Queries for Author

Paper: **IJSEM-D-19-00708**

The proof of your manuscript appears on the following page(s).
Please read the manuscript carefully, checking for accuracy, verifying the reference order and double-checking figures and tables.

During the preparation of your manuscript for publication, the questions listed below have arisen. Please attend to these matters and return the answers to these questions when you return your corrections.

Please note, we will not be able to proceed with your article and publish it in print if these queries have not been addressed.

No	Query
AQ1	Please check and confirm that the authors name and affiliations details are correct.
AQ2	Please check that the values '6085.336 bp, larger than the 5387.817 bp' have been fomatted correctly. Is the decimal point correct?
AQ3	In fig. 1 1. In Sinobacterium norvegicum type strain has been changed from CM4 to 3CM4

INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Lucena *et al.*, *Int. J. Syst. Evol. Microbiol.*
DOI 10.1099/ijsem.0.003906

MICROBIOLOGY SOCIETY

Thalassocella blandensis gen. nov., sp. nov., a novel member of the family *Cellvibrionaceae*

Teresa Lucena[1], David R. Arahal[1], Isabel Sanz-Sáez[2], Silvia G. Acinas[2], Olga Sánchez[3], Rosa Aznar[1], Carlos Pedrós-Alió[4] and María J. Pujalte[1,*]

Abstract

Strain ISS155^T, isolated from surface Mediterranean seawater, has cells that are Gram-reaction-negative, motile, strictly aerobic chemoorganotrophic, oxidase-positive, unable to reduce nitrate to nitrite, and able to grow with cellulose as the sole carbon and energy source. It is mesophilic, neutrophilic, slightly halophilic and has a requirement for sodium and magnesium ions. Its 16S rRNA gene sequence places the strain among members of *Cellvibrionaceae*, in the *Gammaproteobacteria*, with *Agarilytica rhodophyticola* 017^T as closest relative (94.3% similarity). Its major cellular fatty acids are $C_{18:1}$, $C_{16:0}$ and $C_{16:1}$; major phospholipids are phosphatidyl glycerol, phosphatidyl ethanolamine and an unidentified lipid, and the major respiratory quinone is Q8. The genome size is 6.09 Mbp and G+C content is 45.2 mol%. A phylogenomic analysis using UBCG merges strain ISS155^T in a clade with *A. rhodophyticola*, *Teredinibacter turnerae*, *Saccharophagus degradans* and *Agaribacterium haliotis* type strain genomes, all of them possessing a varied array of carbohydrate-active enzymes and the potential for polysaccharide degradation. Average amino acid identity indexes determined against available *Cellvibrionaceae* type strain genomes show that strain ISS155^T is related to them by values lower than 60%, with a maximum of 58% to *A. rhodophyticola* 017^T and 57% to *T. turnerae* T7902^T and *S. degradans* 2-40^T. These results, together with the low 16S rRNA gene sequence similarities and differences in phenotypic profiles, indicate that strain ISS155^T represents a new genus and species in *Cellvibrionaceae*, for which we propose the name *Thalassocella blandensis* gen. nov., sp. nov., and strain ISS155^T (=CECT 9533^T=LMG 31237^T) as the type strain.

The family *Cellvibrionaceae* was established by Spring and colleagues after a comparative genome-based study [1]. It comprises the genera *Cellvibrio*, *Eionea*, *Gilvimarinus*, *Maricurvus*, *Marinagarivonans*, *Marinibactrum*, *Marinimicrobium*, *Pseudomaricurvus*, *Pseudoteredinibacter*, *Saccharophagus*, *Simiduia*, *Teredinibacter* and *Umboniibacter* [2]; while *Agarilytica* [3], *Agaribacterium* [4] and '*Halioxenophilus*' [5] were added recently. Members of genus *Cellvibrio* have a terrestrial origin, related to soil and decaying plant materials; however, *Cellvibrionaceae* are marine bacteria and display a slightly halophilic behaviour. Most species in this family possess a large variety of polysaccharide-degrading abilities and their genomes contain dozens of CAZyme (carbohydrate-active enzyme) genes, enabling the hydrolysis of cellulose, agar, carrageenan, xylan, starch, chitin and several other polysaccharides. One species, *Teredinibacter turnerae*, establishes symbiotic relationships as gill-associated, facultative endosymbiotic bacteria with different members of the bivalve family *Teredinidae*, a group of wood-boring molluscs [6]. The cellulolytic capacity of these animals relays in the enzymatic machinery provided by the associated bacteria [7], which, in addition to its dinitrogen-fixing ability, provides the bacteria with a particular niche in the molluscs. Other species have been isolated from marine algae (*Graciliaria*, *Gelidium*), other molluscs (*Haliotis*, *Umbonium*) or coastal seawater and tidal flat sediments [1].

Here we present the description of a novel genus and species in this family, based on the phenotypic, genomic and

Author affiliations: [1]Departamento de Microbiología y Ecología and Colección Española de Cultivos Tipo (CECT), Universitat de València, Valencia, Spain; [2]Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, ICM-CSIC, Barcelona, Spain; [3] Departament de Genètica i Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; [4]Department of Systems Biology, Centro Nacional de Biotecnología (CNB), CSIC, Madrid, Spain.
***Correspondence:** María J. Pujalte, maria.j.pujalte@uv.es
Keywords: *Thalassocella* ; *Teredinibacter*; *Agarilytica*; *Cellvibrionaceae*; marine bacteria; ANI; AAI.
Abbreviations: AAI, average amino acid identity; ANI, average nucleotide identity; DDH, DNA–DNA hybridization; GSI, gene support index; UBCG, up-to-date bacterial core gene.
The accession numbers of the 16S rRNA gene sequence and the draft genome of *Thalassocella blandensis* ISS155^T are MH732325 and CABFPG01, respectively.
Two supplementary tables and four supplementary figures are available with the online version of this article.

Table 1. Genomes used in the study and their main characteristics

Strain	Accession number	Size (Mbp)	G+C content (mol%)	Protein-encoding genes	rRNA-encoding genes
Thalassocella blandensis ISS155^T	CABFPG01	6.09	45.2	5425	43
Agarylytica rhodophyticola 017^T	NZ_CP020038	6.88	40.9	6307	82
Teredinibacter turnerae T7902^T	ARAH01	5.39	50.8	4886	39
Saccharophagus degradans 2-40^T	NC_007912	5.06	45.8	4504	47
Agaribacterium haliotis feces2^T	NKQJ01	4.17	50.5	3551	39
Marinagarivorans algicola Z1^T	LGAK01	4.06	45.1	3716	46
Umboniibacter marinipuniceus DSM 25080^T	REFJ01	2.69	49.4	2491	68
Gilvimarinus polysaccharolyticus YN3^T	LFIJ01	3.74	49.4	3452	45
Gilvimarinus chinensis DSM 19667^T	ARIX01	4.07	51.2	3774	61
Cellvibrio japonicus Ueda107^T	NC_010995	4.58	52.0	3993	57
Cellvibrio mixtus J3-8^T	ALBT01	5.17	46.7	4695	43
Marinimicrobium koreense DSM 16974^T	RJUK01	3.85	58.8	3248	45
Marinimicrobium agarilyticum DSM 16975^T	AUHU01	4.50	57.9	3861	60
Simiduia agarivorans DSM 21679^T	NC_018868	4.30	55.8	3955	50

phylogenetic study of the free-living strain ISS155^T, a Mediterranean Sea isolate.

Strain ISS155^T was isolated in 2015 from surface seawater at the Northwestern Mediterranean Sea (41° 40′ N 2° 48′ E, in Blanes Bay, Girona, Spain. Isolation medium was based on the marine agar 2216 (Difco) but phosphate was autoclaved separately from agar to avoid hydrogen peroxide formation [8]. The culture was grown at room temperature at the Institut de Ciències del Mar (CSIC; Barcelona, Spain). The almost-complete 16S rRNA gene sequence of strain ISS155^T (deposited under the accession number MH732325) was obtained after DNA extraction using the DNeasy Blood and Tissue kit (Qiagen), following the manufacturer's recommendations and using the modified primers from Page *et al.* [9] 27F (5′-AGRGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTT AYGACTT-3′). ChromasPro 2.1.8 software (Technelysium) was used for manual cleaning and quality control of the sequences. A BLAST search for the closer taxa, based on this sequence, related the strain to *T. turnerae* and other members of the family *Cellvibrionaceae* but the highest sequence similarities found were all below 95 %, suggesting that the strain represented a new taxon in the family. Thus, a study, including phenotypic, genomic and phylogenetic characterization of the strain, was undertaken in order to define its taxonomic position in the family. In this study, *T. turnerae* CECT 9444^T was used for comparative purposes and all available genomes of *Cellvibrionaceae* type strains were retrieved from public databases (Table 1). Strain ISS155^T has been maintained by lyophilization at the Spanish Type Culture Collection (CECT) as CECT 9533^T.

Phenotypic characterization, including morphological, cultural, biochemical, physiological and nutritional screening, was performed by following already described methods [10, 11]. In addition to the tests described above, flagellar arrangement was determined with phase-contrast microscopy (DMRB, Leica) by using the staining technique of Heimbrook *et al.* [12]. Cellulose degradation was tested in shipworm basal medium as described by Distel *et al.* [6] with submerged strips of Whatman filter paper as sole carbon source and incubation at 26 °C. Nitrate reduction was determined in two ways: in Baumann's denitrification medium [13] and using API 20NE strips. The API strips were inoculated with suspensions made on half-strength artificial sea water [13] and the AUX medium of the API 20NE and 50CH/E kits were supplemented with marine cations supplement. For TEM and SEM observations of the morphology and ultrastructure of strain ISS155^T, the strain was grown overnight in marine broth with shaking in the dark. For SEM observations, cells were filtered with a 47 mm polycarbonate membrane filter of 0.2 µm pore size (Millipore) with a peristaltic pump. Filters were fixed in glutaraldehyde with final concentration 3% at room temperature and washed three times with 0.1 M phosphate buffer. A series of sequential ethanol dehydrations were performed for 10 min each (50, 70, 95 and 100%) before drying samples under CO_2 using a critical point drier apparatus (CPD030, Baltec). Samples were gold sputter coated in order to visualize them with SEM Zeiss Merlin Fe apparatus. On the other hand, for TEM observations, the overnight culture was centrifuged at 1000 ***g*** during 15 min and supernatant was discarded. The pellet was fixed with paraformaldehyde at final concentration

2% during 30 min at room temperature. After fixation, pellet was processed as described previously [14] to finally obtain thin sections of the samples that were examined by using TEM (JEM-1400 plus, JEOL). Visualizations were done by the microscopy service of the Universitat Autònoma de Barcelona (http://sct.uab.cat/microscopia/en/content/inici).

Cells of strain ISS155^T were Gram-reaction-negative, rod-shaped and motile by a single polar flagellum, strictly aerobic and chemoorganoheterotrophic, oxidase- and catalase-positive, unable to ferment carbohydrates, and unable to reduce nitrates. Growth in liquid media usually produced a superficial translucent mucous mass. The strain grew well on marine agar and in marine broth, in contrast to *T. turnerae* CECT 9444^T, which was unable to grow on this medium. Colonies on marine agar were non-pigmented, small, regular and transparent. The strain was able to grow on cellulose as the sole carbon source in the liquid medium described by Distel *et al.* after 7 days [6] with only a little delay and without the yellowish colour developed by *T. turnerae* CECT 9444^T, but the degradation of Whatman paper was similar. A thick mucous, translucent layer was formed at the surface of the medium. The strain was also able to hydrolyse gelatin in API 20NE (but it did not grow on marine broth plus 12% gelatin), casein, Tween 80, starch and DNA, but not alginate or agar. Carbon sources sustaining growth on Baumann's basal medium included a few carbohydrates (D-glucose and *N*-acetyl D-glucosamine rendered the highest growth, also, amygdalin and D-glucuronate and D-glycerol gave positive results), organic acids (acetate, pyruvate, fumarate) and amino acids (L-glutamate, L-leucine, L-alanine, L-serine, L-arginine, L-citrulline and L-aspartate). The strain was mesophilic, neutrophilic and slightly halophilic, with an absolute requirement for sodium and magnesium ions for growth.

SEM images of strain ISS155^T cells (Fig. 1) show the absence of membrane blebs characteristic of other members of the order [15, 16]. TEM images (Fig. S1, available in the online version of this article) show the usual profile of the envelope of a Gram-negative bacterium. Additional results of the phenotypic characterization are displayed in the species description.

Fatty acid methyl esters were extracted from strain ISS155^T and *T. turnerae* CECT 9444^T biomass grown in shipworm basal medium with D-glucose [6] at two different incubation times, 72 h and 5 days (26 °C) and also on marine agar for strain ISS155^T. Extracts were prepared according to standard protocols as described for the MIDI Microbial Identification System [17] at the CECT. Cellular fatty acid content was analysed by gas chromatography with an Agilent 6850 chromatographic unit, with the MIDI Microbial Identification System using the TSBA6 method [18] and identified using the Microbial Identification Sherlock software package. Table S1 shows the cellular fatty acids detected in both strains in different media and incubation times. The cellular fatty acids of strain ISS155^T included summed feature 8 ($C_{18:1}\omega7c/\omega6c$;, 29–40%, depending on the media), summed feature 3 ($C_{16:1}\omega7c/\omega6c$, 23–27%, $C_{16:0}$ (23–26%) and smaller amounts of $C_{10:0}$ 3-OH (5–9%) and $C_{10:0}$ (3–4%). Fatty acids $C_{12:0}$ 3OH and $C_{12:0}$ were found only in *T. turnerae* CECT 9444^T, which suggests that they may be discriminative for these two taxa.

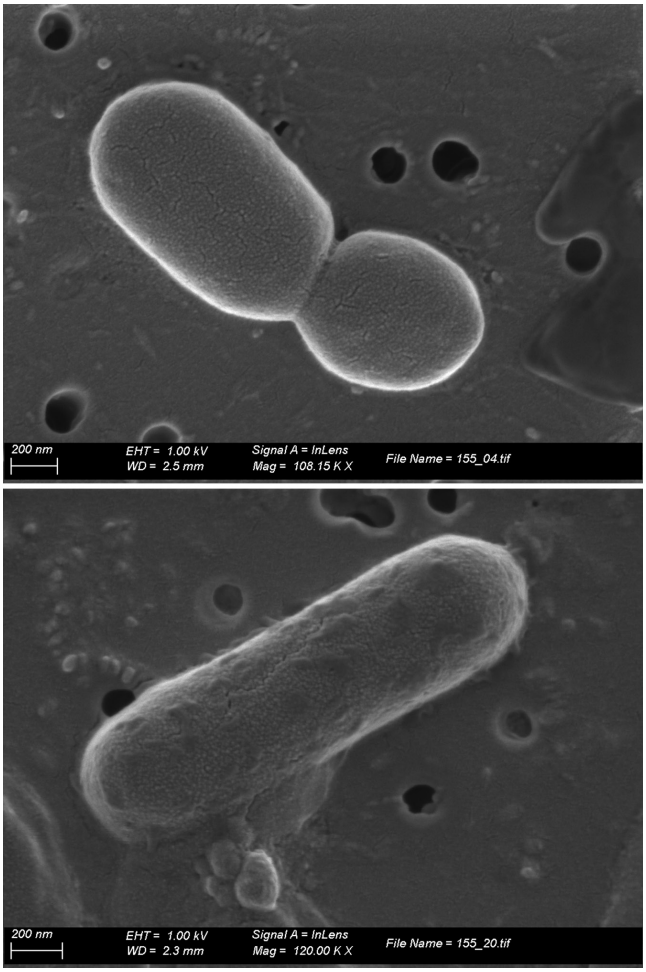


Fig. 1. Scanning electron microscopy images of strain ISS155^T. Samples were gold sputter coated in order to visualize them by SEM. Visualizations were done by the Microscopy Service of the Universitat Autònoma de Barcelona (http://sct.uab.cat/microscopia/en/content/inici).

Analysis of respiratory quinones and major polar lipids were carried out by the Identification Service and Dr. Brian Tindall at the DSMZ (Braunschweig, Germany). Respiratory quinones were extracted from 50 mg freeze-dried cells using hexane and were further purified by a silica-based solid phase extraction. Purified samples were further analysed by HPLC using a reverse-phase column recording absorption spectra; 270 nm for ubiquinones and 326 nm for menaquinones were used for a relative quantification [19, 20]. Major polar lipids were extracted from the same 50 mg freeze-dried cells using a chloroform–methanol–0.3% aqueous NaCl mixture, polar lipids were recovered into the chloroform phase (modified after Bligh and Dyer [21]). Polar lipids were separated by two-dimensional silica gel TLC. The first direction was developed in chloroform–methanol–water, and the second in chloroform–methanol–acetic acid–water. Total lipid material

was detected using molybdatophosphoric acid and specific functional groups detected using spray reagents specific for defined functional groups [19–22]. Further details are available in Fig. S2. Q8 was identified as the major quinone and phosphatidylglycerol and phosphatidylethanolamine were the main identified polar lipids, plus two unidentified lipids, one glycolipid and one phospholipid.

A draft genome of strain ISS155^T was obtained through whole genome sequencing at Centre Nacional d'Anàlisi Genòmica (CNAG; www.cnag.crg.eu/). DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen), following the manufacturer's recommendations. The KAPA Hyper Prep Kit for PCR-free workflows (Roche Kapa Biociences) was used for DNA library preparation with some minor modifications. In brief, 0.5–1.0µg genomic DNA was sheared on a Covaris LE220 Plus in order to reach the fragment sizes of~250–700bp. The sheared DNA was end-repaired, adenylated and ligated to IDT adaptors with unique dual-matched indexes (Integrated DNA Technologies). The adaptor-modified end library was size-selected and purified with AMPure XP beads (Agencourt, Beckman Coulter). Final library size was confirmed on an Agilent 2100 Bioanalyzer with the DNA 7500 assay. The PCR-free library was quantified by Library Quantification Kit for Illumina Platforms (Roche Kapa Biosystems). The libraries were sequenced on HiSeq 2500 apparatus (HiSeq Rapid SBS Kit V2, Illumina) in paired-end mode 2×251+8+8bp. Primary data analysis, image analysis, base-calling and quality-scoring of the run were processed using the manufacturer's software Real Time Analysis (version 1.18.66.3), followed by the generation of FASTQ sequence files.

The reads were analysed for quality control using FASTQC, a common quality control tool developed by Babraham Bioinformatics to check raw sequencing data. After filtering, the remaining reads were assembled using SPAdes 3.9.0 software [23]. A plot (coverage versus length of the contigs) was performed to help in the choice of the parameters for contig filtering. After the filtration of contigs (500 bp long and 10–50×kmer coverage), evaluation of the final assembly against a reference genome was done with the software QUAST version 4.3 [24]. The bioinformatic tool CheckM version 1.0.7 [25] was used to assess the genome quality prior to annotation using Prokka version 1.12 [26] and RAST version 2.0 [27, 28]. The processes of quality assessment of reads, read-processing, assembly and annotation with Prokka was carried out in Linux OS; other tools were accessed online. The minimal standards for the quality of genome sequences and how they can be applied for taxonomic purposes have been observed in this study [29].

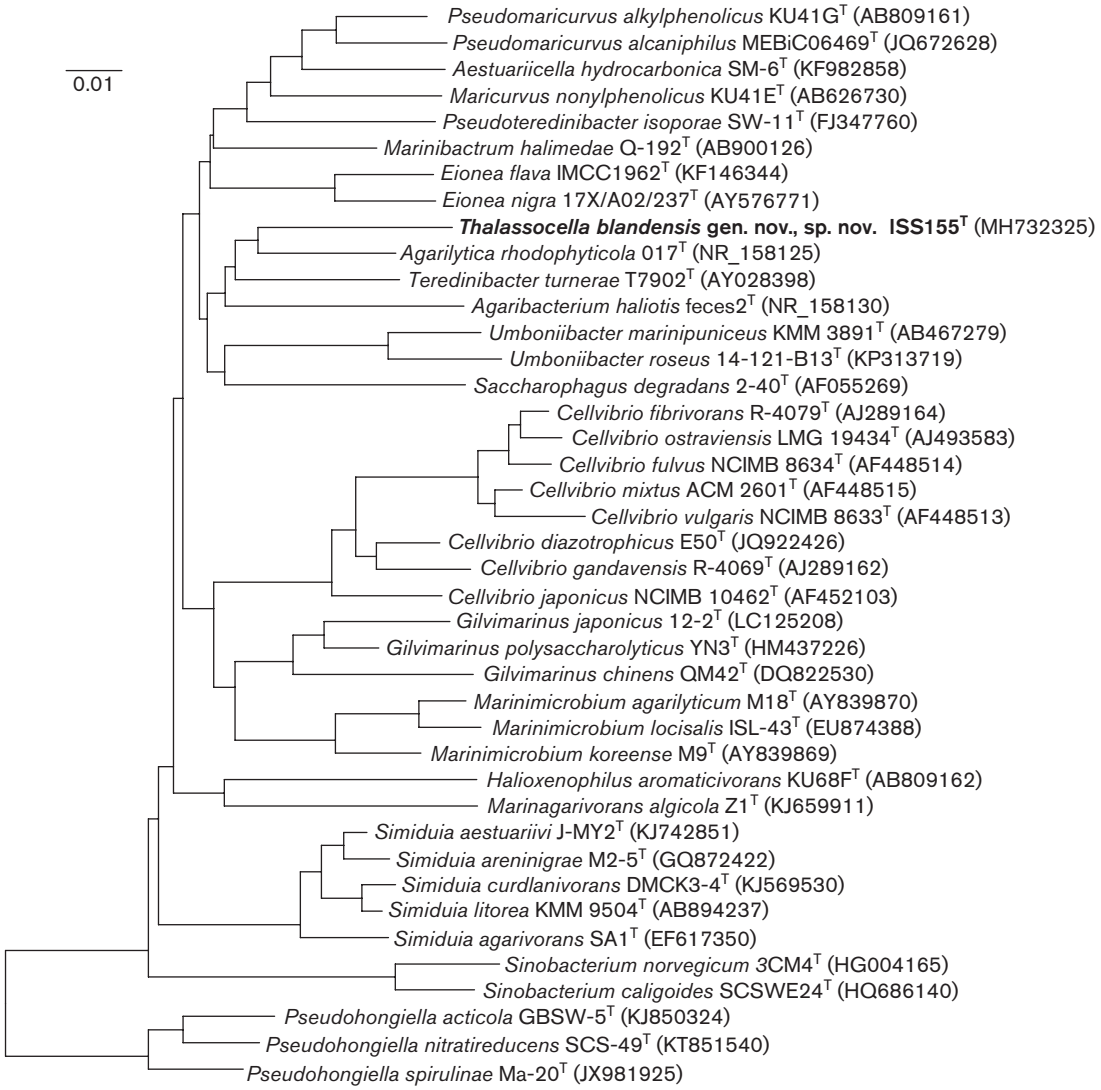
The draft genome of strain ISS155^T had an estimated size of 6085.336 bp, larger than the 5387.817 bp of *T. turnera*e T7902^T, AQ2 but smaller than that of *A. rhodophyticola* 017^T, the largest genome so far known in the family (Table 1). It is composed of 49 contigs with a *N50* value of 590828 nucleotides and final assembly coverage of 200×. CheckM results of contamination and completeness were 1.34 and 99.27%, respectively. It contained 5425 protein-coding sequences and 43 RNA genes (including a single copy of the 16S rRNA gene). The G+C molar content was 45.2 mol%.

The similarity between genomes was assessed using several indices useful for species and genus delineation. Average amino acid identity (AAI) values were calculated with the online server ANI/AAI-Matrix [30]. DNA–DNA hybridization (DDH) values were estimated *in silico* with the Genome-to-Genome Distance Calculator (GGDC 2.1), using the BLAST method and recommended formula 2 [31]; average nucleotide identity (ANI) values according to MUMmer (ANIm) and BLAST (ANIb) were determined in JSpeciesWS [32].

AAI values among all *Cellvibrionaceae* members whose type strain has an available genome are shown in Table 2. Figure S3 shows a distance matrix tree applied to these AAI values using Kostas lab tools [30]. ANIb, ANIm and *in silico* DDH values are shown in Table S2. Consistent with the low AAI values, the genome of strain ISS155^T showed values low enough for the three indexes when compared to other members of the family.

The almost-complete 16S rRNA gene sequence (1399 nt) of strain ISS155^T obtained by PCR amplification was compared by BLAST against the annotated 16S rRNA gene sequence in its genome (1542 nt) resulting in 100% similarity. Similarities to type strains genes of other validly named species were determined by using the appropriate tool in the EzBioCloud. The highest similarity in 16S rRNA gene sequence was found to the type strains of *A. rhodophyticola* (94.3%), *T. turnerae* (93.1%), *Marinibactrum halimedae* (93.1%) and *S. degradans* (92.9%). The sequence was compared with corresponding sequences of the type strains within the *Cellvibrionaceae* using alignments retrieved from SILVA and LTP [33] latest updates as references. When necessary, additional sequences were retrieved from the GenBank/EMBL/DDBJ databases. Alignments were corrected manually based on secondary structure information. Sequence similarities were calculated in ARB based on sequence similarities without the use of an evolutionary substitution model. Phylogenetic analysis using the neighbour-joining method (NJ) was performed using ARB tools [34]. Fig. 2 shows the tree obtained with NJ algorithm, where strain ISS155^T relates to *A. rhodophyticola* and *T. turnerae.*

Phylogenetic relationships of the genomes were also explored with UBCG [35] using default settings. This software tool is available for download at EzBioCloud [36] and employs a set of 92 single-copy core genes commonly present in all bacterial genomes. The estimation of robustness of the nodes is done through the gene support index (GSI), defined as the number of individual gene trees, out of the total genes used, that present the same node. Fig. 3 shows the nucleotide-based UBCG tree, the amino acid-based tree is displayed in Fig. S4. Both phylogenomic trees confirm *A. rhodophyticola* as the nearest phylogenetic neighbour of strain ISS155^T, with a high GSI (59-56). *T. turnerae* and *S. degradans* are close relatives, followed by *Agaribacterium haliotis.* Distances among them, however, are larger than the ones found for same species pairs, *Cellvibrio*, *Gilvimarinus* and *Marinimicrobium* branches.



AQ3 **Fig. 2.** Phylogenetic reconstruction based on the 16S rRNA gene using the neighbour-joining method. Sequence accession numbers are given in parentheses. Bar, number of substitutions per position.

The genome of strain ISS155^T contained genes for β-glucosidase (EC 3.2.1.21; five copies), maltodextrin glucosidase (EC 3.2.1.20), glucan 1–4 α-glucosidase (EC 3.2.1.3), endo 1–4 β-glucanase (EC 3.2.1.4) celA precursor, chitinase (EC 3.2.1.14; 22 copies), endo 1–4 β-xylanase (EC 3.2.1.8), α-L-arabinofuranosidase (EC 3.2.1.55; four copies), glucoamylase (EC 3.2.1.3), pullulanase (EC 3.2.1.41) and other CAZymes, indicating a broad potential for polysaccharide degradation activities. It also contained 31 copies of gene *tonR*, a biopolymer transport-related system, which is also overrepresented in the genomes of *A. rhodophyticola* (45 copies), *S. degradans* (40 copies), *T. turnerae* (33 copies) and, in lower numbers, *Marinagarivorans* (16 copies) and *Agaribacterium* (14 copies). This potential resembles to the one exhibited by other members of *Cellvibrionaceae*. Strain ISS155^T genome lacks the nitrogenase system-related genes that are found in the genomes of *T. turnerae* T7902^T and *A. haliotis* feces2^T.

Other traits predicted from the genome of strain ISS155^T are the presence of secretion systems type IV and VI, genes related to PHB metabolism, a type I restriction-modification system and the absence of CRISPR-Cas systems.

The differences found between strains ISS155^T and *A. rhodophyticola* 017^T genomes, add up to 301 unique genes (147 present in ISS 155^T but absent in *A. rhodophyticola* type strain and 154 present in the later and absent from ISS 155^T genome).

The low 16S rRNA gene sequence similarities displayed by strain ISS155^T indicates that it represents a new taxon allocated phylogenetically within the family *Cellvibrionaceae*.

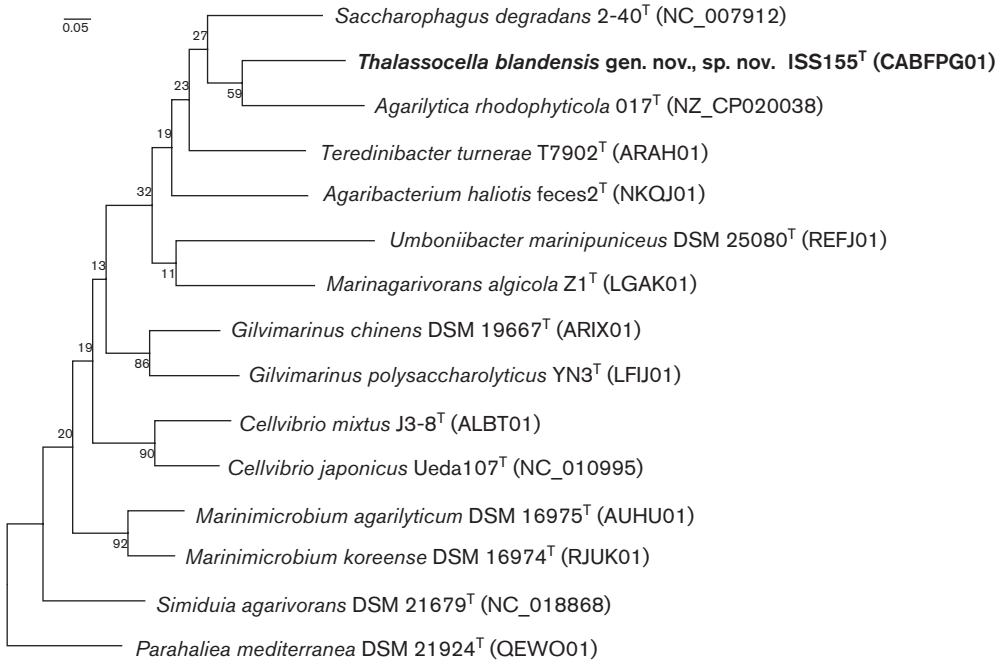


Fig. 3. Phylogenetic tree generated with UBCG [35] by using the nucleotides sequences. The numbers at the nodes indicate the gene support index (maximal value is 92). Genome accession numbers are indicated in parentheses. Bar, 0.05 substitutions per position.

Although similarities lower than 95% for this gene may be indicative of a different generic rank, we also explored genomic and phylogenomic distances among strain ISS155^T and all available type strain genomes in the family in order to substantiate the proposal of a new genus status for the taxon. UBCG trees and, particularly, AAI indexes (Table 2) confirmed what the 16S rRNA had already suggested, strain ISS155^T displays AAI figures lower than 60% with its relatives; *A. rhodophyticola* genome shows the highest value, a 58%, closely followed by those of *T. turnerae* and *S.*

Table 2. Average amino acid identity indexes among genomes of type strains of species in *Cellvibrionaceae*

Bold text, values relating *Thalassocella blandensis* with all other species. Coloured boxes, intrageneric values.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Agaribacterium haliotis* feces2^T													
2	*Agarilytica rhodophyticola* 017^T	54												
3	*Thalassocella blandensis* ISS155^T	**55**	**58**											
4	*Saccharophagus degradans* 2-40^T	55	56	**57**										
5	*Teredinibacter turnerae* T7902^T	55	55	**57**	58									
6	*Marinagarivorans algicola* Z1^T	55	53	**53**	55	54								
7	*Cellvibrio japonicus* Ueda107^T	52	51	**53**	54	54	52							
8	*Cellvibrio mixtus* J3-8^T	51	50	**52**	53	52	51	71						
9	*Gilvimarinus chinensis* DSM 19667^T	53	52	**53**	55	54	53	58	58					
10	*Gilvimarinus polysaccharolyticus* YN3^T	52	51	**53**	54	54	53	59	58	69				
11	*Marinimicrobium agarilyticum* DSM 16975^T	52	51	**53**	54	54	52	61	59	62	62			
12	*Marinimicrobium koreense* DSM 16974^T	52	52	**53**	54	54	53	61	60	62	62	79		
13	*Simiduia agarivorans* DSM 21679^T	52	51	**52**	53	53	52	54	53	54	55	55	56	
14	*Umboniibacter marinipuniceus* DSM 25080^T	49	48	**49**	49	49	49	49	48	50	50	50	50	50

Table 3. Differential characteristics between *Thalassocella blandensis* ISS155^T and its closest phylogenomic relatives

Strains: 1, *Thalassocella blandensis* ISS155^T (data from this study); 2, *Agarilytica rhodophyticola* 017^T [3]; 3, *Teredinibacter turnerae* T7902^T [6]; 4, *Saccharophagus degradans* 2-40^T [14]; 5, *Agaribacterium haliotis* feces2^T [4]. +, Positive; –, negative; ND, not determined. All type strains are Gram-negative rods and positive for the following traits: motility, polar flagellum, salt requirement, mesophilic, neutrophilic, aerobic chemoheterotrophy, production of Q-8 as major respiratory quinone, presence of phosphatidylglicerol and phosphatidylethanolamine among major polar lipids and summed feature 3 and summed feature 8 among major cellular fatty acids. Characteristics highlighted in bold allow differentiation among the five genera.

Characteristics	**1**	**2**	**3**	**4**	**5**
G+C content (mol%)	45.2	40.9	50.8	45.8	50.5
Major polar lipids	PG, PE, L, AL	PG, PE, AL, Ls	PG, PE, PS, ALs, PLs*	PG, PE, PS, DPG, ALs, PLs*	PG, PE, DPG, ALs, Ls
Presence of $C_{12:0}$ (in fatty acid profile)	–	+	+	–[a]	+
Oxidase	+	–	+	+	+
Catalase	+	+	+	+	–
Hydrolysis of:					
Starch	+	+	–	+	–
Cellulose	+	–	+	+	–
Agar	–	+	–	+	+
Alginate	–	–	–	+	ND
Gelatin	+	+	–	–	–
Tween 80	+	–	+*	–*	ND
Nitrogenase[b]	–	–	+	–	+
Pigmentation	None	Yellow	Yellowish (aged)	None	None
NO_3^- reduction to NO_2^-	–	+	ND	+	+
Growth at 40 °C	–	+	–	–	+

*From [39]. *a*, from [40]. *b,* from genomes: *NifK*, *NifD* and *NifH* genes present in *T. turnerae* and *A. haliotis*; nitrogenase activity is experimentally confirmed in *T. turnerae* but has not been experimentally determined in *A. haliotis*.
†PG, phosphatidylglicerol; PE, phosphatidylethanolamine; DPG, diphosphatidylglicerol; PS, phosphatidylserine; L, lipid; PL, phospholipid; AL, unidentified aminolipids.

degradans. These three species are also the closest ones in 16S rRNA gene sequence similarities and in UBCG-based trees. The AAI index has been proposed, in different versions, as a useful parameter for genus delimitation in Bacteria (Proteobacteria) [37, 38]. Despite variations on the exact cut-off value, figures lower than 60% of AAI are considered indicative of strains pertaining to different genera. As it can be observed in the table, AAI values that correspond to species of the same genus are in the range 69–79% (*Cellvibrio*, *Gilvimarinus* and *Marinibacterium* species in Table 2). Thus, all genomic and phylogenomic evidence points to a separate genus status for strain ISS155^T. From a phenotypic point of view, we looked for differences sustaining the proposal of a new genus by carefully comparing its phenotypic (including chemotaxonomic) profile and the descriptions of the closest genera, namely *Agarilytica*, *Teredinibacter*, *Saccharophagus* and *Agaribacterium*. Table 3 shows a selection of the more noticeable differences among these taxa, highlighting the ones that allow differentiation to the genus level. There are important characters differing between strain ISS155^T and its nearest neighbour *Agarilytica*: oxidase reaction, G+C molar content, ability to reduce nitrate to nitrite, pigmentation, the set of polysaccharide degradative capabilities and occurrence of some of the minor cellular fatty acids (see Table 3). In addition, acid production from 5-ketoglutarate, trehalose, cellobiose and starch are negative for *A. rhodophyticola* 017^T and positive for strain ISS155^T. Altogether, these differences allow a clear distinction of both genera. Several other traits allow differentiation from the facultative endosymbiont *Teredinibacter*, from *Saccharophagus* and *Agaribacterium*, as shown in Tables 3 (and S1, for *Teredinibacter*). Thus, we propose to recognize strain ISS155^T as representing a new genus and species in the family *Cellvibrionaceae*, with the name *Thalassocella blandensis* gen. nov., sp. nov. and strain ISS155^T (=CECT 9533^T=LMG 31237^T) as the type strain of the new species.

DESCRIPTION OF *THALASSOCELLA* GEN. NOV.

Thalassocella (Tha.las.so.cel'la. Gr. fem. n. *thalassa*, the sea; L. fem. n. *cella*, chamber and, in biology, a cell. N.L. fem. n. *Thalassocella*, a cell of the sea).

Cells are Gram-negative rods, chemoorganotrophic and aerobic. Oxidase and catalase are positive. Strictly halophilic and mesophilic. Major polar lipids include phosphatidylglycerol and phosphatidylethanolamine. Major cellular fatty acids are $C_{18:1}$ *ω7c*/*ω6c*, $C_{16:0}$ and $C_{16:1}$ω7*c*/ω6*c*. The DNA G+C content is 45.2 mol%. Affiliated to the family *Cellvibrionaceae*, in the *Gammaproteobacteria*. The type species is *Thalassocella blandensis*.

DESCRIPTION OF *THALASSOCELLA BLANDENSIS* SP. NOV.

Thalassocella blandensis (blan.den'sis. N.L. fem. adj. *blandensis* pertaining to *Blande* or *Blanda*, the name the Romans used for the city of Blanes, which has given its name to the Bay of Blanes, where the type strain was isolated).

Cells are Gram-reaction-negative, rod-shaped, 0.5–0.6×1.0–1.3 µm and motile by one polar flagellum. Aerobic chemoorganotroph, unable to ferment carbohydrates, and positive for catalase and oxidase. Growth in liquid medium produces surface mucoid masses that sink with shaking. Colonies in marine agar medium are small (2–3 mm diameter), regular and transparent and non-pigmented. Mesophilic, neutrophilic and slightly halophilic, with optimal growth at 28–37 °C (range, 15–37 °C; no growth, 4 and 40 °C), pH 7–8 (range, pH 6–9; no growth, pH 5; weak growth, pH 10) and 2.5% total salinity (range, 1.5–9.0%). Requires both sodium and magnesium ions for growth. Nitrate is not reduced to nitrite or N_2. Hydrolyses aesculin, casein, gelatin, starch, Tween 80 and DNA, but not alginate or agar. Grows with cellulose as sole carbon source in *Teredinibacter* medium after 7 days' incubation. Negative for arginine dihydrolase, indole production from tryptophan, urease, and β-galactosidase. Sole carbon and energy sources sustaining growth on basal medium agar with D-glucose, *N*-acetyl-D-glucosamine, amygdalin, D-glucuronate, D-glycerol, acetate, pyruvate, fumarate, 3-hydroxybutyrate, L-leucine, L-serine, L-glutamate, L-alanine, L-arginine, L-citruline and L-aspartate. No growth is obtained with the following substrates: D-ribose, L-arabinose, D-xylose, D-fructose, D-galactose, trehalose, D-mannose, L-rhamnose, maltose, cellobiose, sucrose, lactose, melibiose, salicin, D-gluconate, D-galacturonate, D-mannitol, D-sorbitol, *m*-inositol, D-saccharate, propionate, citrate, *t*-aconitate, 2-oxoglutarate, malate, lactate, glycine, L-treonine, L-tyrosine, L-ornithine, 4-aminobutyrate, L-lysine, L-histidine and L-sarcosine. The following carbohydrates are metabolized with acid production in aerobic API 50CH/E tubes: glucose, cellobiose, maltose, trehalose, aesculin, starch, glycogen and gentibiose.

Major polar lipids are phosphatidylglycerol, phosphatidylethanolamine, one unidentified glycolipid, an unidentified phospholipid and two unidentified lipids. Major respiratory quinone is Q8. The main cellular fatty acids include $C_{18:1}$ ω7*c*/ω6*c*, $C_{16:0}$, $C_{16:1}$ ω7*c*/ω6c, $C_{10:0}$ 3-OH and $C_{10:0}$.

Type strain G+C molar content is 45.2 mol% and its genome size is 6.09 Mbp.

Type strain ISS155^T (=CECT 9533^T=LMG 31237^T) was isolated from surface seawater of the western Mediterranean Sea.

Funding information
Sequencing was funded by grant CTM2016-80095-C2-1-R to C. P. A. from the Spanish Ministerio de Economía y Competitividad. Additional funding was obtained by grant CTM2017-87736-R to S. G. A. from the Spanish Ministerio de Economía y Competitividad.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Spring S, Scheuner C, Göker M, Klenk H-P. A taxonomic framework for emerging groups of ecologically important marine *Gammaproteobacteria* based on the reconstruction of evolutionary relationships using genome-scale data. *Front Microbiol* 2015;6:281.
2. Parte AC. LPSN - List of prokaryotic names with standing in Nomenclature (bacterio.net), 20 years on. *Int J Syst Evol Microbiol* 2018;68:1825–1829.
3. Ling S-K, Xia J, Liu Y, Chen G-J, Du Z-J. *Agarilytica rhodophyticola* gen. nov., sp. nov., isolated from *Gracilaria blodgettii*. *Int J Syst Evol Microbiol* 2017;67:3778–3783.
4. Huang Z, Lai Q, Zhang D, Shao Z. *Agaribacterium haliotis* gen. nov., sp. nov., isolated from abalone faeces. *Int J Syst Evol Microbiol* 2017;67:3819–3823.
5. Iwaki H, Yamamoto T, Hasegawa Y. Isolation of marine xylene-utilizing bacteria and characterization of *Halioxenophilus aromaticivorans* gen. nov., sp. nov. and its xylene degradation gene cluster. *FEMS Microbiol Lett* 2018;365:fny042.
6. Distel DL, Morrill W, MacLaren-Toussaint N, Franks D, Waterbury J. *Teredinibacter turnerae* gen. nov., sp. nov., a dinitrogen-fixing, cellulolytic, endosymbiotic gamma-proteobacterium isolated from the gills of wood-boring molluscs (Bivalvia: Teredinidae). *Int J Syst Evol Microbiol* 2002;52:2261–2269.
7. Brito TL, Campos AB, Bastiaan von Meijenfeldt FA, Daniel JP, Ribeiro GB *et al*. The gill-associated microbiome is the main source of wood plant polysaccharide hydrolases and secondary metabolite gene clusters in the mangrove shipworm *Neoteredo reynei*. *PLoS One* 2018;13:e0200437.
8. Tanaka T, Kawasaki K, Daimon S, Kitagawa W, Yamamoto K *et al*. A hidden pitfall in the preparation of agar media undermines microorganism cultivability. *Appl Environ Microbiol* 2014;80:7659–7666.
9. Page KA, Connon SA, Giovannoni SJ. Representative freshwater bacterioplankton isolated from crater lake, Oregon. *Appl Environ Microbiol* 2004;70:6542–6550.
10. Pujalte MJ, Lucena T, Rodrigo-Torres L, Arahal DR. Comparative genomics of *Thalassobius* including the description of *Thalassobius activus* sp. nov., and *Thalassobius autumnalis* sp. nov. *Front Microbiol* 2017;8:2645.
11. Arahal DR, Lucena T, Rodrigo-Torres L, Pujalte MJ. *Ruegeria denitrificans* sp. nov., a marine bacterium in the family *Rhodobacteraceae* with the potential ability for cyanophycin synthesis. *Int J Syst Evol Microbiol* 2018;68:2515–2522.
12. Heimbrook ME, Wang WL, Campbell G. Staining bacterial flagella easily. *J Clin Microbiol* 1989;27:2612–2615.

13. Baumann P, Baumann L. The marine gram-negative eubacteria: genera *Photobacterium, Beneckea, Alteromonas, Pseudomonas* and *Alcaligenes*. In: Starr MP, Stolp H, Trueper HG, Balows A (editors). *The Prokaryotes*, 2. Springer; 1981. pp. 1302–1331.
14. Lee C, Kim JY, Lee WI, Nelson KL, Yoon J *et al*. Bactericidal effect of zero-valent iron nanoparticles on *Escherichia coli*. *Environ Sci Technol* 2008;42:4927–4933.
15. González JM, Mayer F, Moran MA, Hodson RE, Whitman WB. *Microbulbifer hydrolyticus* gen. nov., sp. nov., and *Marinobacterium georgiense* gen. nov., sp. nov., two marine bacteria from a lignin-rich pulp mill waste enrichment community. *Int J Syst Bacteriol* 1997;47:369–376.
16. Ekborg NA, Gonzalez JM, Howard MB, Taylor LE, Hutcheson SW *et al*. *Saccharophagus degradans* gen. nov., sp. nov., a versatile marine degrader of complex polysaccharides. *Int J Syst Evol Microbiol* 2005;55:1545–1549.
17. Sasser M. *Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids*, MIDI Technical Note 101. Newark: DE: MIDI Inc; 1990.
18. MIDI. *Sherlock Microbial Identification System Operating Manual, version 6.1*. Newark, DE: MIDI Inc; 2008.
19. Tindall BJ. A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* 1990;13:128–130.
20. Tindall BJ. Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* 1990;66:199–202.
21. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;37:911–917.
22. Tindall BJ, Sikorski J, Smibert RM, Kreig NR. Phenotypic characterization and the principles of comparative systematics. *In: Methods for General and Molecular Microbiology*, 3rd ed; 2007. pp. 330–393.
23. Nurk S, Bankevich A, Antipov D, Gurevich AA, Korobeynikov A. Assembling genomes and mini-metagenomes from highly chimeric reads. In: Deng M, Jiang R, Sun F, Zhang X (editors). *Research in Computational Molecular Biology. RECOMB 2013. Lecture Notes in Computer Science*, 7821. Berlin, Heidelberg: Springer; 2013.
24. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 2013;29:1072–1075.
25. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 2015;25:1043–1055.
26. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068–2069.
27. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T *et al*. The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 2008;9:75.
28. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ *et al*. The seed and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Res* 2014;42:D206–D214.
29. Chun J, Oren A, Ventosa A, Christensen H, Arahal DR *et al*. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 2018;68:461–466.
30. Rodriguez-R LM, Konstantinidis KT. The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. *PeerJ Preprints*;2016:e1900v1.
31. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 2013;14:60.
32. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* 2016;32:929–931.
33. Yarza P, Ludwig W, Euzéby J, Amann R, Schleifer K-H *et al*. Update of the All-Species living tree project based on 16S and 23S rRNA sequence analyses. *Syst Appl Microbiol* 2010;33:291–299.
34. Ludwig W, Strunk O, Westram R, Richter L, Meier H *et al*. ARB: a software environment for sequence data. *Nucleic Acids Res* 2004;32:1363–1371.
35. Na S-I, Kim YO, Yoon S-H, Ha S-M, Baek I *et al*. UBCG: up-to-date bacterial core gene set and pipeline for phylogenomic tree reconstruction. *J Microbiol* 2018;56:281–285.
36. Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y *et al*. Introducing EzBioCloud: a taxonomically United database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol* 2017;67:1613–1617.
37. Luo C, Rodriguez-R LM, Konstantinidis KT. MyTaxa: an advanced taxonomic classifier for genomic and metagenomic sequences. *Nucleic Acids Res* 2014;42:e73.
38. Wirth JS, Whitman WB. Phylogenomic analyses of a clade within the roseobacter group suggest taxonomic reassignments of species of the genera *Aestuariivita, Citreicella, Loktanella, Nautella, Pelagibaca, Ruegeria, Thalassobius, Thiobacimonas* and *Tropicibacter*, and the proposal of six novel genera. *Int J Syst Evol Microbiol* 2018;68:2393–2411.
39. Chen M-H, Sheu S-Y, Arun AB, Young C-C, Chen CA *et al*. *Pseudoteredinibacter isoporae* gen. nov., sp. nov., a marine bacterium isolated from the reef-building coral *Isopora palifera*. *Int J Syst Evol Microbiol* 2011;61:1887–1893.
40. González JM, Weiner RM. Phylogenetic characterization of marine bacterium strain 2-40, a degrader of complex polysaccharides. *Int J Syst Evol Microbiol* 2000;50 Pt 2:831–834.