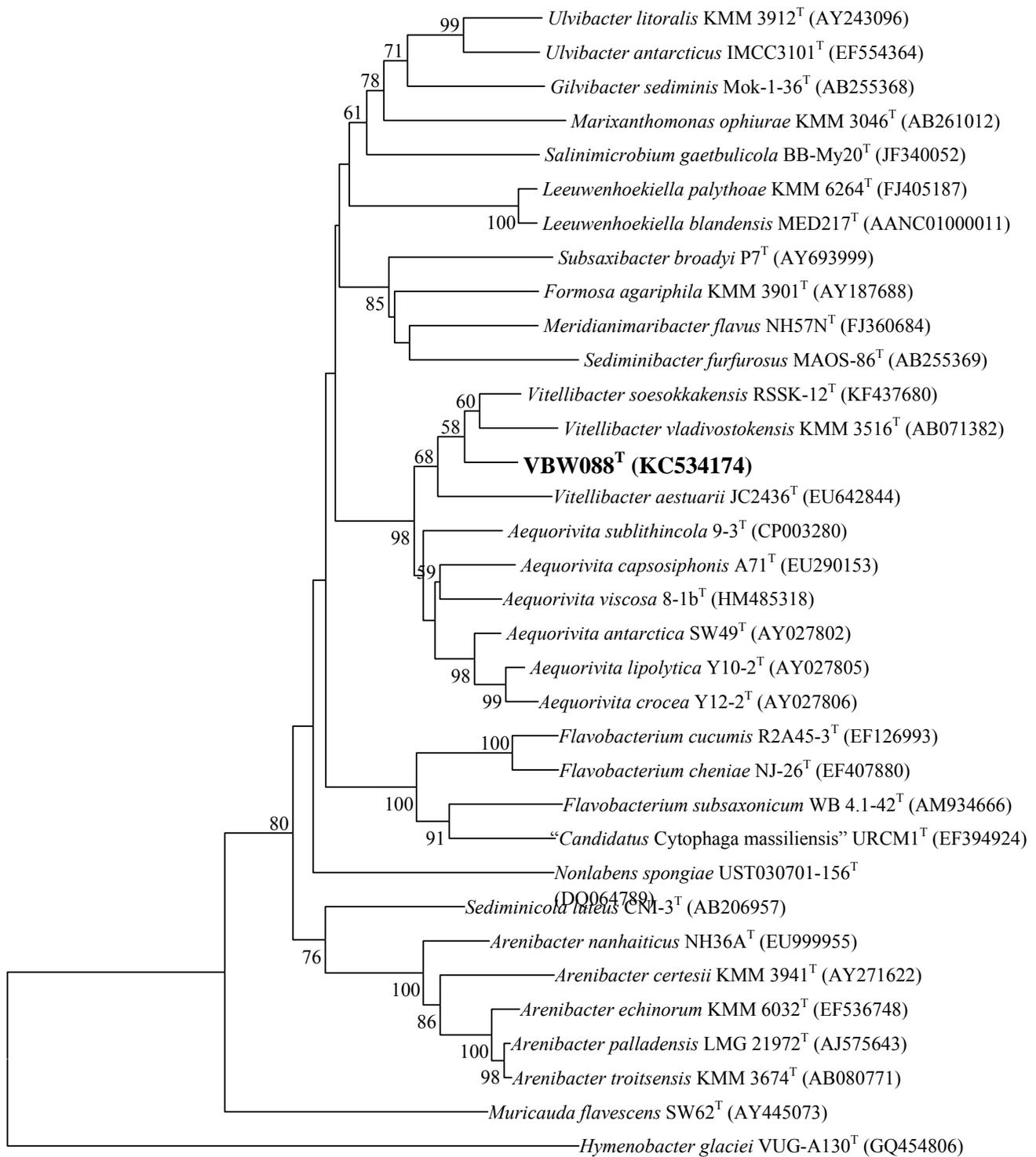
Fatty acid	1	2	3	4
C <sub>16:0</sub>	1.2	1.4	2.9	1.4
C <sub>17:0</sub> 2-OH	1.4	1.4	1.0	-
C <sub>17:0</sub> 3-OH	2.1	1.3	-	-
iso-C <sub>15:0</sub>	33.5	34.6	40.1	36.7
anteiso-C <sub>15:0</sub>	2.6	3.2	4.5	5.4
iso-C <sub>15:0</sub> 3-OH	4.0	4.4	3.3	3.9
iso-C <sub>16:0</sub>	2.4	2.4	1.7	1.7
iso-C <sub>16:0</sub> 3-OH	2.4	2.3	-	1.4
iso-C <sub>17:0</sub> 3-OH	32.0	31.0	25.3	20.8
iso-C <sub>15:1</sub> G	2.6	2.2	1.6	4.7
iso-C <sub>17:1</sub> ω9 <i>c</i>	8.3	7.1	6.8	6.7
Summed feature 3	4.2	5.6	5.6	10.9

Strains: 1, VBW088<sup>T</sup> (present study); 2, *V. soesokkakensis* RSSK-12<sup>T</sup> (present study); 3, *V. vladivostokensis* KMM 3516<sup>T</sup> and 4, *V. aestuarii* JC2436<sup>T</sup> (Park *et al.*, 2014). Media and growth conditions are similar for all the type strains. Summed feature 3 contained C<sub>16:1</sub>ω6*c*/ C<sub>16:1</sub>ω7*c*; Fatty acids amounting ≥1 % of the totals fatty acids are shown.

### Figure legend

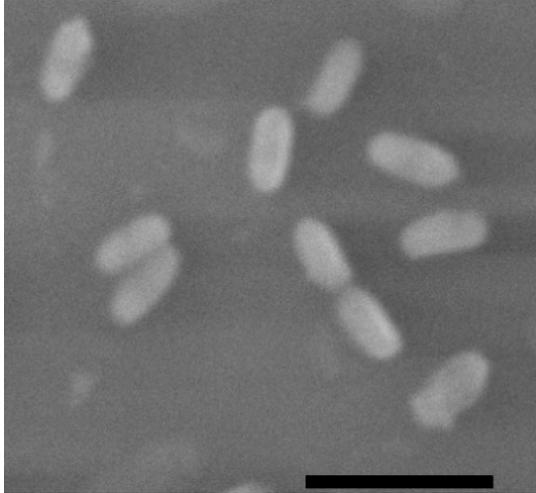
**Fig. 1.** Neighbour-joining tree showing the phylogenetic relationship of strain VBW088<sup>T</sup> and phylogenetically related species based on 16S rRNA gene sequences. Numbers at the nodes are bootstrap values >50 %. *Hymenobacter glaciei* VUG-A130<sup>T</sup> (GQ454806) was used as an out-group. Bar, 0.02 substitutions per nucleotide position.

**Fig. 1.**

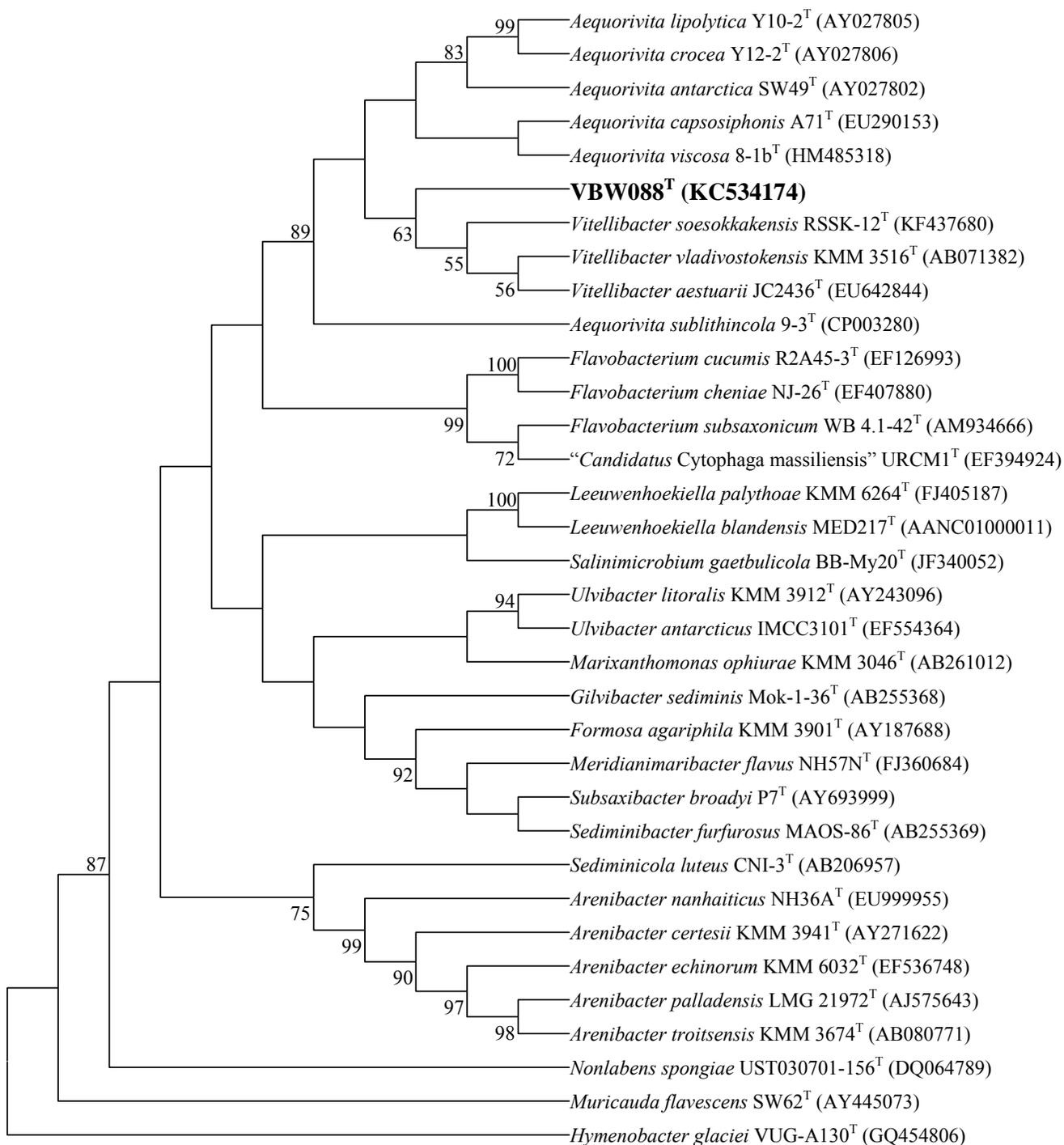


0.02

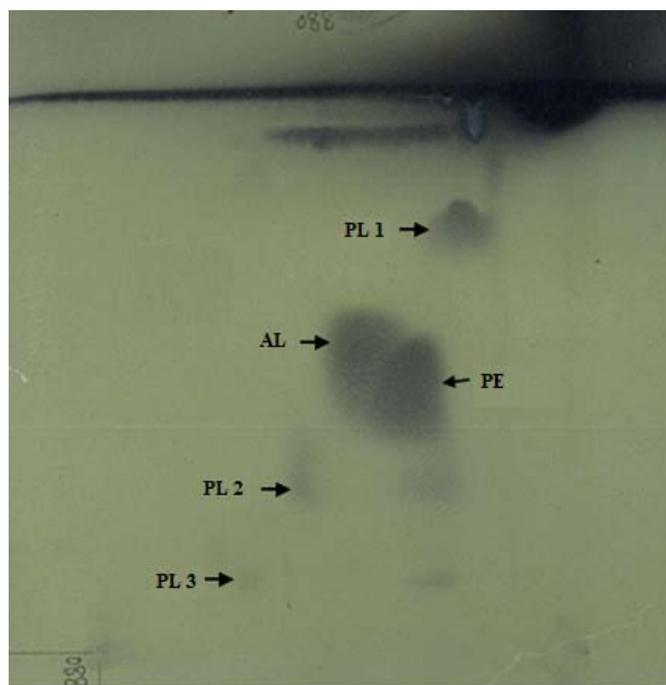
**Supplementary Fig. 1** Scanning electron microscopic picture of strain VBW088<sup>T</sup> showing rod-shaped cells (bar = 2.0  $\mu\text{m}$ )



**Supplementary Fig. 2.** Maximum-parsimony tree based on 16S rRNA gene showing the phylogenetic position of the strain VBW088<sup>T</sup> and related representative taxa.



**Supplementary Fig. 3.** Two-dimensional TLC of polar lipids of the strain VBW088<sup>T</sup>. PL 1-3, unidentified phospholipids; PE, phosphatidyl ethanolamine; AL, unidentified aminolipid



**Supplementary Fig. 4.** UV-VIS absorption spectrum of a methanolic extract of the pigment from strain VBW088<sup>T</sup>

