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First description of French *V. tubiashii* strains pathogenic to mollusk: I. Characterization of isolates and detection during mortality events

Marie-Agnès Travers[a, *, 1], Rachida Mersni Achour[a, b, c, 1], Philippe Haffner[a], Delphine Tourbiez[a], Anne-Laure Cassone[a], Benjamin Morga[a], Ibtissem Doghri[b], Céline Garcia[a], Tristan Renault[a], Ingrid Fruitier-Arnaudin[b, c, 2], Denis Saulnier[a, 3, 2]

[a] Ifremer, SG2M-LGPMM, Laboratoire de Génétique et Pathologie des Mollusques Marins, Avenue de Mus de Loup, 17390 La Tremblade, France
[b] UMR 7266 CNRS-ULR, LIENSs, Equipe Approches Moléculaires, Environnement-Santé, Université de La Rochelle, Avenue Michel Crépeau, 17042 La Rochelle, France
[c] Fédération de Recherche en Environnement et Développement Durable, FR CNRS 3097, Université de La Rochelle, France

[1] Marie-Agnès Travers and Rachida Mersni Achour participated equally to this work and should be considered as primary co-authors.

[2] Ingrid Fruitier-Arnaudin and Denis Saulnier participated equally to this work and should be considered as last co-authors.

[3] Present address: Ifremer, Centre Ifremer du Pacifique, UMR EIO 241 Ecosystèmes Insulaires Océaniens, Tahiti, 98719 Taravao, French Polynesia.

*: Corresponding author : Marie-Agnès Travers, Fax: +33 5 46 76 26 11 ;
email address : marie.agnes.travers@ifremer.fr

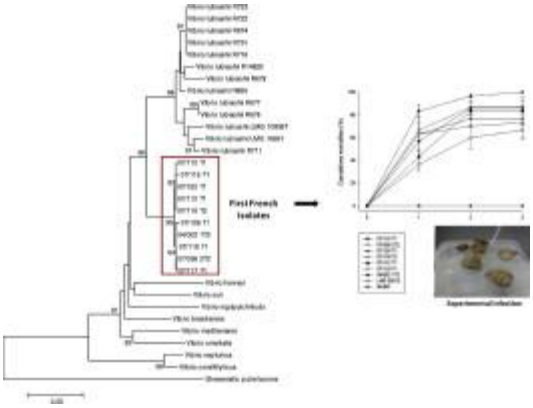
Abstract:

Nine dominant bacterial isolates were obtained from different batches of *Crassostrea gigas* spat experiencing high mortality rates in a French experimental hatchery/nursery in 2007. Using phenotypic analysis combined with multilocus sequence analysis, the isolates were shown to be genetically close to the *Vibrio tubiashii* type strain. Based on (1) analyses of the *recA* gene sequences; (2) the results of DNA–DNA hybridization assays between 07/118 T2 (LMG 27884 = CECT 8426), which is a representative strain, and the *V. tubiashii* type strain (69%); and (3) phenotypic traits, the bacteria were classified in a group close to American *V. tubiashii* strain. Its virulence (70% of mortalities) and the toxicity of the extracellular products of 07/118 T2 was demonstrated (41% of mortalities).

Moreover, a QPCR diagnostic tool targeting the *gyrB* gene was developed to investigate the epidemiological significance of *V. tubiashii* in French oyster mortality outbreaks recorded by the national surveillance network. Of the 21 batches originating from hatcheries, only two were positive, whereas *V. tubiashii* DNA could not be detected in any of the batches of moribund animals collected in field/outdoor facilities.

These results demonstrate the existence of a group of virulent *V. tubiashii* in France that episodically infect *C. gigas*.

Graphical abstract



Highlights

► First detection of *V. tubiashii* during mollusk mortality in France. ► French isolates are phylogenetically close but separated from American ones. ► French isolates are virulent to Pacific oyster and produce toxic ECPs. ► *V. tubiashii* was episodically detected in France since 2004.

Keywords : *Crassostrea gigas* ; Pathogenicity ; Extracellular products ; Polyphasic approach, Real time PCR

1. Introduction

The causes of abnormal mortalities during summer in *Crassostrea gigas* (*C. gigas*) remains unclear although several studies suggest that this phenomenon has a complex etiology involving environmental stressors (oxygen depletion, lack of food, salinity, temperature, industrial pollution, pesticides, aquaculture practices) and biological factors that include pathogens and an altered physiology of the host (Samain et al., 2007). These mortalities can affect both diploid and triploid oysters, their different life stages (Romalde and Barja, 2010; De Decker et al., 2011) both in the field (for juveniles and adults) and in hatcheries (for larval and post-larval stages). Among the biological factors, microorganisms are often associated with oyster diseases. Three infectious microorganisms are frequently described as major pathogens of oysters : herpes-like viruses (Renault et al., 1994; Aguirre-Guzman et al., 2005), parasites (Meyers et al., 1991; Bower et al., 1997; Tun et al., 2008) and bacteria of the genus *Vibrio* which have been described as important causal agents affecting all life stages of bivalve mollusks (Le Roux et al., 2002; Garnier et al., 2008; Beaz-Hidalgo et al., 2010; Saulnier et al., 2010). *V. splendidus* and *V. aestuarianus* are presented as the two main *Vibrio* species associated with summer mortalities of *C. gigas* in France (Lacoste et al., 2001; Le Roux et al., 2002; Garnier et al., 2008). Besides these two major bacterial, another *Vibrio* species was reported by Saulnier et al. (2010) in a large-scale epidemiological study including nine virulent strains that belong to the *V. harveyi* group. The virulence of these strains was investigated by experimental infection on *C. gigas* spat. The results showed a strong correlation between the virulence of *Vibrio* and their capacity to produce metalloprotease-like activity in the culture supernatant, particularly for the strains 07/108 T1 and 07/118 T2. These two strains can secrete a high level of proteases and can induce extremely high levels of mortality just 20 hours post injection. Partial sequence analysis based on 16S rDNA revealed that 07/108 T1 and 07/118 T2 were clustered within the *V. harveyi* group.

Nevertheless, phenotypic analysis and the use of standard molecular techniques are insufficient to classify *Vibrio*, and indeed cannot even be used to identify related species. The identification of vibrios at the family and genus level are habitually based on 16S rDNA gene sequencing, but this method is unable to separate certain closely related species (Gomez-Gil et al., 2004). The association of other analyses, including DNA-DNA hybridization and molecular fingerprinting methods, is useful for *Vibrio* identification. However, these tools are restricted to some reference laboratories and inter-laboratory comparisons of fingerprint patterns are difficult (Thompson et al., 2005).

Multilocus sequence analysis (MLSA) has been adopted to fine-tune the approach and clarify certain doubts concerning *Vibrio* classification. Using this methodology, Thompson et al. have shown that *V. harveyi.* and *V. campbellii* form two separate clusters (Thompson et al., 2007) and *V. communis* and *V. owensii*, which were assigned to the *Harveyi* clade, were recently discovered to be the same species after identification using the MLSA approach (Hoffmann et al., 2012).

The aim of the present study was to refine the taxonomic affiliation of these virulent strains, previously clustered with *V. harveyi*, using a polyphasic approach (phenotypic and genotypic characterization). Secondly, quantitative challenges with live bacteria and *in vitro* culture supernatants were performed with *C. gigas* in order to evaluate their virulence and the toxicity of the extracellular products, respectively. Finally, a quantitative real-time PCR diagnostic tool was developed to screen isolates/DNA collected during the episodes of mortality that have occurred in France since 2004 and evaluate their epidemiological significance.

2. Materials and methods

2.1. Taxonomic affiliation

2.1.1. Bacteria genotyping and phylogenetic analyses (10 isolates)

The strains used in this study are described in Table 1. The predominant bacteria, which have not been yet described, were isolated, as previously done by Saulnier et al. (2010), during the mortalities recorded in oyster hatchery stocks in 2007. Briefly, after 10^{-2} and 10^{-3} dilutions in Sterile Artificial Sea Water (SASW : 2.3 % (w/v) NaCl, 20 mM KCl, 5 mM $MgSO_4$, 2 mM $CaCl_2$), moribund oyster samples (hemolymph for adults and crushed tissues for juveniles) were plated on thiosulfate-citrate-bile salts-sucrose (TCBS, Difco™), Zobell agar (0.4 % peptone, 0.1 % yeast extract, 0.01% ferric citrate and 1.5 % agar in SASW, pH 7.6) or marine agar (Conda) and the predominant bacteria were isolated after 48h at 20°C. Pure cultures of bacterial strains were conserved frozen at -80°C in Zobell broth with glycerol 15%.

For genotyping, DNA was extracted following the FTA® procedure (Whatman). Bacterial isolates were cultured during 24 hours at 20 °C in Zobell broth and 65 µl of bacterial suspension at the stationary phase of *in vitro* culture was deposited on FTA® paper matrix cards according to the manufacturer's instructions. Briefly, a punch from the FTA® Card was washed three times with FTA® Purification Reagent and rinsed with 200 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8). This punch was either directly transferred into a PCR tube, or conserved at -20°C before use.

The 16S rRNA (ribosomal RNA), *ftsZ* (cell division protein, cell division), *pyrH* (uridylate kinase, nucleotide biosynthesis), *recA* (recA protein, DNA repair) and *topA* (topoisomerase I, DNA replication and repair) genes were amplified using the universal bacterial primer pairs listed in supplementary Table 1 and the methods previously described (Thompson et al., 2005). Amplicons with the expected size were purified using a Microcon PCR filter kit (Millipore). Purified PCR products were mixed (final volume 10 µl) with 0.4 µl ABI Prism Big Dye Terminator ready reaction mix (Applied Biosystems®) and 0.75 µM of primer. Cycle sequencing reactions were performed using a Gene Amp PCR System 2700 (Applied Biosystems®) and following the manufacturer's instructions. Separation of the DNA fragments was carried out in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Sequences were aligned using ClustalW http://www.ebi.ac.uk/Tools/msa/clustalw2/ and BioEdit software http://www.mbio.ncsu.edu/bioedit/bioedit.html. Pairwise similarity was calculated with BioEdit.

Phylogenetic trees were built using Mega4 http://www.megasoftware.net/mega4/mega.html. The trees were drawn using the Neighbor-Joining method with the Kimura two-parameter model (Saitou and Nei, 1987) and the maximum parsimony method (Eck and Dayhoff, 1966). Reliability of topologies was assessed by the bootstrap method (Felsenstein, 1985) with 1000 replicates. The Genbank accession numbers for the 16S rDNA, *gyrB*, *recA*, *topA*, *pyrH*, *ftsZ* gene sequences obtained in this study or already published in GenBank are presented in Supplementary Table 3.

2.1.2. DNA-DNA hybridization assays (2 isolates)

DNA-DNA hybridization (DDH) was performed by the BCCM™/LMG laboratory at Gent University. After a genomic DNA extraction according to a modified Wilson protocol (Wilson,

1987), hybridizations were performed in microplates at 40°C (Ezaki et al., 1989) and DNA relatedness values (%) reported were the means of a minimum of 6 hybridizations.

2.1.3. Bacteria phenotypic characterization (10 isolates)

The phenotypes of the selected isolates were characterized in different ways. Bacteria growth on TCBS medium (AES Chemunex), chromID™ Vibrio medium (Biomérieux) and CHROMagar™ Vibrio medium (CHROMagar) was observed. The capacity of bacteria to grow in Zobell broth with 8 and 10% NaCl at 22°C was determined and the ability of bacteria to grow on Zobell agar at 40°C was established.

Antibiograms were carried out using disc diffusion assays on Mueller-Hinton agar (Oxoid) with commercial impregnated discs (Oxoid): Flumequine 30 µg (UB30), Kanamycin 30 µg (K30), Tetracycline 30 µg (TE30), Sulphamethoxazole/trimethoprim 19:1 25 µg (SXT25), Streptomycin 25 µg (S25), Sulphonamides 300 µg (S3), Erythromycin 30 µg (E30), Penicillin 10 UI (6 µg) (P10), Trimethoprim 5 µg (W5), Chloramphenicol 30 µg (C30), Ampicillin 10 µg (AMP10), Gentamicin 30 µg (CN30) or the Vibriostatic agent, Pteridine 150 µg and 10 µg (O129/150 and O129/10). Inhibition was observed after 24 to 48h at 22°C on Zobell agar. API 20E, API 20NE and API 50CH (Biomérieux) were used in accordance with the manufacturer's recommendations. Fresh colonies, obtained from culture bacteria on TSA (Difco™) adjusted to 2% NaCl, were suspended in SASW to obtain about OD_{600} = 0.1. After inoculation, the strips were incubated for 48 h at 22 °C and the results were determined according to the manufacturer's instructions.

2.2. Pathogenicity assays

2.2.1. Bacterial virulence tests (7 isolates)

Bacteria (07/038 2T2, 07/110 T1, 07/112 T1, 07/120 T1, 07/119 T1, 07/118 T2 and 04/002 1T2) were grown at 22 °C for 20 h in marine broth with constant shaking at 40 rpm (Rotator SB3; Stuart). The bacterial concentrations in the cultures were evaluated spectrophotometrically at an optical density (OD) of 600 nm and checked by plating. The cells were centrifuged at 3,200 × g for 10 min, the supernatant discarded and the resulting pellet resuspended in SASW to obtain an $OD_{600\ nm}$ of 1. Ten to twelve-month-old oyster spat were anesthetized for 1 to 2 hours at 22 °C in a magnesium chloride solution ($MgCl_2$, Fluka) at a final concentration of 50 g l^{-1} (1/4: v/v seawater/freshwater) and under aeration. Subsequently, 100 µl of bacterial suspension (10^7 cfu per oyster) was injected into the adductor muscle. A group of oysters were injected with SASW to serve as negative controls. After injection, the oysters were transferred to tanks (triplicates of 10 oysters in 2 liters) filled with UV-treated and 1 µm-filtered seawater at 3.1% salinity and maintained under static conditions at 22 °C with aeration. Mortality was monitored twice a day and any newly dead oysters were removed from the tanks over a four day period. Animals were considered to be dead when the valves did not close following stimulation.

2.2.2. Toxicity and characterization of ECPs from the V. tubiashii 07/118 T2

Bacterial extracellular products (ECPs) from 07/118 T2 were produced in two types of culture medium, plate agar and liquid medium.For culture in liquid medium: bacteria were grown on Luria-Bertani (Difco™) medium supplemented with 1% sodium chloride (LBS-1% NaCl). Briefly 100 µl of a pre-culture of bacteria (OD_{600} of 1) was cultured in 100 ml of LBS medium

in a 500 ml Erlenmeyer flask at 22°C with constant shaking at 100 rpm (Aquatron, INFORS HT). After 24 h of incubation, ECPs were obtained by centrifugation at 3,000 × g for 40 min at 4°C. The supernatant containing the ECPs was filtered at 0.22 µm and then concentrated by lyophilisation. The concentrated sample was resuspended in Tris-HCl buffer (50 mM Tris-HCl, pH 8.0) and dialyzed against distillated water in the first step for 12h and against Tris-HCl buffer for 12h in the second step. Finally, the samples were stored at -80°C until use. For culture on solid medium: ECPs were produced using the cellophane plate technique described by Liu (1957). A volume of 1 ml of exponential phase culture in LBS (OD_{600} of 1, 22°C for 18 h) was spread on a sterile cellophane film overlying LBS agar (LBSA) plates. After 48 h of culture at 22°C, the cells were washed off the cellophane using 10 ml of cold SASW and removed by centrifugation at 3,000 × g (45 min at 4°C). The supernatant containing the ECPs was then treated in the same way as the ECPs from liquid media.

The toxicity of both ECPs was tested as follows: a volume of 100 µl of ECP solution, adjusted in Tris-HCl buffer to a concentration of 5 µg g^{-1} of soft body weight, was injected into the adductor muscle of ten to twelve month-old oyster spat after anesthetizing them (see above). A group of oysters used as controls were injected with SASW and Tris-HCl buffer. Mortality was monitored twice a day and any newly dead oysters were removed from the tanks. ECPs proteins concentration was determined using the method described by Bradford (using Bradford Reagent, from Sigma -Aldrich) with bovine albumin serum as a standard (Sigma-Aldrich).

The proteolytic activity was determined using azocasein (Sigma-Aldrich) as a substrate according to the method of Teo et al (2003) with some modifications. Briefly, 100 µl of a suitable dilution of ECP solution (1/10 v/v) was added to 100 µl of azocasein (5 mg ml^{-1} in 50 mM Tris–HCl, pH 8.0). The mixture was incubated at 22°C for 2 h in darkness and stirred constantly. The reaction was stopped by adding 200 µl of 10% trichloroacetic acid. The mixture was centrifuged at 12,000 × g for 10 min and 50 µl of the supernatant was added to 50 µl of 1M NaOH in 96 well plates (Nunc/Thermo Scientific**)**. The absorbance was measured at 440 nm in a microplate reader (Molecular Devices). One unit of azocaseinase activity was defined as the amount of crude ECPs that caused an increase of 0.01 absorbance unit after a 2 h incubation at 22°C.

The stability of the proteolytic activity was determined by incubating ECPs from LBSA for 24 h and 48 h at 22°C without substrate. After the preincubation, the azocaseinase activity was measured according to the protocol described previously.

The effect of 1,10-phenantroline on proteolytic activity was examined. Stock solutions of 1,10-phenantroline (Sigma-Aldrich) were dissolved in a small volume of absolute ethanol (until total solubilization) and adjusted to the appropriate concentration with Tris-HCl buffer. Briefly, 25 µl of inhibitor, diluted to the appropriate concentrations, was pre-incubated with 25 µl of ECPs for 30 min at 22°C and the proteolytic activity was then measured (see above). Residual azocaseinase activity was calculated on the basis of assays run in the absence of inhibitor.

2.3. Quantitative real-time PCR diagnostic tool

2.3.1. Diagnostic tool development (2 isolates)

Oligonucleotide sequences were derived from the sequence alignment of the *gyrB* (DNA gyrase B subunit, basic replication machinery) gene in different *Vibrio* species, including the *V. tubiashii* type strain and the sequence obtained from the 07/118 T2 and 07/112 T2 strains with the primers listed in supplementary Table 1. Primer 3 software http://frodo.wi.mit.edu/

was used to design a real-time PCR probe (Table S1) and forward and reverse PCR primers. The probes consists of an oligonucleotide dually labeled with a 5‘- reporter dye and the downstream 3‘-quencher dye indicated Table S1 (Eurogentec). The probe and primers were assessed for species specificity using a BLAST search to determine homology to known sequences.

A Real-time PCR assay was conducted on MX3000 and MX3005 Thermocyclers (Agilent) using Brilliant III Ultrafast kit (Stratagene). Each reaction was run in duplicate in a final volume of 20 µl containing various concentrations of a DNA sample (5 µl), 200 nM of each primer and 200 nM of oligonucleotidic probe. The thermal cycle was performed with a two-step PCR protocol: 1 cycle at 95 °C for 3 min followed by 40 cycles at 95 °C for 10 s and 60°C for 20 s. Fluorescence intensity was expressed in delta reporters (dR) after background subtraction. The threshold was set using an amplification based algorithm from the MX3000-3005 software (Stratagene) for the initial plate. Amplicons were sequenced to check primers specificity. Serial dilutions were performed on genomic DNA extracts quantified by spectrophotometry (Nanodrop/Thermo Scientific) and adjusted to 10^8 bacteria/ml = 1.05 ng/µl (for the 5,353 kbp *V. tubiashii* genome).

2.3.2. Screening on DNA isolated from mollusks

Zoosanitary monitoring was performed by the REPAMO network (REseau de PAthologie des MOllusques) between 2003 and 2012. During this period 254 batches of animal samples were collected from abnormal mortality episodes affecting *Crassostrea gigas* oysters, *Haliotis tuberculata* abalone, *Venerupis philippinarum* clams and *Pecten maximus* scallops. Samples from 49 of these batches were analyzed in this work. They were selected to provide a range of origin (hatchery/field), year of sampling, age and ploidy of the animals. The isolation of dominant bacteria was carried out between 2003 and 2012 on animals that were moribund on their arrival in the laboratory. Total DNA from infected animals was systematically extracted between 2003 and 2012. Depending on mollusk species and the age of the animals, different tissues were sampled in sterile conditions for bacteriological analysis: haemolymph was taken for each oyster or abalone; a piece of adductor muscle, gill and mantle mixed together for each scallop; and a mixture of five mantles from individual clams. All these tissues were prepared using five individuals or five pools of animals from each batch, and in each case this corresponded either to an all-adult or all-juvenile age class. Haemolymph was drawn into a syringe from the adductor muscle or pericardial cavity of oysters and from the pericardial cavity of abalone using a 23-gauge needle. Tissues from scallop and clams were homogenized in 100 µl of SASW with a sterile pellet-pestle (Sigma) for 1 minute on average. For larvae and early spat stages (shell size smaller than 3 cm in length), several whole animals were taken and disrupted in SASW as a single pool of individuals for each batch sample.

Bacterial DNA from a log-phase culture was extracted from cultured *Vibrio* either by boiling in 200 µl of ultra pure water (Saulnier et al., 2009) or using the standard QIAmp DNA mini kit (Qiagen) procedure. After extracting DNA according to the manufacturer’s instructions, the pelleted DNAs were resuspended in 100 µl of AE buffer and stored at -20°C. DNA yield and purity were determined by spectrophotometry (Nanodrop/Thermo Scientific). DNA from the crushed tissues of mollusk was extracted using a similar QIAmp DNA mini kit (Qiagen) method.

3. Results

3.1. Genetic and biochemical characterization of bacterial strains isolated in 2004 and 2007

Bacterial strains were isolated during the period in which the REPAMO surveillance network reported high levels of mollusk mortalities in hatcheries. In 2007, in an experimental hatchery/nursery in France, nine batches of oyster juveniles and adults (either diploids or triploids) experienced heavy mortalities. Nine predominant bacterial strains were isolated from these batches on TCBS plates (Table 1). After isolation, the bacteria appeared small (1-2 mm), opaque, regular, white to cream-colored on marine agar and yellow on TCBS after 48 h at 20°C.

Initial phylogenetic analyses based on the 16S rRNA gene sequence of these strains led to their classification in the *V. harveyi* group (Saulnier et al., 2010). One other strain from the French REPAMO bacterial collection (04/002 1T2 = LMG 27885 = CECT 8427) had a similar 16S rRNA gene sequence (more than 99.8% mutual 16S rRNA gene sequence similarity) and was added to the other analyses.

A multilocus sequence analysis based on the conserved genes 16S rRNA, *ftsZ*, *topA*, *pyrH* and *recA* confirmed the tight grouping of these isolates. They were most closely related to the *V. tubiashii* LMG10936 type strain but appeared in a distinct cluster as revealed by the high bootstrap value of 100 (Fig. 1). Phylogenetic analysis with the maximum parsimony or the neighbor joining algorithms produced congruent results revealing the reliability of the obtained phylogenetic tress (Not shown). Similarities between the *V. tubiashii* type strain LMG10936 and the novel isolates were 97-97.5% for 16S rDNA, 98-99% for *ftsZ*, 92-96% for *topA,* and 91-97% for *pyrH* and for *recA* (sequences alignment is presented in Supplementary Table 4).

The *recA* gene showed the highest resolution for differentiating *V. tubiashii* related strains. Phylogenetic analysis of this gene provides strong evidence that the French isolates form a distinct group from the North American ones (Fig. 2), presenting nucleotidic sequence similarities around 94-95% (sequences alignment is presented in Supplementary Table 5).

This classification was confirmed by DNA-DNA hybridization (DDH) of two of the strains that were isolated from diseased oysters in 2007 or diseased abalone in 2004 (Table 2). DDH between the novel isolates 07/118 T2 (= LMG 27884= CECT 8426) and 04/002 1T2 (= LMG 27885 = CECT 8427) indicated 86% genomic similarity, confirming that they belong to the same species. The isolates 07/118 T2 and 04/002 1T2 had 69% DNA-DNA relatedness with the *V. tubiashii* type strain LMG 10936T, which is just under the border value accepted for the delineation of prokaryotic species (Wayne et al., 1987).

Additional tests such as biochemical characterization, temperature and salinity tolerance and antibiotic sensitivity were performed to decide whether to allocate the strains to the *V. tubiashii* species or to a new species.

Of the 31 parameters measured, only two majoritary traits differed (but inconsistently according to the used strains) between the other *V. tubiashii* related strains and our strains (acid production from citrate and cellobiose), clearly demonstrating the relatedness with the *V. tubiashii* species (Table 3). All our isolates were positive for cellobiose utilization while this character appeared variable for *V. tubiashii* strains (Hada et al., 1984). Conversely, citrate utilization is a variable characteristic of our isolates (7 positive / 10 tested strains).

Moreover, our strains were able to produce acid from melibiose, which is one of the specific characteristics of *V. tubiashii*.

In conclusion, with 69% DNA-DNA relatedness with *V. tubiashii* type strains, two phenotypic variables differences revealed by majoritary trait analysis and distinct grouping by MLST analysis, our isolates should be considered as *V. tubiashii* and not as a new species.

3.2. Pathogenicity assays

The pathogenicity of seven French isolates (07/038 2T2, 07/110 T1, 07/112 T1, 07/120 T1, 07/119 T1, 07/118 T2 and 04/002 1T2) was investigated by experimental infections that also included a non virulent strain, LMG20012T (Fig. 3). One year-old oysters were intra muscularly injected with these bacterial suspensions used at the same concentration. After 24 h, cumulative mortalities were comprised between 36.7% and 83.3% indicating that these seven isolates had different degrees of virulence. 07/110 T1, 07/038 2T2, 07/120 T1 and 07/118 T2 were the most virulent and caused more than 60% mortality after 24h. 07/112 T1, 07/119 T1 and 04/002 1T2 caused less than 60% mortality at 24 h but all the isolates induced more than 60% mortality after 3 days. In some moribund oysters, several predominant bacterial isolates were obtained in muscle and haemolymph tissues. All bacterial isolates were found to belong to *V. tubiashii* species using our *gyrB*-QPCR diagnosis assay.

In parallel, the toxicity of ECPs from 07/118 T2 produced on agar plates (LBSA) and in liquid medium (LBS) was tested by experimental infections. A mortality of 41% was observed two days after injecting oysters with ECPs produced on LBSA (Fig. 4A). However, no mortality was observed with ECPs produced in LBS. Similarly, no mortality of control oysters was observed during the experiments with Tris-HCl buffer or SASW.

3.3. Characterization of proteolytic activity of 07/118 T2 ECPs

The proteolytic activity of ECPs from 07/118 T2 (in liquid and solid medium) was severely reduced by metalloprotease inhibitor (1, 10-phenanthroline), with residual activities of 5% and 25% for ECPs produced on LBS and LBSA, respectively (Fig. 4). For the stability of ECPs produced on LBSA, 80% of the protease activities was maintained 48 hours post-incubation at 22°C (Fig. 4 B).

3.4. Development of a QPCR assay and hatchery- and field-samples screening

The gene encoding gyrase B (*gyrB*) in the 07/112 T2 and 07/118 T2 strains was sequenced (KF270490 and KF270491 respectively). After sequence alignments with the *V. tubiashii* type strain and closely related strains (*V. hepatarius, V. orientalis, V. brasiliensis, V. coralliilyticus, V. neptunius, V harveyi* and *V. xuii*), species-specific (*gyrB*) primers and probes were designed. The *gyrB* primers and probes were designed to amplify all *V. tubiashii* isolates.

The concentration of the primers and probes was optimized at 200 nM. Using these conditions, excellent real time PCR efficiencies were obtained by 10-fold dilutions of 07/118 T2 and *V. tubiashii* type strain DNAs, 99.2 % and 96.3 % respectively (Fig. S1). Finally, the specificity was investigated on different bacterial DNA extracts (Table 1). Among 20 tested strains including 7 type strains belonging to different *Vibrio* species, only all tested *V*.

tubiashii strains (n=13, including the type strain) were found positive. By considering Ct above cycle 38, our QPCR assay appeared to be specific and able to detect more than 10^3 bacteria/ml (Fig. S1).

Using this new diagnostic tool, the French LNR hatchery and field DNA collection were screened. These DNA were extracted from oysters tissues sampled in a context of declared mortalities analyzed by the REPAMO network. Of the 49 batches of animals (379 samples), only DNA from two batches were positive with the *V. tubiashii* diagnostic tool. These batches came from two different French hatcheries (2 positive batches from the 21 analysed) (Table S2). On the 28 batches of moribund animals from the field collection, all were negative for the presence of *V. tubiashii* DNA.

4. Discussion

The aims of the present study were (1) to fine-tune the taxonomic affiliation of virulent strains collected during an episode of high level *Crassostrea gigas* mortality in France using a polyphasic approach (phenotypic and genotypic characterization); (2) to evaluate both their virulence and the toxicity of their extracellular products; and (3) to investigate the geographical distribution of these bacteria.

The strains used in this study were isolated from an experimental hatchery/nursery in France in 2007, during a period of heavy oyster mortalities, under the auspices of the French institutional surveillance network, REPAMO.

Hatchery systems provide ideal conditions for the growth of larvae and bacteria multiplication (density, temperature, organic matter load, etc.) (Prado et al., 2005). Furthermore, farmed animals are monitored daily, allowing precise observations of mortality and investigations into pathogens. Hatcheries are therefore ideal places for the isolation of bacteria and the description of new bacterial species (Sugumar et al., 1998; Estes et al., 2004; Gay et al., 2004; Labreuche et al., 2006a; Elston et al., 2008).

For mollusks pathogens, the investigation of the pathogenic potential of a particular strain is still in its infancy due to the absence of animal models, cloned or knock-out animals for *in vivo* studies, or the availability of culture cell lines for *in vitro* assays. Furthermore, the virulence of *Vibrio* species is known to involve many virulence factors and different pathways (Shinoda and Miyoshi, 2011) and there is a lack of standardized *in vitro* protocols for virulence phenotype characterization.

In an *in vivo* assay, bacteria can be injected into animal muscle (injection (Gay et al., 2004)), put into seawater surrounding the animals after a culture phase (immersion or bath challenge (Travers et al., 2008; Segarra et al., 2010)), or transmitted by cohabitation challenges (De Decker and Saulnier, 2011). Even if they rely on the injection of large quantities of cultured bacteria, bypass the penetration step and consequently the first lines of defense, injection protocols are still the most reproducible and widely-used methodology for initial *Vibrio* challenges (Labreuche et al., 2006a; Gagnaire et al., 2007; Garnier et al., 2007; Williams et al., 2009). In this study, this protocol was applied to our isolated strains and allows the demonstration of the high degree of virulence of the strains into oyster tissues (between 36.7 and 83.3% of mortality as soon as 24 hours post-injection). This mortality rate is comparable to the one induced by other *Vibrio* species when used at same dose that the present study: *V. aestuarianus* (02/041) induced 62.5% of mortality eight days post-infection (Labreuche et al., 2006a) and the co-infection of a mix of two *Vibrio* (*V. splendidus* LGP 32 and *V.*

aestuarianus 02/041) gave a mean mortality rate of 88.9% five days post-infection (De Decker and Saulnier, 2011).

After isolating the bacteria and demonstrating their high level of virulence, a precise classification was needed. Initial identification of strains isolated in 2007, based on partial 16S rDNA analysis, led to the affiliation of these strains to the *V. harveyi* species (Saulnier et al., 2010). It is difficult to classify them as part of the *Vibrio* clades, and particularly the *V. harveyi* clade, based on 16S rDNA analysis. A reliable classification needs to be supported by sequence analyses of multiple housekeeping genes (Thompson et al., 2005). Here, this analysis with 16S rDNA, *ftsZ*, *topA*, *pyrH* and *recA* showed a tight grouping of 07/118 T2 with other French isolates and close to the *V. tubiashii* type strain, but clearly distinct from *V. tubiashii* group. This was confirmed by the DNA-DNA hybridization of two selected strains (04/002 1T2 and 07/118 T2) and phenotypic characterization based on 31 different parameters (salt, temperature and antibiotic sensitivity, sugar utilization, enzymatic activities). Although DNA-DNA hybridization with the *V. tubiashii* type strain was below 70% (69%), as biochemical assays do not allow a clear distinction between our isolates and the *V. tubiashii* strains, we propose to affiliate the 07/118 T2 (LMG 27884 = CECT 8426T) strain and other proximate isolates to a group belonging to the *V. tubiashii* species.

V. tubiashii, misclassified until 1984, was first implicated in bivalve mortalities on the east coast of the United States (Tubiash et al., 1965; Tubiash et al., 1970; Hada et al., 1984). In the eighties, in England, three virulent isolates of *V. tubiashii* were described that were pathogenic for *Crassostrea gigas* and *Ostrea edulis* larvae (Jeffries, 1982). More recently, in 2006 and 2007, some *V. tubiashii* strains were detected on the north American Pacific coast that caused a serious loss of hatchery production (Estes et al., 2004; Elston et al., 2008). The associated disease was characterized by a rapid appearance of symptoms resulting in reduced larval motility and necrosis in extensive soft tissue (Kothary et al., 2001).

It is important to note that our isolates are close but clearly separated to those from the USA. Interestingly, a strain isolated during a mortality episode of *Haliotis tuberculata* abalone (Normandy, 2004; 04/002 1T2 = LMG 27885 = CECT 8427) clusters with other french *V. tubiashii*. *V. tubiashii* has therefore been present in France since at least 2004. This strain can also induce high mortality in *C. gigas* oysters after intramuscular injection. In contrast to work on other *Vibrio* demonstrating a specific virulence for their natural hosts (Saulnier et al., 2000; Travers et al., 2009), one *V. tubiashii* virulent strain seems to affect both oysters and abalone, even if virulence has not yet experimentally demonstrated on abalone.

Vibrio tubiashii is a pathogen that is known to affect hatchery and nursery production and cause serious financial losses. Specific and sensitive detection tools are therefore necessary. A previously described, real time PCR assay targeting the *V. tubiashii vtpA* gene (Gharaibeh et al., 2009) was tested but we encountered specificity issues (data not shown). Taqman real time PCR based on the *dnaJ* sequence has also been used to detect and quantify *V. aestuarianus* (Saulnier et al., 2009). This technique is sensitive, rapid and reproducible, irrespective of the nature of the sample (seawater or oyster homogenate samples). In this paper, a taqman real time PCR diagnostic tool was developed to investigate the distribution of *V. tubiashii* in France and its potential implication in mollusk mortalities. Based on the hypothesis that a pathogen implicated in a mortality event is present in high amounts, a cut-off was fixed in our QPCR assay to ensure specificity rather than sensitivity. In spite of this, using these protocols we could detect as little as 10^3 bacteria/ ml, allowing a reliable detection of this pathogen.

The production of extracellular enzymes such as proteases by pathogenic *Vibrio* has been widely observed (Shinoda and Miyoshi, 2011). These proteases, which are often from the family of zinc metalloproteases, are known to be one of the most toxic factors to the host (Delston et al., 2003; Teo et al., 2003; Binesse et al., 2008; Hasegawa et al., 2008;

Labreuche et al., 2010) particularly for *V. tubiashii.* Hasegawa et al. showed that *V. tubiashii* 's ECPs were toxic to *C. gigas* larvae, and identified virulence factors in these ECPs: the metalloprotease *VtpA* and the hemolysin *VthA* (Hasegawa et al., 2008).

Besides the infection of oysters by french *V. tubiashii,* ECPs from the 07/118 T2 strain produced either from plate agar or from liquid medium were tested for toxicity on oysters by experimental injection.

The stability test showed that 80% of protease activity is maintained after 48 h pointing out its potential role in the infection process and the colonization step in particular since metalloproteases are known to degrade the extracellular matrix of a variety of host and tissues even if we cannot exclude the existence of other virulence factors also acting. Interestingly, only ECPs from culture on agar plates (LBSA) showed toxicity. However, differences in protease composition and quantity produced could be suggested, supported by the differential effect of a specific inhibitor such as 1,10-phenanthroline on residual protease activity. This type of difference in bacterial protein composition after culture in liquid or solid medium has been reported previously (Cheung and Fischetti, 1988; McCarter and Silverman, 1990). Culture on solid medium favors biofilm formation and microenvironments that can influence bacterial metabolite production (Cheung and Fischetti, 1988). Bacterial adherence is an important step in local multiplication and infection. Lorian proposed that *in vitro* systems providing a solid support surface for the growth of bacteria reflect more accurately *in vivo* conditions (Lorian, 1989). The toxicity of *V. tubiashii* ECPs produced only on agar plates may indicate the importance of adherence in *V. tubiashii* virulence factor production and in consequence in the infection cycle.

It should be noticed that protease activity was only characterized by testing azocasein as a non-specific substrate, as already reported in other studies (Delston et al., 2003; Teo et al., 2003; Labreuche et al., 2006b; Hasegawa et al., 2008), and this was completed using inhibitor assays. A more specific cocktail of inhibitors and substrates should be developed to obtain a more precise composition of the ECPs protease.

The French survey network allows the isolation of dominant strains from haemolymph or tissue samples, but also the conservation of bacteria in cryoprotectant as well as bacterial DNA. With the support of the LNR (National Reference Laboratory for mollusk disease), we were able to screen the different bacterial isolates and also the DNA library, thus avoiding the isolation biases (only the predominant strains isolated on TCBS at 20°C are conserved).

A *gyrB* gene was the target of the screening and primers were designed to amplify DNA from all strains belonging to *V. tubiashii* species. Using this approach, we detected *V. tubiashii* DNA in a strain isolated during 2 episodes of hatchery mortality in 2008. This strain was also isolated in 10 hatchery batches affected by mortalities, 9 from 2007 and 1 from 2004. In conclusion, we have evidence of the presence of high amounts of *V. tubiashii* during 12 episodes of mortality in French hatcheries since 2004. None of the field samples tested revealed for instance the presence of such high amounts of *V. tubiashii*.

5. Conclusions

Based on their biochemical and genetic characterization, nine predominant bacterial strains, isolated from a French experimental hatchery/nursery during mortalities events, were classified as a novel group belonging to *V. tubiashii* species*.* Two reference strains, isolated from diseased abalone and diseased oysters in 2004 and 2007, respectively, were described for this group. The significant pathogenicity of different isolates and the toxicity of the extracellular products of 07/118 T2 were confirmed by experimental challenge. Further

studies will be required to complete our knowledge on the pathogenic factors secreted by these strains of *V. tubiashii* and their mechanisms of action.

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Figures

Figure 1: Neighbor-joining phylogenetic tree showing the phylogenetic position of *V. tubiashii* strains, based on concatenated *16S rDNA*, *ftsZ*, *pyrH*, *recA* and *topA* gene sequences (2307 bp). Phylogenetic analyses were conducted in MEGA4. The optimal tree with the sum of branch length = 2.23671430 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Nodes without a number are of low significance (<50%).

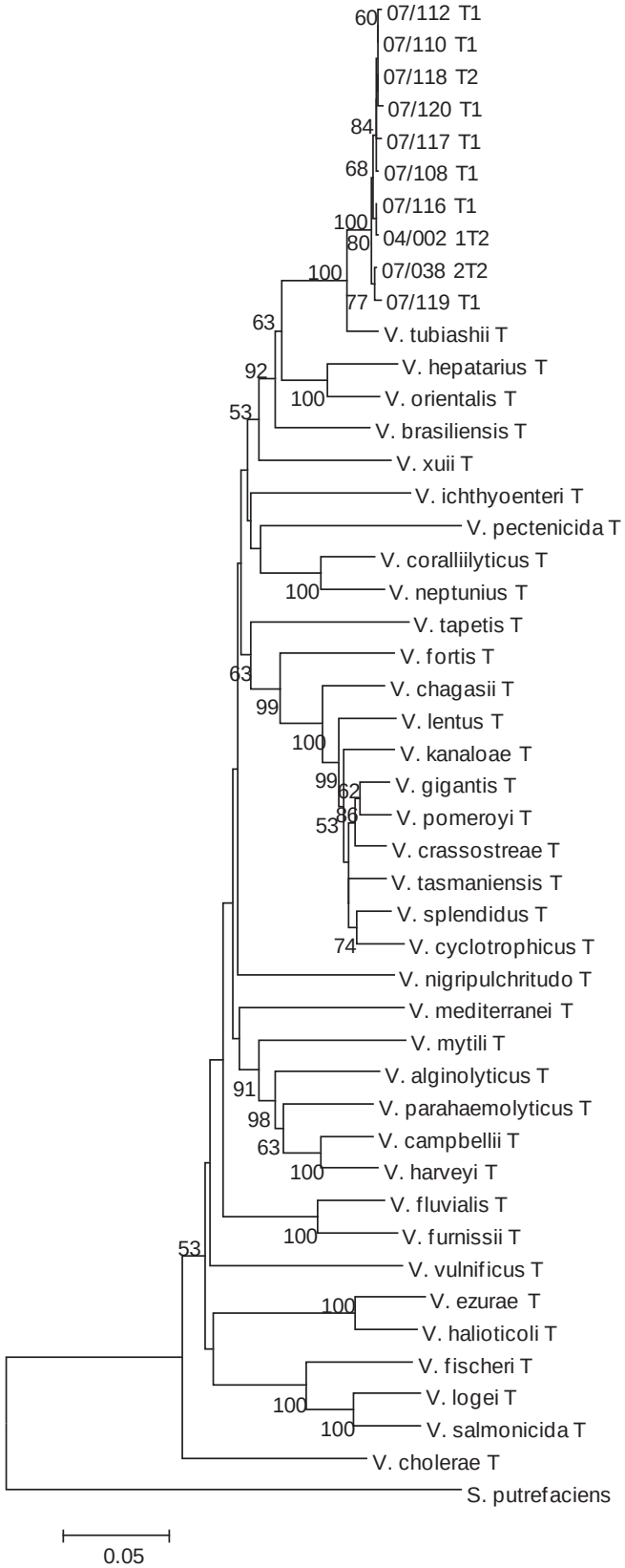


Figure 2: Neighbor-joining phylogenetic tree showing the phylogenetic position of *V. tubiashii* strains, based on *recA* sequences (357 bp). Phylogenetic analyses were conducted in MEGA4. The optimal tree with the sum of branch length = 1.103893013 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Nodes without a number are of low significance (<50%).

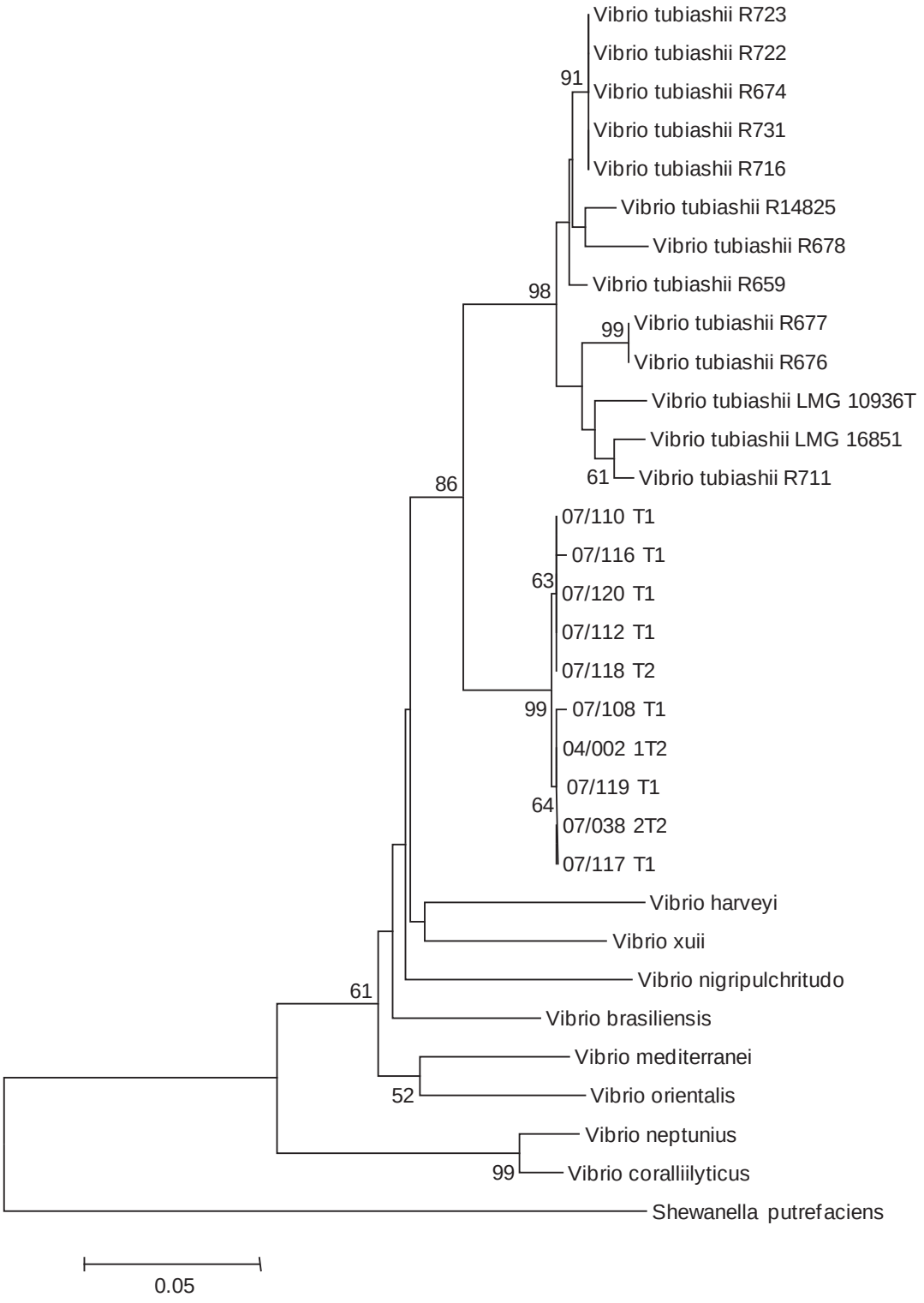


Figure 3: Cumulative mortalities recorded during experimental infections performed on adult oysters. Bacterial strains were grown for 24 h in Zobell broth before resuspension into artificial and sterile seawater (SASW) and DO_{600} adjustment to 1. 100µl of bacterial suspension were injected into anesthetized oysters. The control corresponds to the injection in similar conditions of sterile SASW (▲) or injection of non virulent *V. tasmaniensis* LMG20012 (×) adjusted to DO_{600}=1. Seven strains of *V. tubiashii* were tested (07/038 2T2, 07/110 T1, 07/112 T1, 07/118 T2, 07/119 T1, 07/120 T1 and 04/002 1T2). Standard deviations are represented by bars (n=3 biological replicates).

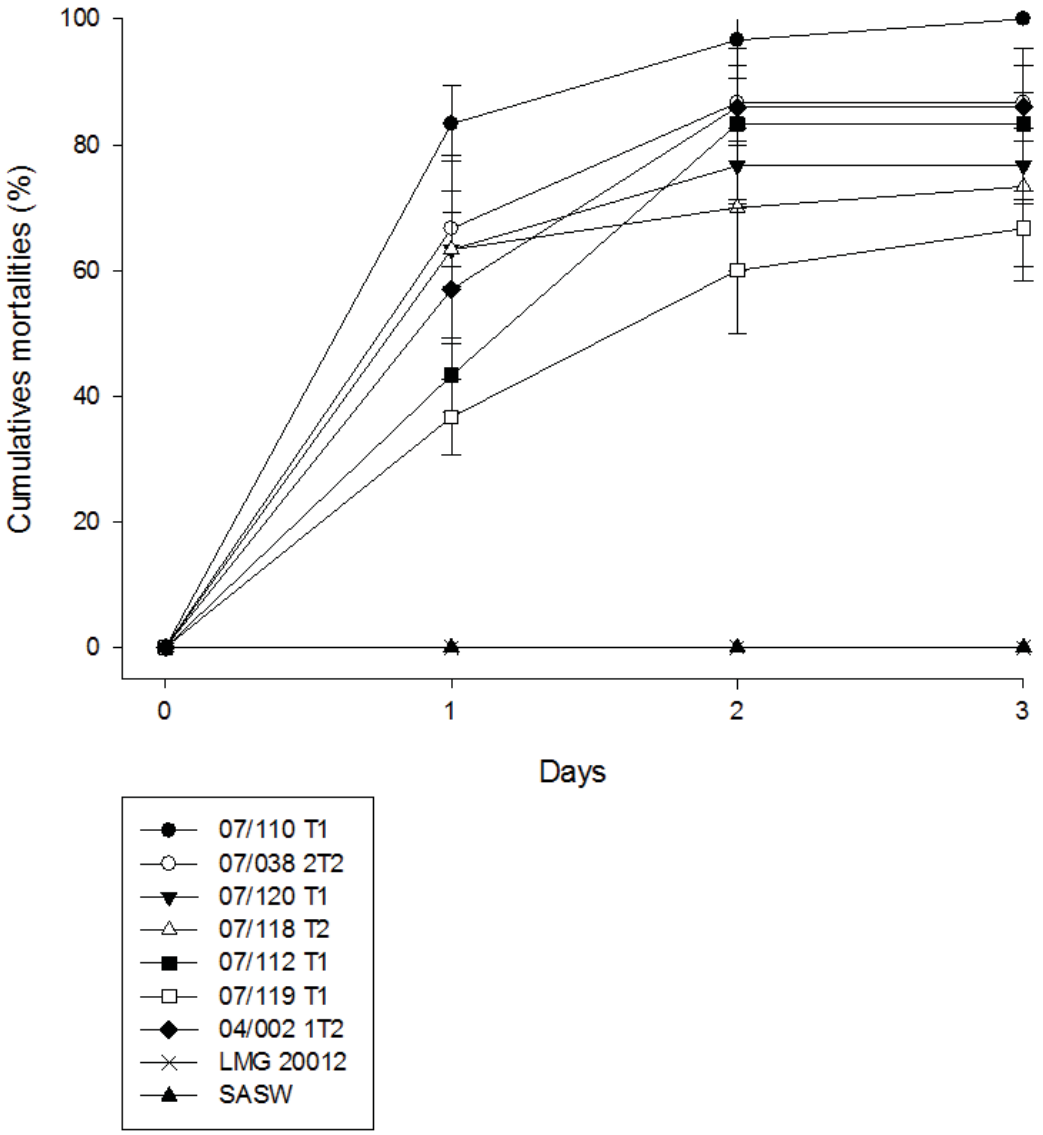


Figure 4: (**A) Cumulative mortalities of juvenile oysters following intramuscular injection of *V. tubiashii* (07/118 T2= LMG 27884= CECT 8426) ECPs produced on LBSA and LBS medium and *in vitro* stability of protease activity (azocaseinase activity).**

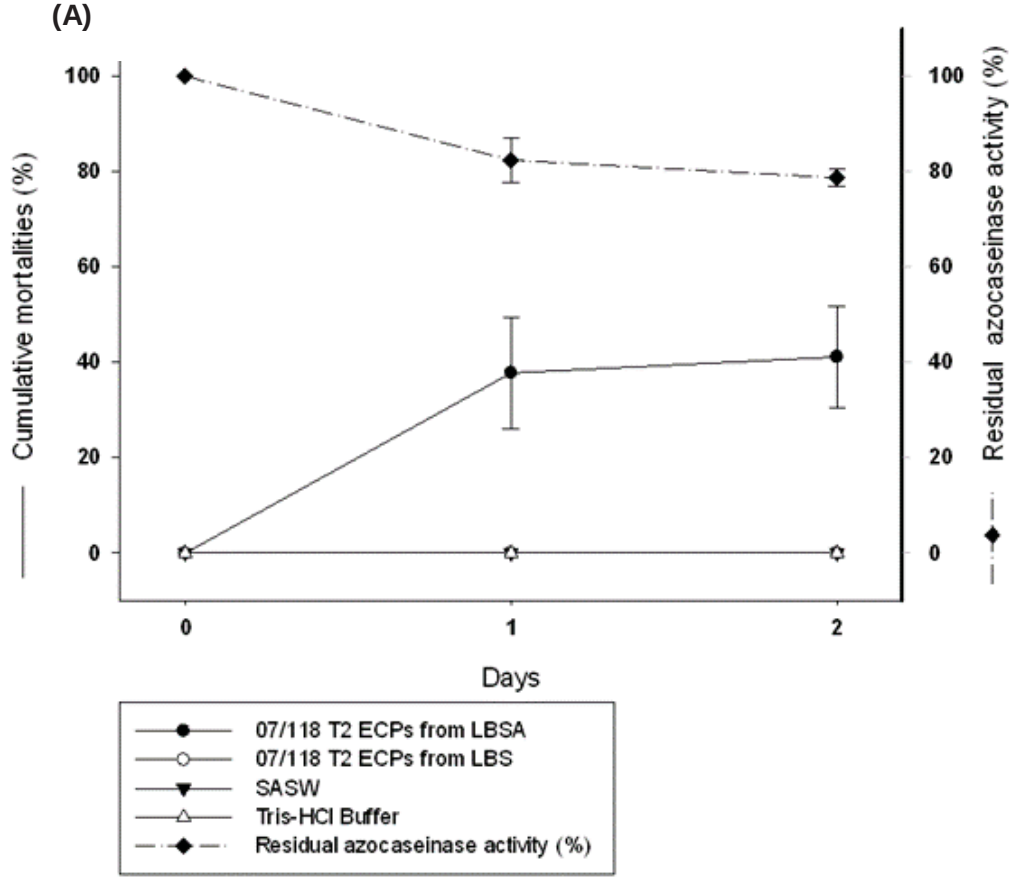
The primary axis corresponds to cumulative mortalities recorded during experimental infections performed on juvenile oysters after injection of 07/118 T2 ECPs produced in LBS and on LBSA medium (solid curves). 100 µl of ECPs (5µg g^{-1} of proteins) were injected into anesthetized oysters. The control corresponds to the injection in similar condition of artificial and sterile seawater (SASW) and Tris- HCl buffer (50 mM pH 8).

The secondary axis corresponds to the stability of protease activity measured *in vitro* (dashed line curve). 100 µl of 07/118T2 ECPs produced on LBSA were incubated at 22°C for two days. The azocaseinase activity (after 24 h and 48 h) was measured using an azocasein assay. Standard deviations are represented by bars (n=2 biological replicates).

(B) Effect of one metalloprotease inhibitor on azocaseinase activity of 07/118 T2 ECPs produced on LBSA and LBS medium.

[a] Final concentration of the inhibitor in the assay mixture.

(A)



(B)

	07/118 T2 ECPs produced in LBS	07/118 T2 ECPs produced on LBSA
% of residual activity after preincubation with 1,10 phenanthroline (10mM) [a]	5.06 ± 0.5	25.8 ± 5.8

Table 1: Bacterial strains used and their sources, as well as QPCR *gyrB* assays for the different *Vibrio* species. QPCR were performed twice on 5 µl of boiled bacteria or on 25 ng of genomic DNA.

Strains	References	Source and date of isolation	QPCR results (+/-)
04/002 1T2	(Saulnier et al., 2010)	Diseased spat abalone (*Haliotis tuberculata*), Cotentin, France, January 2004	+
07/038 2T2	(Saulnier et al., 2010)	Diseased diploid spat oysters, (*Crassostrea gigas*), Ronce les Bains, France, June 2007	+
07/108 T1	Present study	Diseased diploid spat oysters, (*Crassostrea gigas*), Ronce les Bains, France, August 2007	+
07/110 T1	(Saulnier et al., 2010)	Diseased diploid spat oysters, (*Crassostrea gigas*), Ronce les Bains, France, August 2007	+
07/112 T1	Present study	Diseased diploid spat oysters, (*Crassostrea gigas*), Ronce les Bains, France, August 2007	+
07/116 T1	Present study	Diseased triploid spat oysters, (*Crassostrea gigas*), Ronce les Bains, France, August 2007	+
07/117 T1	(Saulnier et al., 2010)	Diseased triploid spat oysters, (*Crassostrea gigas*), Ronce les Bains, France, August 2007	+
07/118 T2	Present study	Diseased diploid spat oysters, (*Crassostrea gigas*), Ronce les Bains, France, August 2007	+
07/119 T1	(Saulnier et al., 2010)	Diseased diploid spat oysters, (*Crassostrea gigas*), Ronce les Bains, France, August 2007	+
07/120 T1	Present study	Diseased diploid spat oysters, (*Crassostrea gigas*), Ronce les Bains, France, August 2007	+
V. tubiashii			
LMG 10936T		Hardclam (*Mercenaria mercenaria*), moribund larvae, Milford, USA	+
LMG 11229		Moribund juvenile oyster (*Crassostrea virginica*), Milford, USA	+
LMG 16851		Sea bream , Greece	+
V. brasiliensis			
LMG 20546T		Bivalve larvae (*Nodipecten nodosus*), Brazil	-
V. coralliilyticus			
DSMZ 19607T		Diseased coral (*Pocillopora damicornis*),Indian Ocean near Zanzibar	-
V. hepatarius			
LMG 20362T		*Litopenaeus vannamei*, Manglaralto, Ecuador	-
V. neptunius			
LMG 20536T		Bivalve larvae (*Nodipecten nodosus*), Brazil	-
V. nereis			
LMG 3895T		Seawater enriched with propanol, Hawaii, United States	-
V. orientalis			
LMG 7897T		Seawater, Yello sea, China	-
V. tasmaniensis			

LMG 20012T	Atlantic salmon (*Salmosalar*), Tasmania Australia	ND
V. xuii		
DSMZ 17185T	Shrimp culture water, China	-

ND: not done

Table 2: DNA-DNA hybridizations
The DNA-DNA relatedness percentages are the mean of minimum 6 hybridization assays. The values given between brackets are the differences between the reciprocal values.

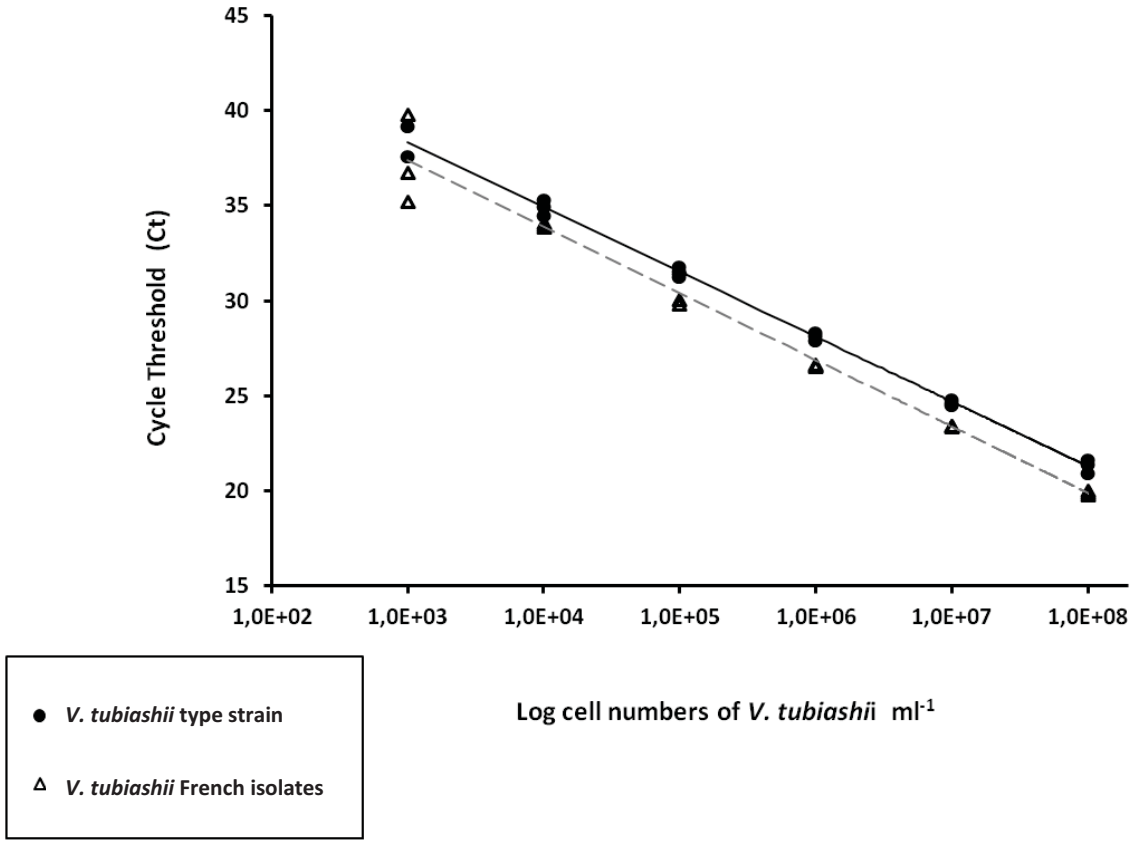
	Vibrio sp. 04/002 1T2 LMG 27885	*Vibrio* sp. 07/118 T2 LMG 27884	*V. tubiashii* LMG 10936T	*V. hepatarius* LMG 20362T
Vibrio sp. 04/002 1T2 LMG 27885	100			
Vibrio sp. 07/118 T2 LMG 27884	86 (11)	100		
V. tubiashii LMG 10936T	69 (12)	69 (13)	100	
V. hepatarius LMG 20362T	21 (4)	22 (3)	24 (10)	100

Table 3: Phenotypic characteristics of *Vibrio* sp. isolated from *C. gigas*

Traits were determined using API20E, API20NE and API50CH, and standard growth assays under various culture conditions (NaCl, temperature, and presence of an antibiotic). Symbols are: +: positive; -: negative. For the ten French isolates of *V. tubiashii* tested (column 1) and for the three *V. tubiashii* (column 2: LMG 10936T, LMG 11229 and LMG 16851), dominant traits are indicated and traits which differ among isolates are given in parentheses.

UB30: Flumequine 30 µg, E30: Erythromycin 30 µg, C30: Chloramphenicol 30 µg, CN30: Gentamicin 30 µg, K30: Kanamycin 30 µg, TE30: Tetracycline 30 µg, SXT25: Sulphamethoxazole/trimethoprim 19:1 25 µg, S25: Streptomycin 25 µg, S3: Sulphonamides 300 µg, W5: Trimethoprim 5 µg, O129/150 and O129/10: *vibrio* static agent, pteridine 150 µg and 10 µg, respectively, P10: Penicillin 10 UI (6 µg), AMP10: Ampicillin 10 µg

	Vibrio sp. = *V. tubiashii*	*V. tubiashii*	*V. hepatarius* LMG 20362T	*V. xuii* DSMZ 17185T	*V. neptunius* LMG 20536T	*V. orientalis* LMG 7897T	*V. brasiliensis* DSMZ 17184T	*V. nereis* LMG 3895T	*V. coralliilyticus* DSMZ 19607T
Growth in NaCl at									
8% (w/v)	- (+)	-	+	+	+	-	+	+	+
10% (w/v)	-	-	-	+	-	-	-	+	-
Growth at									
40°C	-	-	-	+	-	-	+	+	+
Antibiotic sensitivity									
UB30	+(-)	+	+	+	+	+	+	+	+
E30, C30, CN30	+	+	+	+	+	+	+	+	+
K30, TE30, SXT25, S25, S3, W5, O129/150, O129/10	+	+	+	-	+	+	+	+	+
P10, AMP10	+	+	+	+	-	+	+	+	-
Acid production from									
L-arabinose	-	-	-	+	-	-	-	-	-
D-ribose	+	+	+	+	+	+	+	+	-
Mannitol	+	+	+	+	-	+	+	-	+
Salicin	- (+)	-	-	-	-	-	+	-	-
Cellobiose	+	- (+)	+	+	-	+	+	-	-
Melibiose	+	+	-	-	-	-	-	-	-
Citrate	+(-)	-	+	+	+	-	+	-	+
Presence of									
β-galactosidase (ONPG)	+	+	+	-	-	+	+	-	+
Arginine Dihydrolase	+	+(-)	+	+	-	-	+	+	-
Lysine Decarboxylase	-	-	+	-	-	+	-	-	-
Gelatinase	+	+	+	-	+	+	+	-	+
Galactosidase	+	+	+	-	-	+	+	-	+
Production of									
Indole	+	+	+	+	-	+	+	+	+
Nitrate	+	+	+	+	-	+	+	+	+



Supplementary Fig. S1. QPCR *gyrB* diagnostic tool
Standard curves for *V. tubiashii* type strain and French isolates using QPCR in 10 fold dilutions of genomic DNA. Standard curves were generated by plotting the threshold cycle (Ct) versus the log cell number of bacteria value. Each plot corresponds to the individual values of Ct obtained from a representative experiment of all experiments performed independently. The linear regression represented by the solid line, was obtained by collectively treating all the data of *V. tubiashii* with an r^2 of 0.996 and a PCR reaction efficacy of 96.3%. The linear regression represented by the dotted line was obtained by collectively treating all the data of *V. tubiashii* with an r^2 of 0.988 and a PCR reaction efficacy of 99.2%.

Supplementary Table 1: Primers used for DNA sequencing and QPCR development

Gene	Primers	Sequence	Annealing temperature	Amplicon size (bp)	Application
16s rRNA	SAdir S17rev	AGAGTTTGATCATGGCTCAGA GTTACCTTGTTACGACTT	50 °C	1 500	PCR amplification and sequencing (Thompson et al., 2007)
ftsZ	VftsZ75F VftsZ800R	GCTGTTGAACACATGGTACG GCACCAGCAAGATCGATATC	50 °C	750	
pyrH	VpyrH80F VpyrH530R	GATCGTATGGCTCAAGAAG TAGGCATTTTGTGGTCACG	50°C	450	
topA	VtopA400F VtopA1200R	GAGATCATCGGTGGTGATG GAAGGACGAATCGCTTCGTG	50°C	800	
recA	VrecA130F VrecA720R	GTCTACCAATGGGTCGTATC GCCATTGTAGCTGTACCAAG	50°C	600	
gyrB	VgyrB274F VgyrB1171R	GAAGTTATCATGACGGTACTTC CCTTTACGACGAGTCATTTC	55°C	900	
gyrB	gyrB94F gyrB296R gyrBprobe203	TTGGTGAATCTGAGCAAACG CGCTTGGATACCACCTTCAT TCTGGCCAAGTGAAGAGACA (5'Rox - 3'BHQ-2)	60°C	202	QPCR diagnostic (this study)

Supplementary Table 2: QPCR diagnostics (detection of *V. tubiashii* with *gyrB* taqman assay) performed on DNA extracts from oyster tissues sampled during mortality events (REPAMO network). QPCR were performed on 25 ng of total DNA and considered as positive when Ct values were greater than 36. Batch names (sampling year / sampling number) and origin are indicated in the first columns.

Batch	Origin	Number of positive samples (*V. tubiashii*)
2008/011	Hatchery/nursery	0/2
2008/062	Hatchery/nursery	0/20
2008/082	Hatchery/nursery	0/4
2008/104	Hatchery/nursery	1/1
2008/105	Hatchery/nursery	0/5
2008/110	Hatchery/nursery	0/5
2008/132	Hatchery/nursery	1/6
2008/139	Hatchery/nursery	0/6
2008/165	Hatchery/nursery	0/3
2008/166	Hatchery/nursery	0/3
2009/051	Hatchery/nursery	0/3
2009/052	Hatchery/nursery	0/3
2009/066	Hatchery/nursery	0/6
2009/067	Hatchery/nursery	0/2
2009/109	Hatchery/nursery	0/11
2009/143	Hatchery/nursery	0/2
2010/080	Hatchery/nursery	0/1
2010/125	Hatchery/nursery	0/2
2011/009	Hatchery/nursery	0/4
2011/010	Hatchery/nursery	0/4
2011/176	Hatchery/nursery	0/6
2008/076	Field – juveniles 3n	0/10
2008/077	Field – juveniles 3n	0/10
2008/075	Field – juveniles 3n	0/10
2008/102	Field – adults 2n	0/10
2008/080	Field – spat 3n	0/10
2008/081	Field – juveniles 2n	0/10
2008/090	Field – juveniles 3n	0/10
2008/092	Field – spat 2n	0/10
2008/093	Field – spat 3n	0/10

2008/098	Field – juveniles 2n	0/10
2008/097	Field – juveniles 2n	0/10
2008/085	Field – spat 3n	0/10
2008/086	Field – juveniles 2n	0/10
2008/087	Field – spat 3n	0/10
2008/088	Field – spat 2n	0/10
2008/096	Field – juveniles 3n	0/10
2008/089	Field – spat 2n	0/10
2008/091	Field – juveniles 3n	0/10
2008/074	Field – juveniles 3n	0/10
2008/079	Field – spat 2n	0/10
2008/083	Field – juveniles 2n	0/10
2008/084	Field – spat 2n	0/10
2008/108	Field – spat 2n	0/10
2012/080	Field - adults	0/10
2012/112	Field - adults	0/10
2012/067	Field - adults	0/10
2012/099	Field - adults	0/10
2012/114	Field - adults	0/10

Supplementary Table 3: Genbank accession numbers

	16s rDNA	ftsZ	pyrH	topA	recA	gyrB
V. alginolyticus LMG 4409T	X74690	EF027344	JN408273	DQ907472	AJ842373	
V. brasiliensis LMG20546T	AJ316172	DQ907335	HQ452398	DQ907473	HM771379	HM771364
V. campbelli LMG11216T	X56575	DQ907337	EF596641	DQ907475	EF596670	
V. chagasii LMG21353T	AJ316199	DQ996590	EU118252	DQ481649	AJ580874	
V. cholerae CECT 514T	X74695	HE805627	FM202582	HE805631	FM204835	
V. coralliilyticus LMG20984T	AJ440005	DQ907341	GU266292	EF114213	JN039156	AB298210
V. crassostreae LMG22240T	AJ582808	DQ481624	EU871948	DQ481650	EU541594	
V. cyclitrophicus LMG21359T	U57919	DQ481625	EU871958	DQ481651	AJ842405	
V. ezurae LMG19970T	AY426980	DQ907343	EU871949	DQ907481	AJ842413	
V. fisheri LMG4414T	X70640	DQ907344	EU185914	DQ907482	AJ842417	
V. fluvialis LMG7894T	X74703	DQ907345	JN426808	DQ907483	AJ580892	
*V. fortis*LMG 21557T	AJ514916	DQ907346	EU251629	DQ907484	AJ842422	
V. furnisii LMG 7910T	X74704	EF027345	JF316672	DQ907485	AJ842427	
V. gigantis LMG22741T	AJ582810	DQ481629	EU871951	DQ481655	EU541593	
V. halioticoli LMG18542T	AB000390	DQ907349	EU871952	DQ907487	AJ842430	
V. harveyi LMG4044T	X74706	DQ907350	EU118238	DQ907488	EF596430	EF596215
V. hepatarius LMG20362T	AJ345063	DQ907352	JF316674	DQ907491	AJ842444	AB298222
V. ichthyoenteri LMG19664T	AJ437192	DQ907354	HM771375	DQ907493	AJ842446	
V. kanaloae LMG20539T	AJ316193	DQ481631	FN908851	DQ481657	AJ842450	
V. lentus LMG21034T	AM162659	DQ914234	EU871959	DQ914237	AJ842452	
V. logei LMG21012T	AJ437616	DQ907355	EF380234	DQ907494	AJ842457	
V. mediterranei LMG11258T	HM771351	DQ907356	GU266288	DQ907495	AJ842459	
V. mytili LMG19157T	X99761	DQ907358	GU266287	DQ907499	AJ842472	
V. neptunius LMG20536T	AJ316171	DQ907361	GU266291	DQ907503	AJ842478	AB298234
V. nigripulchritudo LMG3896T	X74717	EF027347	GU266290	DQ907505	AJ842480	
V. orientalis LMG7897T	X74719	DQ907365	EU118243	DQ907507	AJ842485	EF380260
V. parahaemolyticus LMG2850T	X74720	DQ907367	GU266286	DQ907509	AJ842490	
V. pectenicida LMG19642T	Y13830	DQ907368	JN039143	DQ907510	AJ842491	
V. pomeroyi LMG20357T	AJ491290	DQ481634	EU871960	DQ481660	AJ842497	
V. salmonicida NCIMB2262T	X70643	DQ907375	EU118245	DQ907517	EF380243	
V. splendidus LMG19031T	X74724	DQ481635	EU118241	DQ481661	AJ842511	
V. tapetis LMG19706T	Y08430	DQ907379	HE795189	DQ907520	AJ842514	
V. tasmaniensis LMG20012T	AJ514912	DQ481636	EU871961	DQ481662	AJ842515	
V. tubiashii LMG10936T	X74725	DQ907381	JF316670	DQ907521	AJ842518	
V. vulnificus LMG13545T	X76333	DQ907382	GQ382226	DQ907522	AJ580890	
V. xuii LMG21346T	AJ316181	DQ907384	GU266284	DQ907524	AJ842529	AB298254

S. putrefaciens CN-32	AF136269	NC_009438 (493799-494392)	NC_009438 (1531099-1531836)	NC_009438 (2590448-2593090)	NC_009438 (3182123-3183196)	
Vibrio sp = V. tubiashii						
04/002 1T2 (=LMG 27884 = CECT 8426)	KF270470	KF270480	KF270492	KF270460	KF270502	
07/038 T1	KF270471	KF270481	KF270493	KF270461	KF270503	
07/108 T1	KF270472	KF270482	KF270494	KF270462	KF270504	
07/110 T1	KF270473	KF270483	KF270495	KF270463	KF270505	
07/112 T1	KF270474	KF270484	KF270496	KF270464	KF270506	KF270490
07/116 T1	KF270475	KF270485	KF270497	KF270465	KF270507	
07/117 T1	KF270476	KF270486	KF270498	KF270466	KF270508	
07/118 T2 (=LMG 27884 = CECT 8426)	KF270477	KF270487	KF270499	KF270467	KF270509	KF270491
07/119 T1	KF270478	KF270488	KF270500	KF270468	KF270510	
07/120 T1	KF270479	KF270489	KF270501	KF270469	KF270511	
V. tubiashii						
LMG16851					AJ842520	
R659					EU717015	
R674					EU717030	
R676					EU717031	
R677					EU717032	
R678					EU717033	
R711					EU717060	
R716					EU717064	
R722					EU717068	
R723					EU717069	
R731					EU717073	
R14825					AJ842522	

S. putrefaciens CN-32	AF136269	NC_009438 (493799-494392)	NC_009438 (1531099-1531836)	NC_009438 (2590448-2593090)	NC_009438 (3182123-3183196)	
Vibrio sp = V. tubiashii						
04/002 1T2 (=LMG 27884 = CECT 8426)	KF270470	KF270480	KF270492	KF270460	KF270502	
07/038 T1	KF270471	KF270481	KF270493	KF270461	KF270503	
07/108 T1	KF270472	KF270482	KF270494	KF270462	KF270504	
07/110 T1	KF270473	KF270483	KF270495	KF270463	KF270505	
07/112 T1	KF270474	KF270484	KF270496	KF270464	KF270506	KF270490
07/116 T1	KF270475	KF270485	KF270497	KF270465	KF270507	
07/117 T1	KF270476	KF270486	KF270498	KF270466	KF270508	
07/118 T2 (=LMG 27884 = CECT 8426)	KF270477	KF270487	KF270499	KF270467	KF270509	KF270491
07/119 T1	KF270478	KF270488	KF270500	KF270468	KF270510	
07/120 T1	KF270479	KF270489	KF270501	KF270469	KF270511	
V. tubiashii						
LMG16851					AJ842520	
R659					EU717015	
R674					EU717030	
R676					EU717031	
R677					EU717032	
R678					EU717033	
R711					EU717060	
R716					EU717064	
R722					EU717068	
R723					EU717069	
R731					EU717073	
R14825					AJ842522	