Vibrio bivalvicida sp. nov., a novel larval pathogen for bivalve molluscs reared in hatchery.

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ABSTRACT

Three isolates were obtained in cultures of carpet shell clam (*Ruditapes decussatus*), reared in a bivalve hatchery (Galicia, NW Spain), from different sources: healthy broodstock, moribund larvae and the seawater corresponding to that larval tank. All isolates were studied by a polyphasic approach, including a phylogenetic analysis based on concatenated sequences of the five housekeeping genes *fts*Z, *gyr*B, *pyr*H, *rec*A and *rpo*A, that supported their inclusion into the Orientalis clade of the genus *Vibrio*, forming a tight group separated from the closest relatives: *V. tubiashii* subsp. *europaensis*, *V. tubiashii* subsp. *tubiashii* and *V. orientalis*. Percentages of genomic resemblance, including average nucleotide identity, DNA–DNA hybridization and *in silico* genome–to–genome comparison, between the type strain and the closest relatives were below values for species delineation and confirmed the taxonomic position of the new species, which could be differentiated from the related taxa on the basis of several phenotypic and chemotaxonomic features including FAME and MALDI–TOF–MS. The pathogenicity of the new species was demonstrated in larvae of *Ruditapes decussatus*, *R. philippinarum*, *Ostrea edulis* and *Donax trunculus*. Results demonstrated that strains analysed represent a novel species into the Orientalis clade of the genus *Vibrio*, for which the name *Vibrio bivalvicida* sp. nov. is proposed. with 605^{T} (= CECT 8855^{T}= CAIM 1904^{T}) as the type strain.

Keywords: *Vibrio bivalvicida* sp. *nov*.; Orientalis clade; Bivalve hatchery; Larval pathogen; ANI, DDH.

The genus *Vibrio* is the largest member of the family *Vibrionaceae* and comprises more than 123 bacterial species (http://www.bacterio.net/vibrio.html) clustered in different clades [21]. Vibriosis caused by some *Vibrio* species represents the main bottleneck of the production process on bivalve hatcheries leading to high larval mortality rates and the rapid loss of production batches [15, 17]. Orientalis clade has a relevant significance for bivalve aquaculture since it includes some of the most well–known larval pathogens, such as *V. tubiashii* subsp. *tubiashii* or the recently described *V. tubiashii* subsp. *europaensis*, whose broad action range was reported in larvae of different species and even in ten to twelve–month–old spat *Crassostrea gigas* [15, 29]. The remainder species of the Orientalis clade, including *V. brasiliensis, V. caribbeanicus, V. hepatarius V. orientalis, V. sinaloensis* and *V. crosai*, were also isolated from aquatic environments [5, 6, 8, 26, 27, 31].

In this study, we report a detailed taxonomic characterization of three isolates obtained from cultures of carpet shell clam (*Ruditapes decussatus*) reared in a bivalve hatchery (Galicia, NW Spain). Results have demonstrated that they constitute a new species within the genus *Vibrio*, namely into the Orientalis clade, with special significance for shellfish aquaculture because its pathogenicity was demonstrated in larvae of several bivalve species.

Microbiological samples were obtained from cultures of carpet shell clam carried out in a bivalve hatchery located in Galicia (NW Spain) and they were processed following Prado et al. [16]. Strain 194 (= CECT 8856= CAIM 1905) was isolated from healthy broodstock. Isolates 605^T (= CECT 8855^T= CAIM 1904^T) and 603 were obtained from a culture tank affected by an episode of mortality larval, namely 605^T was isolated from *R. decussatus* larvae and 603 from seawater used in the same culture tank. All isolates were cultured in Marine Agar (MA; Difco) for 24 h at 25ºC and they were analysed for their phenotypic properties. Phenotypic profiles were obtained using a set of tests [15, 16, 17] that comprised classical procedures, including the utilization of 46 sole carbon sources, as well as the miniaturized systems API ZYM, API 20E and API 50CH (bioMérieux) to determine the enzymatic activities, additional phenotypic features and the fermentation of carbon sources respectively. Antibiograms were carried out by disc diffusion method on Müeller–Hinton Agar (Oxoid) supplemented with 1% NaCl (MHA–1) using antibiotic impregnated discs (Oxoid) with: florfenicol (FFC, 30 µg), flumequine (UB30, 30 µg), sulphamethoxazole/trimethoprim (SXT25, 25 µg), ampicillin (AMP10, 10 µg), amoxicillin (AML25, 25 µg), chloramphenicol (C30, 30 µg), tetracycline (TE30, 30 µg), erythromycin (E15, 15 µg), enrofloxacine (ENR5, 5µg), cephalotin (KF30, 30 µg) and nitrofurantoin (F300, 300 µg). After incubation (24 h and 25ºC) the zones of inhibition around the disc were measured and compared against recognised zone size ranges established by the manufacturer for specific antimicrobial agents.

The three isolates shared the main properties of the genus *Vibrio* [12]. They were motile rods, facultative anaerobic, Gram–negative and oxidase positive, susceptible to the vibriostatic agent O/129, capable of reducing nitrates to nitrites, and to grow on TCBS forming yellow colonies. These isolates showed high phenotypical homogeneity, although variable reactions (Table 1) were observed for hydrolysis of esculine, activity N–acetyl–β–glucosaminidase, utilization of salicine, and fermentation of gentiobiose and arbutine (positive for isolates 605^T and 603). They were susceptible to FFC30, UB30, SXT25, TE30, F300 and C30 whereas they showed resistance to E15, KF30, AMP10, AML25 and ENR5.

Repetitive extragenic palindromic (REP–PCR) and enterobacterial repetitive intergenic consensus (ERIC–PCR) techniques were used to discriminate bacterial strains. For genotyping, the three isolates and the related species were amplified by ERIC–PCR and REP–PCR, as previously described Rodríguez et al. [19]. On the basis of the results obtained, including their DNA fingerprints (Fig. S1) and phenotypic profiles, isolates 605^T and 603 were considered as clones of the same strain, which have sense since they were isolated in the same sampling day but from differences sources in the same tank (larvae and seawater). Therefore, only the isolates 605^T and 194 were selected as the representative strains in the description of the new taxon.

Genomic DNA extraction, PCR amplification and sequencing of the 16S rRNA gene, amplification of the housekeeping genes *rec*A (RNA recombinase alpha subunit), *rpo*A (RNA polymerase alpha subunit), *pyr*H (uridine monophosphate kinase), *gyr*B (gyrase beta subunit) and *fts*Z (cell–division protein) used for the multilocus sequence analysis (MLSA) were performed following Prado et al. [15]. Sequences were analysed with the Lasergene Seqman (DNAstar) and identified using the EzTaxon–e server [9] and FASTA [14]. Phylogenetic analysis based on the individual and concatenated sequences were performed using MEGA 5.2 software [23] and the distance matrices were calculated using Lasergene MegAlign (DNAstar) after multiple alignment of data by ClustalW [28]. Distances and clustering with the Neighbour Joining (NJ) and Maximum Likelihood (ML) algorithms were determined using bootstrap values based on 1000 replications. The sequences of the closest *Vibrio* species were obtained from GenBank. Sequences were deposited in the DDBJ/EMBL/GenBank database and the accession numbers are listed in Table S1.

Phylogenetic analysis based on the 16S rRNA gene sequences (>1459 bp) using the NJ and ML algorithms (Fig. S2) indicated that strains 605^T and 194 constituted an independent and tight group within the genus *Vibrio*. On the basis of these 16S rDNA sequences, both strains showed 100% similarity among them and the closest species were *V. tubiashii* subsp. *europaensis* PP–638^T (98.80%), *V. crosai* CAIM 1437^T (98.79–98.77%), *V. inhibens* BFLP–10^T (98.22–98.20%), *V. tubiashii* subsp. *tubiashii* ATCC 19109^T (98.22–98.19%), *V.* sagamiensis LC2–047^T

(98.15%), *V. owensii* DY05^T (98.10%) and *V. orientalis* CIP102891^T (98.05–98.01%). Most of the percentages were below than the threshold value (98.70%) proposed by Stackebrandt and Ebers [22].

Partial sequences of the housekeeping genes *fts*Z (566–599 bp), *gyr*B (1059–1077 bp), *pyr*H (497–537 bp), *rec*A (791–823 bp) and *rpo*A (874–879 bp) demonstrated the strength of the new cluster in the phylogenetic tree of each individual gene (Fig. S3). In all cases the bivalve pathogens *V. tubiashii* subsp. *europaensis* PP–638^T and *V. tubiashii* subsp. *tubiashii* ATCC 19109^T were the closest relatives according to the phylogenetic analysis.

Phylogenetic trees based on concatenated sequences (3077 bp) of the five housekeeping genes (*rec*A, *rpo*A, pyrH, *gyr*B and *fts*Z) were constructed using the NJ and ML methods (Fig. 1). Results supported the tight of the new group and clustered the new species into the Orientalis clade. Moreover, MLSA confirmed the close relationship between the two strains and the type strains of the related subspecies of *V. tubiashii*. Indeed, the highest range of similarities between the strains of the new species and the closest relatives using the alignment corresponding to the concatenated sequences were 94.20% with *V. tubiashii* subsp. *europaensis*, 94.10% for *V. tubiashii* subsp. *tubiashii* and 90.60% with *V. orientalis*. Although some recombination events were detected in different housekeeping genes (data not shown) these percentages were lower than the value proposed by Thompson et al. [25] for species delimitation within the genus *Vibrio* (95%).

The DNA–DNA hybridization (DDH) experiments were carried out by the hydroxyapatite method in microtiter plates following Ziemke et al. [32] with a hybridization temperature of 60ºC. Strain 605^T was hybridized with 194, and the degree of the homology was 87.76%. DDH values between 605^T and the closest relatives *V. tubiashii* subsp. *tubiashii* CECT 4196^T, *V. tubiashii* subsp. *europaensis* PP–638^T and *V. orientalis* CECT 629^T were 60.33%, 59.64% and 45.96% respectively. All values were clearly below 70% DNA–DNA reassociation proposed by Wayne et al. [30] as the limit for species definition.

The genome of the type strain 605^T was sequenced in an Illumina MiSeq sequencer by Sistemas Genómicos (Valencia, Spain) with a 100X coverage. Genome assembly was performed using SPAdes 3.6 [13]. The resulting draft genome sequence was annotated with the Rapid Annotations using Subsystems Technology (RAST Server) [1]. The subsequent assembly produced 139 contigs (N50= 192254 bp, G+C: 42.29%), 4554 coding sequences (cds) from 4947346 bp genome length. The genome sequence data of the type strain 605^T has been deposited at DDBJ/EMBL/GenBank under the accession number LLEI00000000.

DDH was also calculated *in silico* by the Genome–to–Genome Distance Calculator (GGDC 2.0) using the BLAST+ method [11]. Results were based on recommended formula 2 (identities/HSP length), which is independent of genome length and is thus robust against the use of incomplete draft genomes. Comparison with

the draft genome of the type strain 605^T yielded percentages of 29.90±2.45 with *V. tubiashii* subsp. *tubiashii* ATCC 19109^T (CP009354, CP009355) and 21.60±2.34 with *V. orientalis* CIP 102891^T (ACZV00000000). According to Thompson et al. [24], strains from the same species share 70% *in silico* GGDC, confirming the taxonomic position of the new species observed above.

Calculation of ANI according to MUMmer (ANIm) and BLAST (ANIb) was performed in JSpeciesWS [18] with the genome sequences used previously for *in silico* DDH. We have determined that the ANIb and ANIm between the type strain 605^T and *V. tubiashii* ATCC 19109^T was 85.21% and 87.70% respectively, and 77.35% and 84.64% with *V. orientalis* CIP 102891^T. In all cases these values were clearly below the 95% threshold proposed [18, 24].

Several differentiating phenotypic features were found between the novel species and the closest relatives (Table 1). They can be differentiated from *V. tubiashii* subsp. *tubiashii* CECT 4196^T, *V. tubiashii* subsp. *europaensis* PP–638^T and *V. orientalis* CECT 629^T by the negative results obtained for fermentation of D–mannitol and the utilization of gluconic acid and D–mannitol whereas the closest relatives were sensitive to AMP10 and did not ferment the D–raffinose. Moreover, the ONPG test and fermentation of arbutine was negative for *V. tubiashii* subsp. *tubiashii* CECT 4196^T and *V. tubiashii* subsp. *europaensis* PP–638^T.

MALDI–TOF profile of the type strain 605^T was obtained at CECT (Spanish Type Culture Collection) following the method proposed by Maier et al. [10]. The protein analysis of 605^T confirmed the distance from the closest species *V. tubiashii* subsp. *europaensis* PP–638^T (score=2.394), *V. tubiashii* subsp. *tubiashii* CECT 4196^T (score=2.263) and *V. orientalis* CECT 629^T (score=2.249) in the database.

Fatty acid methyl esters (FAME) were identified according to the Microbial Identifications Systems (MIDI), as described by Sasser [20], using 24 hours cultures from Trypticase Soy Agar (Pronadisa, Lab Conda) supplemented with 0.5% (w/v) NaCl incubated at 25ºC. The majority fatty acids were similar in all cases although differences in their percentages were observed (Table 2). Indeed, the type strain 605^T showed higher values than the closest relatives for $C_{16:0,}$ $C_{12:0}$ and Summed Feature 8 ($C_{18:1\omega7c}$ and/or $C_{18:1\omega6c}$) and lower for Summed feature 2 ($C_{14:0}$ 3–OH and/or iso–$C_{16:1}$ I) and Summed feature 3 ($C_{16:1\omega7c}$ and/or $C_{16:1\omega6c}$).

Biofilm formation as measured putative pathogenicity was assessed using the crystal violet (CV) staining method [2]. Strains 605^T and *V. tubiashii* subsp. *europaensis* PP–638^T were grown in Marine Broth (MB, Difco) and transferred (0.1%, v/v) into borosilicate (Pyrex) glass, polypropylene and polystyrene tubes and incubated 24 h at 25°C. The biofilm attached to the test tube wall was stained with CV (0.2% v/v) and the bound dye was eluted with 95% ethanol (v/v) and measured by spectroscopy at 580 nm. An average of the biofilm quantification was obtained from two independent experiments per strain with three replicates each one. Strain 605^T formed a stronger

biofilm and on all surfaces in comparison with *V. tubiashii* subsp. *europaensis* PP–638^T (Fig. 2). Moreover, the biofilm of the strain 605^T attached to the wall of polystyrene and glass tubes was higher than polypropylene. Biofilm formation has been related with the survival, virulence and stress resistance of *Vibrio* spp. [4]. Biofilm formation constitutes an important risk for the hatchery environment since results demonstrated that the new species is an effective colonizer on different surfaces.

The pathogenic activity of the strains 605^T and 194 was evaluated as previously described Prado et al. [15]. Larvae (10 to 15 days old) of flat oyster (*Ostrea edulis*), Manila clam (*R. philippinarum*), wedge–shell clam (*Donax trunculus*) and the original host, carpet shell clam were inoculated individually at a final concentration of 10^6 CFU ml^{-1}. Challenges were performed in triplicate and wells without bacteria were included as negative control in all experiments. Larvae were checked by inverted microscopy (Olympus CK40, Japan) and most of them showed the typical signs of disease after 48 h i.e. an important reduction in the larval motility, erratic swimming or closing of the valves and even a few larvae were dead (data not shown). Bacterial infections led to high mortality rates (>96%) at 72 h (Table S2) in all experimental challenges. In the negative controls, mortalities were lower than 3.3% (Table S2). Strains were re–isolated from inoculated wells after each challenge. Virulence assays demonstrated the pathogenic potential of the new taxon for the larvae regardless of the bivalve species analysed. Similar results were obtained in other larval pathogens belonging to Orientalis clade, as *V. tubiashii* subsp *tubiashii* or *V. tubiashii* subsp. *europaensis* [3, 7, 15, 29], supporting the broad action range to kill the larvae of different bivalve species.

The genotypic analyses showed that these strains form a stable group into the Orientalis clade and they can be differentiated from the related species according to their phenotypic and genotypic traits. Results obtained from the polyphasic analysis, including MLSA, DDH, ANI, chemotaxonomic techniques (MALDI–TOF–MS and FAME) and phenotypic tests have demonstrated that the strains analysed constituted a novel species within the genus *Vibrio*, for which the name *Vibrio bivalvicida* sp. *nov*. is proposed with 605^T (= CECT 8855^T= CAIM 1904^T) as the type strain.

Description of *Vibrio bivalvicida* sp. *nov*.

Vibrio bivalvicida [bi.val.vi.ci'da. N.L. pl. neut. n. *Bivalvia*, scientific name of a class of molluscs; L. suff. *–cida* (from L. v. *caedo* to cut or kill) murderer, killer; N.L. n. *bivalvicida* bivalve–killer].

Cell are Gram–negative rods, motile and facultative anaerobic. Colonies are round with smooth margins, greenish in color and nonswarming on MA plates. Strains grow on TCBS and form yellow colonies after 24 h at 25 ºC.

Growth occurs between 15–35 ºC from 1 to 6% NaCl (optimum conditions 25ºC at 3% NaCl) but not at 0.5% or 8% NaCl. Glucose metabolism is fermentative without gas production. Strains reduce nitrates to nitrites and oxidase – catalase – tests are positive. Isolates are sensitive to the vibriostatic agent O/129 (150 μg) and they are arginine dihydrolase positive and negative for lysine and ornithine decarboxylases, production of H_2S, urease and acid production from inositol, sorbitol, rhamnose, melibiose and arabinose. All strains are positive for indole production, methyl red, Simmon's citrate, acid production from sucrose and hydrolise gelatin, Tween 80, DNA and starch.

All strains can use as sole carbon source glutamic acid, D–ribose, L–serine, glucose, β–hydroxybutyric acid, lactic acid, succinic acid, propionic acid, malic acid, glycerol, L–threonine, D–galactose, fumaric acid, D–alanine, sodium acetate, maltose, D–fructose, piruvate, L–histidine, N–acetylglucosamine, glicine, ornithine, D–mannose, L–citruline, L–arginine, citric acid and tyrosine. Isolates do not able to use the following carbon sources: melibiose, gluconic acid, aspartic acid, sucrose, D–mannitol, lactose, D–sacaric acid, amygdalin, D–sorbitol, arabinose, myoinositol, trans aconitic acid, putrescine, L–leucine, D–xylose, lysine, γ–aminobutyric acid and L–rhamnose.

All strains ferment D–ribose, D–galactose, D–glucose, D–fructose, D–mannose, N–acetylglucosamine, esculin, salicin, D–cellobiose, D–maltose, D–melibiose, sucrose, D–trehalose, D–raffinose, starch, glycogen and potassium 2–keto–gluconate. None of the strains ferment glycerol, erythritol, D–arabinose, L–arabinose, D–xylose, L–xylose, D–adonitol, methyl–βD–xylopyranoside, L–sorbose, L–rhamnose, dulcitol, inositol, D–mannitol, D–sorbitol, methyl–αD–mannopyranoside, methyl–αD–glucopyranoside, amygdalin, D–lactose, inulin, D–melezitose, xylitol, D–turanose, D–lyxose, D–tagatose, D–fucose, L–fucose, D–arabitol, L–arabitol, potassium gluconate and potassium 5–ketogluconate.

Enzymatic activity is positive to alkaline phosphatase, esterase lipase (C 8), lipase (C 14), leucine arylamidase and negative to esterase (C 4), valine arylamidase, cystine arylamidase, trypsin, α–chymotrypsin, acid phosphatase, naphthol–AS–β1–phosphohydrolase, α–galactosidase, β–galactosidase, β–glucuronidase, α–glucosidase, β–glucosidase, α–mannosidase and α–fucosidase.

The major cellular fatty acids are summed feature 3 (comprising $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$), $C_{16:0}$, summed feature 8 ($C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$), $C_{14:0,}$ $C_{12:0,}$ $C_{12:0}$ 3–OH and Summed feature 2 ($C_{14:0}$ 3–OH and/or iso–$C_{16:1}$ I).

The type strain 605^T (= CECT 8855^T= CAIM 1904^T) was isolated during episodes of mortality affecting larval cultures of carpet shell clam in a shellfish hatchery located in Galicia (NW Spain) (43º32'14'' N / 7º2'35'' O). The isolate 194 (= CECT 8856= CAIM 1905) was obtained from gonad samples of a healthy breeder taken in the same hatchery

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Figure legends.

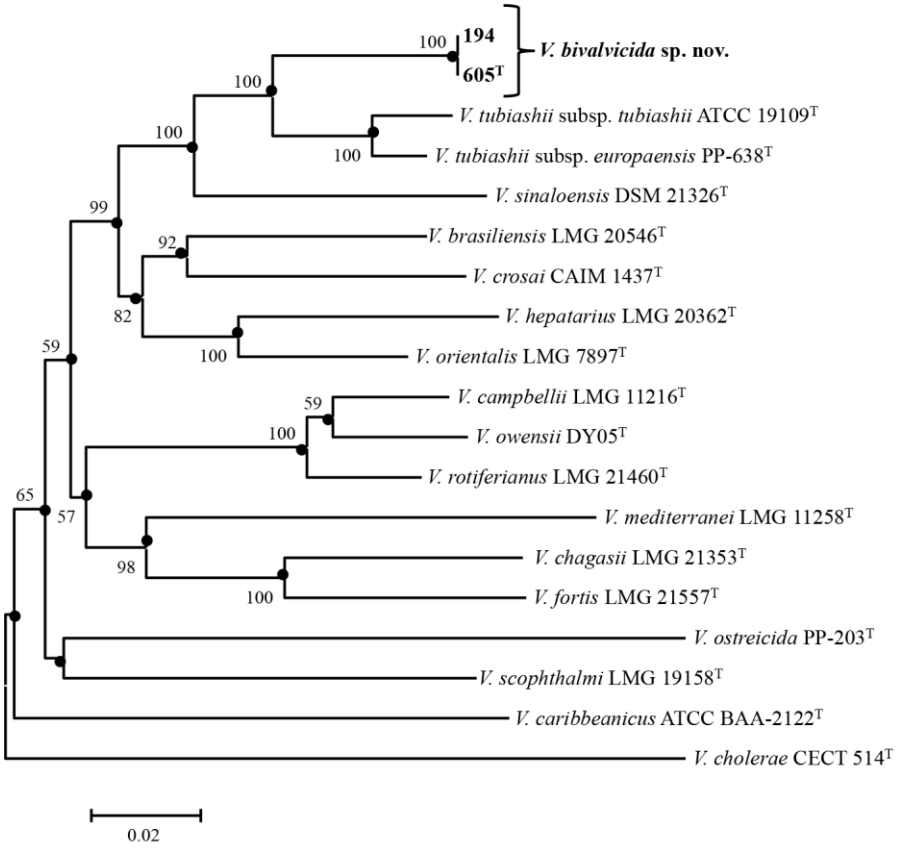

Fig. 1. Phylogenetic tree based on concatenated sequences of five housekeeping genes *fts*Z, *gyr*B, *pyr*H, *rec*A and *rpo*A obtained by NJ method. *Vibrio cholerae* CECT 514^T was used as outgroup. Sequence data are given in Supplementary Table S1. Horizontal branch lenghts are proportional to evolutionary divergence. Bootstrap (≥ 50%) from 1000 replicates appears next to the corresponding branch. Circles indicate that corresponding nodes were coincident in tree generated with ML algorithm. Bar, 0.02 substitutions per nucleotide position.

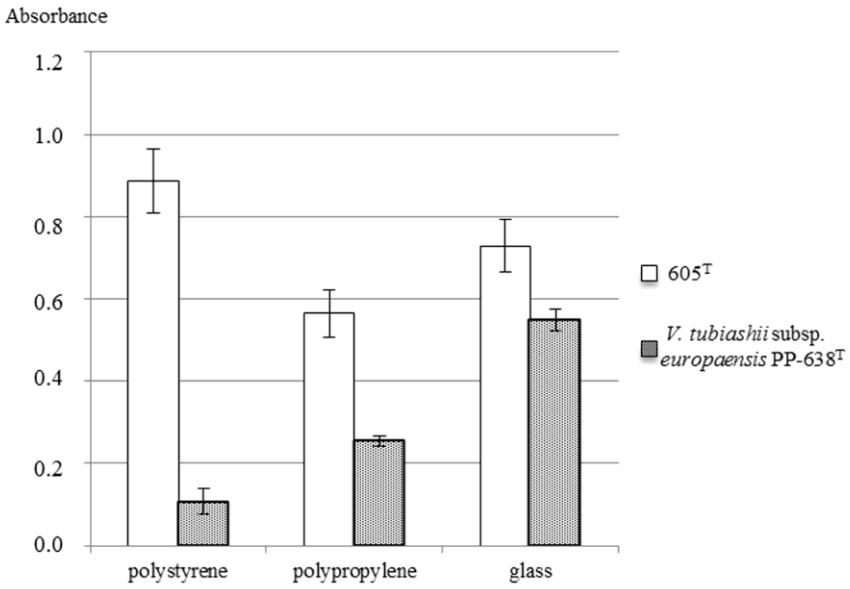

Fig. 2. Quantification of biofilm formed by the type strain 605^T and the closest relative *V. tubiashii* subsp. *europaensis* PP–638^T on different materials.

Table 1. Phenotypic features for distinguishing *V. bivalvicida* sp. nov. from related *Vibrio* species. Taxa are indicated as: 1, *V. bivalvicida* sp. nov. (3 isolates); 2, *V. tubiashii* subsp *europaensis* PP–638^T; 3, *V. tubiashii* subsp. *tubiashii* CECT 4196^T; 4, *V. orientalis* CECT 629^T. All data were obtained in this study.

Characteristics:	**1**	**2**	**3**	**4**
Arginine dihydrolase	+	+	+	–
Lysine decarboxylase	–	–	–	+
ONPG	–	+	+	–
AMP10	R	S	S	S
Growth at:				
4ºC	–	–	–	+
35ºC	+	–	+	+
8% NaCl	–	–	–	+
Enzymatic activities:				
Esterase lipase (C8)	+	+	+	–
Lipase (C14)	+	+	–	–
Valine arylamidase	–	–	+	+
Cystine arylamidase	–	–	+	–
Acid phosphatase	–	–	–	+
Naphthol–AS–β1–phosphohydrolase	–	–	+	+
β–galactosidase	–	–	+	–
Fermentation of:				
D–mannitol	–	+	+	+
D–raffinose	+	–	–	–
Utilization of:				
Glutamic acid	+	+	+	–
Glucose	+	+	–	+
Melibiose	–	+	–	–
Gluconic acid	–	+	+	+
D–galactose	+	+	+	–
Aspartic acid	–	–	+	+
Sacarose	–	+	–	+
D–manitol	–	+	+	+
Glycine	+	+	+	–
D–mannose	+	+	–	+
L–citruline	+	+	+	–
Citric acid	+	+	+	–
Myoinositol	–	–	–	+
Putrescine	–	–	–	+
γ–aminobutiric acid	–	–	–	+
Tyrosine	+	+	+	–

+, positive; –, negative; R, resistant; S, susceptible.

Table 2. Fatty acid composition (%) of *V. bivalvicida* sp. nov. and the related *Vibrio* species.

All data were obtained in this study.

Major fatty	*V. bivalvicida* sp. *nov*		*V. tubiashii* subsp.	*V. tubiashii* subsp.	*V. orientalis*
Acids	605^T	194	*tubiashii* CECT 4196^T	*europaensis* PP–638^T	CECT 629^T
$C_{16:0}$	23.0	20.9	15.8	17.4	19.9
$C_{14:0}$	7.0	5.2	6.3	5.7	8.7
$C_{12:0}$	3.6	2.0	2.8	2.1	3.4
$C_{18:0}$	tr	1.2	tr	tr	2.1
$C_{12:0}$ 3–OH	1.9	1.5	2.1	1.4	1.6
iso–$C_{16:0}$	tr	1.6	4.5	4.2	tr
$C_{17:1\omega8c}$	tr	1.7	tr	1.1	tr
iso–$C_{13:0}$	tr	1.1	tr	1.2	tr
iso–$C_{17:0}$	tr	1.1	1.0	1.4	tr
iso–$C_{15:0}$	tr	1.2	1.1	1.3	ND
iso–$C_{14:0}$	tr	tr	1.2	1.0	tr
anteiso–$C_{15:0}$	tr	tr	tr	1.0	tr
Summed features*:					
2	1.6	1.4	2.0	1.7	2.5
3	37.4	38.0	40.0	38.0	41.7
8	17.8	13.3	16.2	14.5	14.2

tr: trace quantities (≤1.00 %); ND, not detected.

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 2 consisted of $C_{14:0}$ 3–OH and/or iso–$C_{16:1}$ I; Summed feature 3 consisted of $C_{16:1\omega7c}$ and/or $C_{16:1\omega6c}$ and Summed feature 8 consisted of $C_{18:1\omega7c}$ and/or $C_{18:1\omega6c}$.