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Characterization of pathogenic vibrios isolated from bivalve hatcheries in Galicia, NW Atlantic coast of Spain. Description of *Vibrio tubiashii* subsp. *europaensis* subsp. nov

Susana Prado*[,1], Javier Dubert[1], Juan L. Barja

Departamento de Microbiología y Parasitología, CIBUS—Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, 15782, Spain

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ABSTRACT

The taxonomic position of the bivalve pathogen PP-638 was studied together with five similar isolates. The strains were isolated from flat oyster (*Ostrea edulis*) and Manila clam (*Venerupis philippinarum*) cultures during outbreaks of disease in two shellfish hatcheries (Galicia, NW Spain). The pathogenicity, previously established for PP-638, was demonstrated with all isolates and for several bivalve species, including the original hosts. On the basis of phenotypic characterization and 16S rRNA gene sequences, a tight group was defined within the genus *Vibrio*. Multilocus sequence analysis (MLSA) based on concatenated sequences of the 16S rRNA gene and the five housekeeping genes *rec*A, *rpo*A, *pyr*H, *gyr*B and *fts*Z revealed that these strains form a cluster within the Orientalis clade, close to the species *Vibrio tubiashii*. The results of MLSA, the DDH rate and the phenotypic differences with the type strain of *V. tubiashii* supported the differentiation of the Galician isolates as a new subspecies within *V. tubiashii*, for which the name *V. tubiashii* subsp. *europaensis* subsp. nov. is proposed (type strain PP-638^T = CECT 8136^T = DSM 7349^T) The emended description of *V. tubiashii* is included. The pathogenicity assays widen the host range of *V. tubiashii* to add two unreported species, *Venerupis decussata* and *Donax trunculus*, and the described as relatively resistant species *V. philippinarum*.

Hatchery production is currently the only sustainable method of obtaining bivalve spat for the aquaculture industry. Outbreaks of bacterial disease caused by some *Vibrio* species represent the main bottleneck in the production process in bivalve hatcheries. Vibriosis, which is characterized by a sudden large reduction in larval motility and by detached vela and necrotic soft tissue, leads to high mortality rates of more than 90% in 24 – 48 h [14].

The genus *Vibrio* is the largest member of the family *Vibrionaceae* and comprises more than 90 bacterial species (http://www.bacterio.net/vibrio.html), classified in 17 clades [20]. Some vibrios have been described as opportunistic pathogens in bivalve aquaculture and may proliferate under culture conditions and kill bivalve larvae [4,8,10,14,16]. The species *Vibrio tubiashii* [6] was recognized almost fifty years ago as a significant pathogen of bivalve larvae [28,29], causing "bacillary necrosis". In the last years, Elston et al. (2008) have demonstrated the emerging resurgence of *V. tubiashii* infections in US with dramatic impact on intensive shellfish production.

In this study, we report the taxonomic characterization of six bacterial isolates obtained from bivalve larvae reared in two Galician hatcheries during outbreaks of disease. The pathogenicity, previously demonstrated with strain PP-638 [14], was extended now to the other isolates and with different bivalve species. The phenotypic and genotypic results showed that this group constitutes a new subspecies within the species *V. tubiashii*, in the Orientalis clade, with special significance for shellfish aquaculture.

Microbiological samples were obtained during episodes of mortality that affected bivalve cultures in two hatcheries, and they were processed as in the previous study [14]. All strains included in the present study were isolated from Thiosulphate-Citrate-Bile-Sucrose plates (TCBS, Oxoid). In Hatchery A, isolates PP-654 and PP-660 were obtained from flat oyster larvae and PP-638^T and PP-635 were isolated from the seawater used in the same culture. Strains PP2-843 and PP2-978 were isolated from small spat (≥1000 μm) of Manila clam (*Venerupis philippinarum*) reared in Hatchery B. All isolates were cultured in Marine Agar (MA,

Abbreviations: DDH, DNA – DNA hybridization; CECT, Colección Española de Cultivos Tipo, Spain; FAME, fatty acid methyl esters; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MLSA, multilocus sequence analysis.

* Corresponding author. Tel.: +34 881816911; fax: +34 881816966.
E-mail address: ssn.prado@gmail.com (S. Prado).

[1] These authors have contributed equally to this work.

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Pronadisa, Lab Conda) for 24 h at 25 °C, and the phenotypic characterization was carried out as previously described [14]. Utilization of 46 sole carbon sources was evaluated in plates containing basal medium and the certain carbon source [3]. Enzymatic activities and additional phenotypic features were determined with API ZYM and API 20E identification kits (bioMérieux) respectively, with the bacteria suspended in saline solution (NaCl 0.85%). Fermentation of carbon sources was tested with API 50CH strips (bioMérieux), with slight modifications [15].

Analysis of FAME was performed according to the Microbial Identifications Systems (MIDI) described by Sasser [18]. The six isolates and the reference type strains *V. tubiashii* CECT 4196^T and *Vibrio orientalis* CECT 629^T were cultured previously for 24 h at 25 °C on Trypticase Soy Agar (Pronadisa, Lab Conda) supplemented with 0.5% NaCl (w/v).

The six isolates shared the main phenotypic features of the genus *Vibrio* [13]: motile rods, facultatively anaerobic, Gram-negative, oxidase and catalase positive, susceptible to the vibriostatic agent O/129, able to reduce nitrates to nitrites and to grow on TCBS. These strains showed high phenotypic homogeneity, with variable reactions only for Simmon's citrate (positive for strains PP-660, PP2-843 and PP2-978) and acid production from melibiose (positive for the isolates PP-635 and PP-654).

The fatty acid profiles of the six isolates were very similar. The major fatty acids were summed feature 3 (39.6 ± 1.6, comprising 16:1ω7c and/or 16:1ω6c), 16:0 (17.0 ± 1.9%), summed feature 8 (14.0 ± 2.0%, comprising 18:1ω7c and/or 18:1ω6c), 14:0 (6.1 ± 0.4%) and 16:0-iso (3.4 ± 0.8%) (the complete profile is shown in Table S1).

DNA was extracted and purified from pure isolates by using the Instagene kit (Bio-Rad). A fragment (>1450 bp) of the 16S rRNA gene was amplified and sequenced from the extracted DNA by using bacterial universal primers specific to the 16S rRNA gene (27F and 1510R) [11]. The genes encoding RNA recombinase alpha subunit (*rec*A), RNA polymerase alpha subunit (*rpo*A), uridine monophosphate kinase (*pyr*H), gyrase beta subunit (*gyr*B) and a cell-division protein (*fts*Z) were used for MLSA. The amplification of the housekeeping genes were carried out as previously described [19,26,27,30], and in few cases the annealing temperature was adjusted to provide specific amplification. All the genes were amplified using Ready To Go PCR-beads (Amersham Pharmacia Biotech). The sequences obtained were analyzed using the Lasergene Seqman program (DNASTAR). Comparative analysis was carried out using the EzTaxon-e server [9] and BLAST [1]. Phylogenetic analysis based on the individual and concatenated sequences were performed using MEGA 5.2 software [23], after multiple alignment of data by ClustalW [25]. Distances and clustering with the Neighbour Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) algorithms were determined using bootstrap values based on 1000 replications. The sequence data of the closest *Vibrio* species were obtained from GenBank and the online taxonomic scheme for vibrios (http://www2.ioc.fiocruz.br/vibrio/AVib/Species.html). Sequences were deposited in the DDBJ/EMBL/GenBank database and the accession numbers are listed in Table S2.

The enterobacterial repetitive intergenic consensus (ERIC-PCR) and repetitive extragenic palindromic (REP-PCR) techniques were used to discriminate bacterial strains. For genotyping, the six isolates and the two reference strains *V. tubiashii* CECT 4196^T and *V. orientalis* CECT 629^T were amplified by ERIC-PCR and REP-PCR, as previously described Rodríguez et al. [17].

The DNA fingerprints (Fig. S1) revealed that Galician isolates are different from the type strains of *V. tubiashii* and *V. orientalis*. Two profiles were differentiated in relation to the origin (Hatchery A and B), and the isolates PP-638 and PP2-843 were selected as representative strains of each clone.

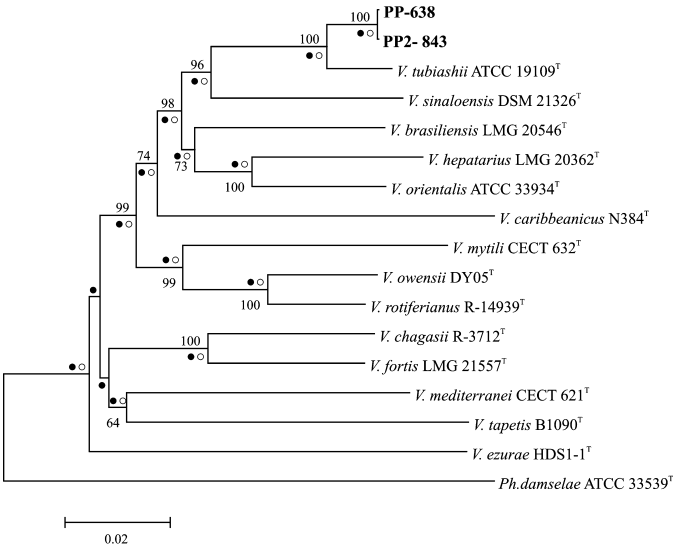

Fig. 1. Phylogenetic tree based on concatenated sequences of five housekeeping genes *rec*A, *rpo*A, *pyr*H, *gyr*B and *fts*Z and the 16S rDNA obtained by the NJ method. *Photobacterium damselae* was used as outgroup. Sequence data are given in Table S2. Horizontal branch lenghts are proportional to evolutionary divergence. Bootstrap (≥50%) from 1000 replicates appears next to the corresponding branch. Species belonging to Orientalis clade were included in the grey box. Circles indicate that corresponding nodes were recovered in trees generated with MP method (open circles) and ML method (filled circles). Bar, 0.02 substitutions per nucleotide position.

Phylogenetic analysis based on the 16S rRNA gene sequences using the NJ, MP and ML algorithms (Fig. S2) indicated that strains PP-638 and PP2-843 formed a cluster within the genus *Vibrio*. On the basis of these 16S rDNA sequences, the species closest to strain PP-638^T were *V. orientalis* CIP 102891^T (98.46%), *Vibrio mytili* CECT 632^T (98.26%), *Vibrio owensii* DY05^T (98.24%), *Vibrio tapetis* CECT 4600^T (98.23%) and *V. tubiashii* ATCC 19109^T (98.01%). These percentages are all lower than the threshold value (98.70%) proposed by Stackebrandt and Ebbers [22] for defining new species.

The new cluster was maintained in the individual phylogenetic tree of each housekeeping gene (Fig. S3). However, the bivalve pathogen *V. tubiashii* was the most closely related species according to the phylogenetic analysis and the similarities of all the housekeeping gene sequences: *rec*A (95.6 – 95.8%, range of lengths, 817 – 822 bp), *rpo*A (99.1 – 99.2%, 860 – 925 bp), *pyr*H (97.8 – 97.9%; 496 – 560 bp), *gyr*B (96.4%, 1050 – 1109 bp) and *fts*Z (99.6 – 99.7%, 570 – 575 bp). These values were above the species limits established for some of these genes: 98% for *rpo*A, 94% for *rec*A and *pyr*H [26].

The phylogenetic trees based on concatenated sequences (4878 bp) of the 16S rRNA and the five housekeeping genes *fts*Z, *gyr*B, *pyr*H, *rec*A and *rpo*A, determined using NJ, MP and ML algorithms (Fig. 1), confirmed the close relationship between the Galician isolates and *V. tubiashii*. Distance matrices for the MSLA combined sequence dataset (data not shown) provided additional evidences, with similarities of 97.6% with the type strain of *V. tubiashii*, clearly higher than the value proposed by Thompson et al. [24] for delimiting the species of genus *Vibrio* (95%). The similarities with the other members of the Orientalis clade were in the range 90.9 – 93.0%, within the specific concatenated identity between species established by Sawabe et al. [20] for the Orientalis clade (88.8 – 93.7%) using eight gene MLSA.

The DDH experiments were carried out by the hydroxyapatite method in microtitre plates [31]. The experiments were performed by duplicate. Strain PP-638 was hybridized with the isolate PP2-843, and the reference strains *V. tubiashii* CECT 4196^T and *V. orientalis* CECT 629^T. The DDH reassociation value within the cluster, between PP-638 and PP2-843, was 87.63%. The degree of