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A Large-Scale Epidemiological Study to Identify Bacteria Pathogenic to Pacific Oyster *Crassostrea gigas* and Correlation Between Virulence and Metalloprotease-like Activity

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Abstract A 4-year bacteriological survey (2003-2007) of four molluscs cultivated in France and faced with mortality episodes was performed by the French shellfish pathology network. The more abundant bacteria isolated during 92 mortality episodes, occurring mainly in Pacific oyster *Crassostrea gigas*, were identified by genotyping methods. It allowed us both to confirm the representativeness of *Vibrio splendidus* and *Vibrio aestuarianus* bacterial strains and to identify both a large number of *Vibrio harveyi*-related strains mainly detected during 2007 oyster mortality outbreaks and to a lesser extent bacterial strains identified as *Shewanella colwelliana*. Because metalloprotease has been reported to constitute a virulence factor in a few *Vibrio* strains pathogenic for *C. gigas*, several bacterial strains isolated in this study were screened to evaluate their pathogenicity in *C. gigas* spat by experimental infection and their ability to produce metalloprotease-like activity in the culture supernatant fluids. A high level (84%) of concordant results between azocaseinase activities and virulence of strains was obtained in this study. Because bacterial metalloprotease activities appeared as a common feature of pathogenic bacteria strains associated with mortality events of *C. gigas* reared in France, this phenotypic test could be useful for the evaluation of virulence in bacterial strains associated with such mortality episodes.

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Introduction

Vibrio are ubiquitous marine bacteria that represent a major source of concern in aquaculture due to the increasing number of strains and species found to be pathogenic for fish and shellfish. Such pathogens can cause severe production losses in the industry. The main aquaculture activity in France is the rearing of Pacific oysters *Crassostrea gigas*, but it has recurrently suffered large-scale summer mortality phenomena over the last 15 years [1]. In particular, *V. aestuarianus* [2, 3] and *V. splendidus* [4, 5] have been reported to be associated with summer mortality of oyster spat reared in open sea tidal areas and, to a lesser extent, in older animals [6]. Some bacterial isolates related to these *Vibrio* species were demonstrated to be pathogenic to *C. gigas* under experimental conditions [3, 5, 7]. Despite these findings, the aetiology of summer mortalities seems to be multifactorial, arising as a result of complex interactions between the physiological and/or genetic status of the host, the environment and known or as yet unidentified pathogen/s [7, 8]. Indeed, another infective agent—a herpes-like virus—has also been associated with mortality episodes, although not systematically [8]. Besides *V. aestuarianus* and *V. splendidus*, another bacterial species not yet reported in France, *V. tubiashii*, has been found to be responsible for a serious disease in North American hatchery-reared *Crassostrea virginica* oysters [9] and *C. gigas* [10, 11].

Various extracellular proteases that play an important role in the pathogenesis of bacterial infections have been described in *Vibrio* isolated from seawater, fish and shellfish [12–16]. Proteases may be involved in tissue invasion and destruction, host defence evasion and its modulation [17]. Many proteases belong to the metalloprotease family [18]. Those produced by human patho-

genic bacteria, including *V. cholerae* and *V. vulnificus*, have been studied extensively, evidencing a broad range of pathological actions [19]. Metalloproteases have been also described in some *V. tubiashii*, *V. aestuarianus* and *V. splendidus* strains pathogenic to Pacific oyster *C. gigas* [20–22]. It has been recently shown by mutagenesis that those produced by two strains related to *V. tubiashii* and *V. splendidus* are an essential determinant of lethality when extracellular products are injected into *C. gigas* oysters [9, 21, 23, 24].

In previous studies, *V. splendidus* and *V. aestuarianus* strains associated with mortality episodes of cultivated *C. gigas* oysters in France were isolated in (1) sentinel animals that had been kept in experimental cohabitation in an aquarium with presumed diseased animals collected from areas where mortality episodes were noted [25], or (2) in temporally and spatially limited mortality outbreaks [3, 4]. The first aim of the present study was to increase the number of identified bacteria associated with mortality episodes, mainly in cultivated Pacific oysters but also in three other mollusc species cultivated in France. This was done during a 4-year bacteriological survey, allowing us to evaluate the representativeness of *V. splendidus* and *V. aestuarianus* bacterial strains. The second aim of the present study was to screen several of the bacterial strains found to be more frequently associated with mollusc disease outbreaks, to evaluate both their pathogenicity in *C. gigas* spat by experimental infection and their ability to produce metalloprotease-like activity in culture supernatant fluids. Results from these investigations allowed us to correlate metalloprotease activity and virulence capacity.

Materials and Methods

Sampling and Bacterial Isolation

Abnormal mortality in mollusc aquaculture is defined by European Directive 95/70/EC as a sudden mortality affecting approximately 15% of stocks and occurring over a short period between two inspections (within 15 days). In practice, the study of abnormal mortality in France is the subject of passive monitoring: mollusc mortality cases are reported by farmers to the competent authority and the French network of shellfish pathology, known as REPAMO, records and performs analyses on the animals. Mortality rate is estimated by counting a proportion of live and dead animals in three randomly chosen bags per oyster lease, three randomly chosen tanks in hatcheries-nurseries or in three different squares for clam and scallop beds.

Over 4 years of zoosanitary monitoring performed by the REPAMO network (2003, 2004, 2005 and 2007), 92 batches of animal samples were collected from abnormal mortality episodes affecting four cultivated mollusc species. Samples were mainly Pacific oysters *C. gigas* (80% of samples), but also scallops *Pecten maximus* (3%), clams *Ruditapes philippinarum* (5%) and abalone *Haliotis tuberculata* (12%). The affected animals mainly came from private farms, in 36 different locations along the Atlantic, English Channel or Mediterranean coasts of France; 47% of oyster samples came from two IFREMER experimental facilities. Animals presumed to be diseased were shipped at 4°C by express courier (<24 h) to the IFREMER pathology laboratory. Bacterial isolations were only made from animals that were still alive on arrival. Generally, two apparently healthy animals and at least three diseased animals exhibiting a weakness in adductor muscle tonicity were examined and analysed by bacteriological methods. In order to prevent any risk of bacterial contaminations from diseased to apparently healthy animals, each group of animals was shipped in a different plastic bag. Depending on mollusc species and 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 corresponded either to an all-adult or all-juvenile age class. Haemolymph was withdrawn into a syringe from the adductor muscle or pericardial cavity of oysters and from the pericardial cavity of abalones, using a 23-gauge needle. Tissues from scallop and clams were homogenized in 100 µl Sterile Artificial Sea Water diluent (SASW 2.3% NaCl, 20 mM KCl, 5 mM $MgSO_4$ and 2 mM $CaCl_2$) with a sterile pellet-pestle (Sigma) for 1 min 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.

Tissue samples diluted in SASW (10 to ×1,000) were spread on thiosulfate-citrate-bile salts-sucrose (TCBS, Difco) agar in Petri dishes. After incubation of plates at 20°C for at least 4 days, each macroscopically different bacterial isolate found in abundance (more than 10^4 CFU/ml) was re-plated on nutrient Petri dishes to check for purity. Each isolate was identified by eight figures, the first two indicating the year of batch reception, the next three indicating the batch number and the last ones giving the sample tissue number followed by the colony type (T) number.

Genotyping

Bacterial isolates were cultured overnight at 20°C in marine broth (Difco), and 65 µl of bacterial suspension were deposited on FTA® paper matrix cards according to the

manufacturer's instructions (Whatman) for nucleic acids extraction and storage. The 16S rRNA and *gyr*B genes were amplified using universal bacterial primer pairs SAdir (5′-AGAGTTTGATCATGGCTCAGA-3′), S17rev (5′-GTTAC CTTGTTACGACTT-3′) and Up1E (5′-GAAGTCATCAT GACCGTTCTGCAYGCNGGNGGNAARTTYRA-3′), UP2R (5′-AGCAGGGTACGGATGTGCGAGCCRTC NACRTCNGCRTCNGYCAT-3′), following methods described by Lambert et al. [26] and Yamamoto and Harayama [27], respectively. The 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), 0.75 μM forward primer using SAdir for the 16S rRNA sequencing reaction and Up1S 5′-GAAGTCATCATGACCGTTCTGCA-3' for *gyr*B sequencing. Cycle sequencing reactions were performed using a Gene Amp PCR System 2700 (Applied Biosystems) following manufacturer's instructions. Separation of the DNA fragments was carried out in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Sequences were then compared against those of the type strains of validly published species using Eztaxon [28]. Sequences from bacterial isolates and closed type strains were aligned with BioEdit [29] and phylogenetic trees were built using Mega4 [30] and the neighbour-joining method with the Kimura's two-parameter model. Reliability of topologies was assessed by the bootstrap method [31] with 1,000 replicates.

Metalloprotease-like Activity in Culture Supernatant Fluids

Proteolytic activity in culture supernatant fluids (CSF) was determined using azocasein (Sigma Chemical Co., St. Louis, MO) as a substrate, according to the method of Teo et al., [32] with slight modifications. Briefly, pure bacterial culture was incubated in Marine Broth (Difco) at 20°C for 48 h on a shaker to yield stationary phase. Bacterial concentrations ranged between 5×10^7 and 2×10^8 CFU/ml according to bacterial strains, as evaluated by the plate-counting method using appropriate dilution. Culture supernatant fluids were harvested by centrifugation (3,200×*g*, 20°C, 10 min), and 250 μl CSF were added to 250 μl azocasein (5 mg/ml) prepared in 50 mM Tris–HCl buffer (pH 8.0). The mixture was incubated at 37°C for 2 h and the reaction stopped by adding 500 μl cold 10% trichloroacetic acid. After centrifugation at 12,000×*g* and 4°C for 5 min, 500 μl of the supernatant were mixed with 500 μl 1 M NaOH and the absorbance measured at 440 nm. One unit of protease activity was defined as the amount of enzyme that caused an increase of 0.01 absorbance unit after 2 h of incubation at 37°C [32].

To confirm metalloprotease-like activity in culture supernatants, CSF were assayed with azocasein under the conditions described above, adding a CSF preincubation step at 20°C for 30 min in the presence of metalloprotease inhibitors 8 mM 10-phenanthroline or 40 mM EGTA [32].

Experimental Infections

Bacteria were grown at 20°C for 20 h in marine broth under constant shaking. The bacterial culture concentrations were evaluated spectrometrically at an optical density (OD) of 600 nm. Cells were centrifuged at 3,200×*g* for 10 min, the supernatant discarded and the resulting pellet resuspended in SASW to obtain an OD of 1. Ten- to twelve-month-old oyster spat were anaesthetised for 1 to 2 h at 20°C in a solution of magnesium chloride (28 g/L $MgCl_2$) supplemented with phytoplankton (*Isochrysis galbata* and *Chaetoceros calcitrans*) under aeration. Subsequently, 100 μL of bacterial suspension were injected into the adductor muscle. A group of oysters were injected with SASW to serve as negative controls. Virulent *V. splendidus* LGP32 strain [5] and *V. aestuarianus* 02/041 strain [3] isolated in experimental cohabitation trials and from the IFREMER experimental hatchery at Argenton (Brittany, France), respectively, were used as positive controls. After injection, oysters were transferred to tanks (20 to 30 oysters in 2 L) filled with 1 μm-filtered seawater at 3.1% of salinity and maintained under static conditions at 20°C with aeration. Mortality was monitored daily and any newly dead oysters were removed from the tanks over a 7-day period.

Results

Phylogenetic Analysis

A total of 183 bacterial isolates were obtained from four cultivated mollusc species collected during mortality episodes (n=92). All isolates were found dominant by the plate culture method at ×1,000 dilution. They were mainly isolated from diseased animals, in contrast to results with apparently healthy animals originating from a same batch, where no cultivable bacteria were observed at the same dilution. Small-subunit ribosomal RNA genes of these isolates were partially sequenced (around 700 bp). Sequence comparisons between isolates of a single tissue sample revealed that identical nucleotide sequences could be obtained, revealing the existence of 147 tissue sample-specific strains. Phylogenetic analysis (Fig. 1) of 120 strains with high quality sequences revealed that most of these strains clustered with reference strains of bacterial species related to polyphyletic groups of *Vibrio splendidus* (38 strains) or *Vibrio harveyi* (32), and monophyletic groups of *V. aestuarianus* (18), *Shewanella colwelliana* (9), *V. chagasii* (5), *V. fortis* (4) and *V. tapetis* (3). To define

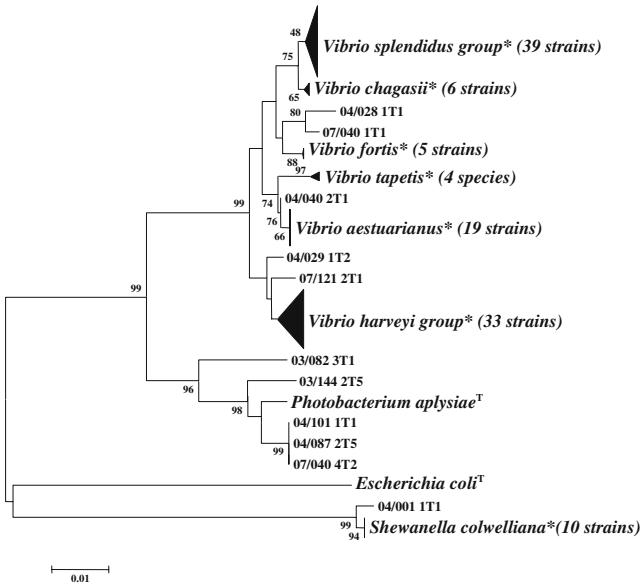

Figure 1 Phylogenetic tree of partial 16S rDNA from bacteria isolated from four cultivated mollusc species (mainly *C. gigas*), collected in the context of mortalities (92 episodes) and *Vibrio* reference strains. A total of 652 gap-free sites were analysed. The evolutionary distances were computed using the neighbour-joining method. Bootstrap values (1,000 replicates) greater than 50% are indicated. *Aserisk* denotes a reference type strain that was included to identify the strains of this study

the taxonomic affiliation of *V. splendidus*-related strains, a phylogenetic analysis was performed on the *gyrB* gene (Fig. 2) using 15 representative strains obtained in Fig. 1. Twelve of these strains were affiliated to the *V. splendidus* type strain, one (05/013 2T1) to the *V. crassostreae* type strain and one (04/112 1T1 strain) was found in the cluster composed of *V. lentus*, *V. tasmaniensis*, *V. kanaloae* and *V. pomeroyi* type strains. An additional strain (03/126 1T2) was found not to be affiliated to any *Vibrio* species yet described.

Strain Distribution

A total of 115 strains were isolated from *C. gigas* collected during 73 mortality episodes between 2003 and 2007, mainly during the hot season between May and October (Table 1). Among these, 63 strains (isolated from 39 mortality episodes) originated from 21 private farms in different localities and 52 strains (isolated from 34 mortality episodes) from experimental facilities of IFREMER in Bouin (Vendée) and La Tremblade (Charente Maritime) (Fig. 3). The strains encountered most frequently belonged to the *V. splendidus* group, *V. harveyi* group or *V. aestuarianus*: representing 32/115 (28%), 27/115 (23%) and 25/115 (22%) of the total strains, respectively. Analysis of the distribution of the strains obtained from private farms along the French coasts, according to the type of production structure, revealed that *V. splendidus* was detected in both tidal areas and indoor facilities at 33% and 27%, respectively. In contrast, *V. aestuarianus* was mainly found in open sea intertidal areas (11 over 12 strains). A discrepancy between these two *Vibrio* species was also noticed according to oyster age. In contrast to *V. splendidus*, which was found in oysters of all ages, *V. aestuarianus* strains were only found in juvenile or adult animals. The same tendency was seen in the IFREMER experimental facilities (11 over 13 strains). Study of the geographical distribution of *V. splendidus*-related bacterial strains revealed that these strains were present all along the Atlantic, English Channel and Mediterranean coasts of France (data not shown). Surprisingly, *V. aestuarianus* was present along the whole Atlantic coast and English Channel but was not detected in oysters cultivated on the Mediterranean. Among the other bacterial species most frequently detected in *C. gigas*, 11 *V. harveyi*-related and 8 *S. colwelliana* strains were isolated from oysters sampled on private farms, mainly from indoor facilities (hatcheries and nurseries); 33% and 20% detection frequencies were shown for these species, respectively, mainly found to be associated with mortality events affecting earlier life stages (24% and 17% respectively). *Vibrio harveyi*-related strains were also frequently detected in young animals bred in the IFREMER experimental facilities (50%), in contrast to *S. colwelliana*, for which only one strain was isolated out of 32. Interestingly, only two *V. harveyi* strains were detected between 2003 and 2005, whereas 25 new ones were isolated in 2007. Reports of *V. harveyi* strains were confined to three open sea rearing sites on the Mediterranean coast and Arcachon Bay

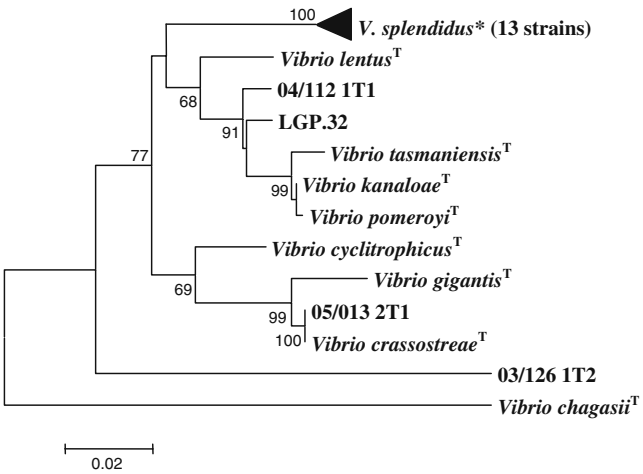

Figure 2 Phylogenetic tree of partial *gyrB* gene from bacteria isolated from three bivalve mollusc species collected in a context of mortalities (19 episodes) and reference *Vibrio* strains, based on the analysis of 700 gap-free sites. The evolutionary distances were computed using neighbour-joining method and Kimura's two-parameter model. Bootstrap values (1,000 replicates) greater than 50% are indicated. *Asterisk* denotes a reference type strain that was included to identify the strains of this study

Table 1 Number of bacterial strains isolated from *C. gigas* during 73 mortality episodes between 2004 and 2007 and distribution according to production type (private farms or experimental facilities), rearing practices (open sea tidal areas or indoor facilities), age of animals (larvae and young spat of less than 3 cm in size were treated in pools for bacteriological analysis, juveniles of more than 3 cm in size and adults were treated individually using haemolymph, see "Material and Methods)", season (the cold season is between October and March) and bacterial species

	Private farms						Experimental facilities	
	Tidal areas	Indoor facilities	Larvae and young spat	Juveniles and adults	Cold season	Warm season	Larvae and young spat	Juveniles and adults
Number of total bacterial strains and percentage representation	48	15	29	34	5	58	32	20
Vibrio splendidus group	**16 (33%)**	**4 (27%)**	**8 (27%)**	**12 (25%)**	3	**17 (29%)**	**6 (19%)**	**6 (30%)**
V. aestuarianus	**11 (23%)**	1	0	**12 (35%)**	1	**11 (19%)**	2	**11 (55%)**
V. harveyi group	6 (13%)	**5 (33%)**	**7 (24%)**	4 (12%)		**11 (19%)**	**16 (50%)**	0
Shewanella colwelliania	5 (10%)	**3 (20%)**	5 (17%)	3	1	7 (12%)	1	1
V. chagasii	0	2 (13%)	2	0	0	2	2	0
V. fortis	3	0	2	1	0	3	2	0
V. tapetis	3	0	2	1	0	3	0	0
Non identified species	4	0	3	1	0	4	3	2 (10%)

More frequently encountered bacterial strains (>19%) are represented in bold type

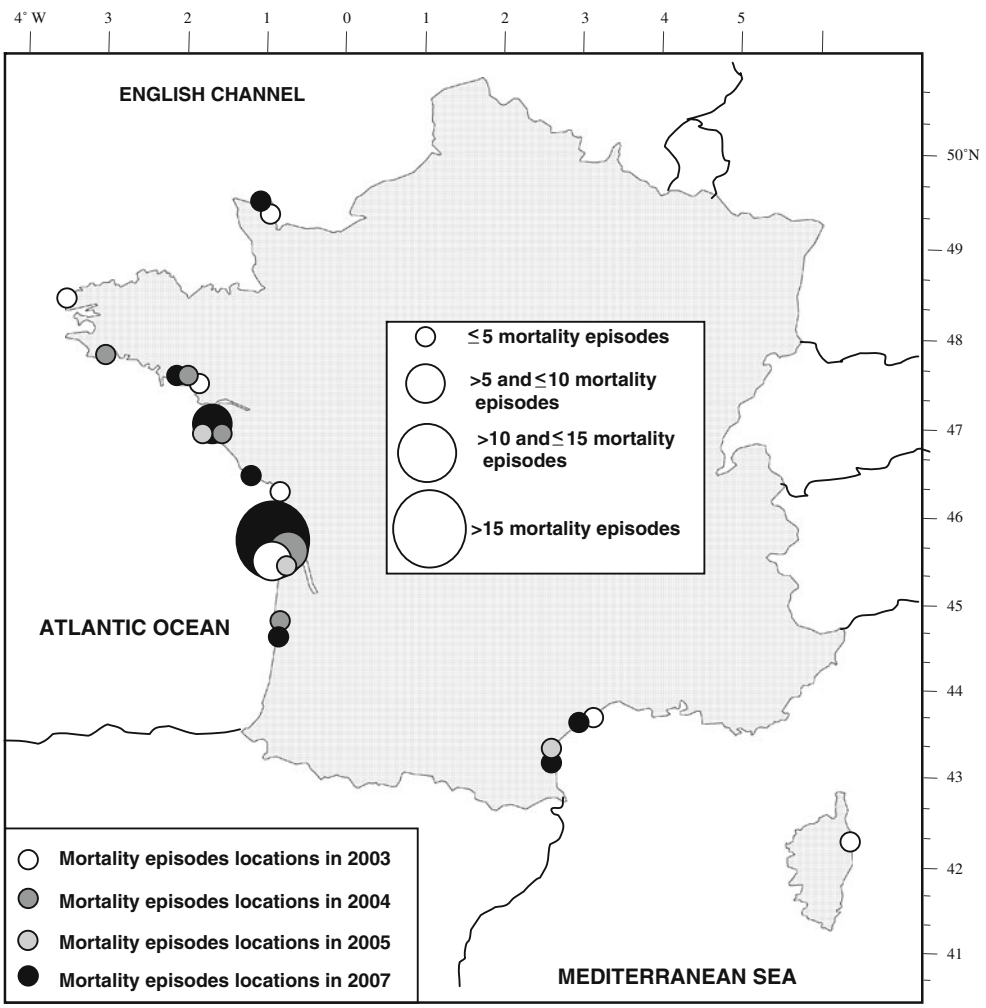

Figure 3 Distribution of the 73 *C. gigas* mortality episodes between 2003 and 2007 in both private farms and Ifremer experimental facilities

Table 2 Classification of bacterial strains isolated from scallops *Pecten maximus*, clams *Ruditapes philippinarum* and abalones *Haliotis tuberculata* according to more frequently detected bacterial species

	Number of batches collected during mortality episodes	Number of strains isolated	Number of bacterial strains identified			
			V. splendidus	*V. tapetis*	*V. harveyi*	*Shewanella colwelliana*
Pecten maximus	3	6	3	1	0	1
Ruditapes philliparum	5	12	7	0	0	3
Haliotis tuberculata	11	14	4	0	6	2

(6 strains), two private hatcheries or nurseries (5 strains) and the IFREMER experimental facilities (16 strains). Other bacterial species detected less frequently were *V. chagasii*, *V. fortis* and *V. tapetis*.

Another 32 bacterial strains were isolated from other mollusc species of economic interest in the context of 19 mortality events that occurred between 2003 and 2007 in Brittany, Normandy and Vendée. The most frequently detected bacterial species were *V. splendidus* and *S. colwelliana* (Table 2) found in scallops *P. maximus*, clams *R. philippinarum* and abalones *H. tuberculata*, representing 62% of total strains. Furthermore, *H. tuberculata* was frequently found to be infected with *V. harveyi* strains (six out of 14).

Considering all the tissue samples analysed, some cases of co-infection by multiple bacterial species were noted. In particular, the presence of both *V. aestuarianus* and *V. splendidus* strains in the same sample was found nine times among the 92 batches of animals analysed.

Strain Screening for Virulence in *C. gigas* and Metalloprotease Activity

Several strains were selected from the more representative phylogenetic clades found in this study, corresponding to bacteria isolated during mortality events in four cultivated bivalve molluscs (Table 3): 14 strains belonged to the

Table 3 Characteristics of field isolates and *Vibrio* reference strains used in this study for the screening of strain virulence and proteolytic activity

Vibrio species	Strain denomination	Source and context of isolation	Virulence		Protease activity
V. splendidus group			1	2	Metalloproteinase-like
V. lentus	04/112 1T1	*C. gigas* oyster spat, 2004 mortality episode(m.e) in an indoor breeding structure, Charente-Maritime (CM), France	++	J2	+ (61 μ/ml)
V. lentus	LGP32	*C. gigas* oyster spat, 2002 m.e. in Ifremer experimental facilities, La Tremblade, CM, France, Gay et al. 2002	+	J2	++ (120 μ/ml)
V. splendidus	03/028 6T1	*C. gigas* oyster spat, 2003 m.e in an indoor breeding structure, CM, France	−		+ (64 μ/ml)
V. splendidus	03/013 4T1	*R. philippinarum* clam spat, 2003 m.e in an indoor breeding structure, Bouin, Vendée, France	+	J3	++ (156 μ/ml) (1)
V. crassotreae	05/013 2T1	*C. gigas* oyster spat, 2005 m.e. in an open sea rearing farm, Leucate lagoon, Southern France	−		++ (141 μ/ml)
V. splendidus	05/103 2T1	*P. maximus* scallop spat, 2005 m.e. in an open sea rearing farm, Morlaix Bay, Brittany , France	++	J1	+++ (205 μ/ml)
V. splendidus	03/126 1T2	*C. gigas* oyster spat, 2003 m.e. in an open sea rearing farm, Urbino lagoon, Corsica , Southern France	+	J5	+++ (264 μ/ml)
V. splendidus	04/113 1T1	*C. gigas* oyster spat, 2004 m.e. in an indoor breeding structure, CM, France	++	J1	++ (149 μ/ml) (1)
V. splendidus	03/024 5T6	*R. philippinarum* clam adult, 2003 m.e. in an open sea rearing farm, Rance, Brittany , France	++	J1	+++ (252 μ/ml)
V. splendidus	03/025 1T1	*R. philippinarum* clam adult, 2003 m.e. in an open sea rearing farm, Rance, Brittany , France	++	J1	+++ (253 μ/ml)
V. splendidus	03/011 1T4	*C. gigas* oyster spat, 2003 m.e. in an indoor breeding structure, CM, France	−		− (0 μ/ml)
V. splendidus	07/040 2T2	*C. gigas* oyster spat, 2007 m.e. in an open sea rearing farm, Thau lagoon, Southern France	++	J2	++ (123 μ/ml)
V. splendidus	07/111 T1	*C. gigas* oyster spat, 2007 m.e. in an indoor breeding structure, CM, France	−		++ (127 μ/ml)

Table 3 (continued)

Vibrio species	Strain denomination	Source and context of isolation	Virulence		Protease activity
V. tasmaniensis	LMG20012	Atlantic salmon (*Salmo salar* L.), Tasmania, Australia, Thompson et al. 2003	−		− (0 μ/ml)
V. harveyi group					
V. harveyi	07/108 1T1	*C. gigas* oyster spat, 2007 m.e. in an indoor breeding structure, CM, France	++	<20 h	++++ (300 u/ml)
V. harveyi	07/115 T2	*C. gigas* oyster spat, 2007 m.e. in an indoor breeding structure, CM, France	++	<20 h	++ (173 u/ml) (1)
V. harveyi	07/116 1T1	*C. gigas* oyster spat, 2007 m.e. in an indoor breeding structure, CM, France	++	<20 h	++ (183 u/ml) (1)
V. harveyi	07/118 T2	*C. gigas* oyster spat, 2007 m.e. in an indoor breeding structure, CM, France	++	<20 h	+++ (204 u/ml) (1)
V. harveyi	07/119 1T1	*C. gigas* oyster spat, 2007 m.e. in an indoor breeding structure, CM, France	++	<20 h	++ (116 u/ml)
V. harveyi	07/121 1T1	*C. gigas* oyster spat, 2007 m.e. in an indoor breeding structure, CM, France	++	<20 h	++ (185 u/ml) (1)
V. harveyi	03/082 2T1	*C. gigas* oyster spat, 2003 m.e. in an open sea rearing farm, Thau lagoon, Southern France	+	J1	− (3 u/ml)
V. harveyi	04/002 1T2	*H. tuberculata* spat, 2004 m.e. in a hatchery, Normandy, France	++	<20 h	++ (137 u/ml)
V. harveyi	04/107 1T1	*H. tuberculata* juvenile, 2004 m.e. in an indoor breeding structure, Blainville-sur-Mer, Normandy, France	++	J2	− (12 u/ml)
V. aestuarianus	05/091 1T1	*C. gigas* oyster spat, 2005 m.e. in an indoor breeding structure, CM, France, Saulnier et al, 2009	++	J2	+ (85 u/ml)
V. aestuarianus	04/021 3T1	*C. gigas* oyster spat, 2004 m.e. in an indoor breeding structure, CM, France, Saulnier et al, 2009	++	J3	++ (185 u/ml)
V. aestuarianus	04/047 3T1	*C. gigas* oyster spat, 2004 m.e. in an indoor breeding structure, CM, France, Saulnier et al, 2009	++	J5	+++ (209 u/ml)
V. aestuarianus	03/011 1T1	*C. gigas* oyster spat, 2003 m.e. in an indoor breeding structure, CM, France, Saulnier et al, 2009	++	J3	++ (120 u/ml))
V. aestuarianus	03/146 2T1	*C. gigas* oyster adult, 2003 m.e. in an open sea rearing farm, Angoulin, CM, France, Saulnier et al, 2009	++	J3	++ (172 u/ml)
V. aestuarianus	04/055 2T3	*C. gigas* oyster adult, 2004 m.e. in an open sea rearing farm, Arcachon Bay, Gironde, France, Saulnier et al, 2009	++	J3	+ (96 u/ml)
V. aestuarianus	07/083 1T1	*C. gigas* oyster adult, 2007 m.e. in an open sea rearing farm, Marennes-Oléron Bay, CM, France, Saulnier et al, 2009	++	J3	++ (189 u/ml)
V. aestuarianus	07/046 1T1	*C. gigas* oyster adult, 2007 m.e. in an open sea rearing farm, Olonne, Vendée, France, Saulnier et al, 2009	++	J2	++ (183 u/ml)
V. aestuarianus	07/080 1T2	*C. gigas* oyster adult, 2007 m.e. in an open sea rearing farm, Marennes-Oléron Bay, CM, France, Saulnier et al, 2009	++	J2	+ (83 u/ml)
V. aestuarianus	02/041	*C. gigas* oyster adult, 2002 m.e. in a hatchery, Argenton, Finistère, France, Garnier et al. 2007	++	J2	++ (173 u/ml)
S. colwelliana	07/050 1T2	*C. gigas* oyster spat, 2007 m.e. in an open sea rearing farm, Bay of Morbihan, Brittany, France	−		- (7 u/ml)
S. colwelliana	07/079 2T2	*C. gigas* oyster adult, 2007 m.e. in an open sea rearing farm, Marennes-Oléron Bay, CM, France	−		− (12 u/ml)
S. colwelliana	07/082 T2	*C. gigas* oyster spat, 2007 m.e. in a hatchery, Bourgneuf Bay, Vendée, France	+	J2	− (8 u/ml)
S. colwelliana	07/085 1T1	*C. gigas* oyster spat, 2007 m.e. in a hatchery, Bourgneuf Bay, Vendée, France	−		− (23 u/ml)
S. colwelliana	07/086 1T1	*C. gigas* oyster spat, 2007 m.e. in a hatchery, Bourgneuf Bay, Vendée, France	−		− (32 u/ml)

Virulence of bacterial strains was evaluated by experimental infection of apparently healthy oyster spat. Column 1: highly virulent strains, denoted "++", were defined by cumulative mortalities up to 60% after a 7-day post-injection survey. Avirulent strains, denoted "−", exhibited cumulative mortalities below 20% after a 7-day post injection survey. Bacterial strains displaying intermediate virulence are denoted "+". Column 2: indicates the day where the peak of mortality was recorded. Metalloprotease activity was calculated as the difference between azocaseinase activity obtained in the absence or presence of phenanthrolein. Positive metalloprotease activity was defined beyond the threshold of 50 μ/ml. For the majority of bacterial strains, metalloprotease activity was sensitive to phenanthrolein treatment (more than 90% of inhibition). Nevertheless, metalloprotease activity **(1)** of some particular strains was partially inactivated (between 50% and 70% according to bacterial strain)

Table 4 Contingency table showing the number of bacterial strains characterized according their ability to produce metalloprotease-like enzyme or not in culture supernants, given with their virulence status evaluated by experimental infection (See Table 3)

		Metalloprotease-like activity		
		+	–	
Virulence status	+	26 (90%)	3	76%
	–	3 (10%)	6	24%
		76%	24%	

V. splendidus group, nine to the *V. harveyi* group, ten *V. aestuarianus* strains and five *S. colwelliana* strains. *V. splendidus*-related strains were mainly those typed by the *gyrB* phylogenetic approach, whereas strains related to other well represented phylogenetic clades were selected arbitrarily from our bacteria collection. All tested *V. aestuarianus* and *V. harveyi* strains were found virulent by experimental injection of oysters with live bacteria whereas *S. colwelliana* strains were mainly avirulent, except for one strain (07/082 T2). Virulence was variable among *V. splendidus*-related strains. In particular, 4 field isolates plus one *V. tasmaniensis* type strain were found to be non-pathogenic. The peak of mortality induced by virulent strains was generally seen between the first and second day of infection challenge for *V. splendidus*-related strains, between days 2 and 3 for *V. aestuarianus* strains and in the first 20 h post-challenge for *V. harveyi* strains.

Metalloprotease-like activities of culture supernatants and virulence status were compared for each of the tested strains, whatever their species, and a contingency table was built (Table 4): 76% of strains displayed metalloprotease-like activities or virulence capacities, whereas 24% were found negative for both tests and a correlation was revealed. Concordant results were obtained for 32 strains out of 38 (84%), with 26 and six strains found positive and negative for the two tests, respectively. Divergent results between the two tests were obtained for *V. splendidus*-related strains (3 strains), *V. harveyi*-related strains (two strains) and S. *colwelliana* (one strain), but not for *V. aestuarianus* strains. Finally, 90% of metalloprotease positive strains were found to be virulent.

Synergistic Virulence Effect of *Vibrio* Co-infection

Following the evidence for cases of natural co-infection by *V. aestuarianus* and *V. splendidus*, well characterized LGP32 and 02/041 strains were injected together to study their pathogenicity and compare it with injection of single strain bacterial injection at the same dose (Fig. 4). The use of a mixture of LGP32 and 02/041 strains at a dose of 5 10^7 CFU oyster^{-1} for each strain gave a mortality rate of 88.9%, which was higher than the mortality rate obtained when bacteria were injected alone at the same dose i.e. 10^8 CFU oyster^{-1} (60 and 53.3%, respectively). Furthermore, lowering the injected dose of the LGP32 and 02/041 mixture by a factor of 10 or 100 resulted in lower but significant cumulative mortalities, with rates of 84.7 and 34.4%, respectively, whereas a 1/100 dilution factor removed the pathogenicity of these strains when they were used individually (data not shown). Furthermore, whatever the bacteria used in experimental challenges, we verified with the plate counting method that haemolymph samples from some freshly dead oysters harboured dominant bacteria belonging to the same species as those injected, fulfilling one of the Koch's postulates (data not shown).

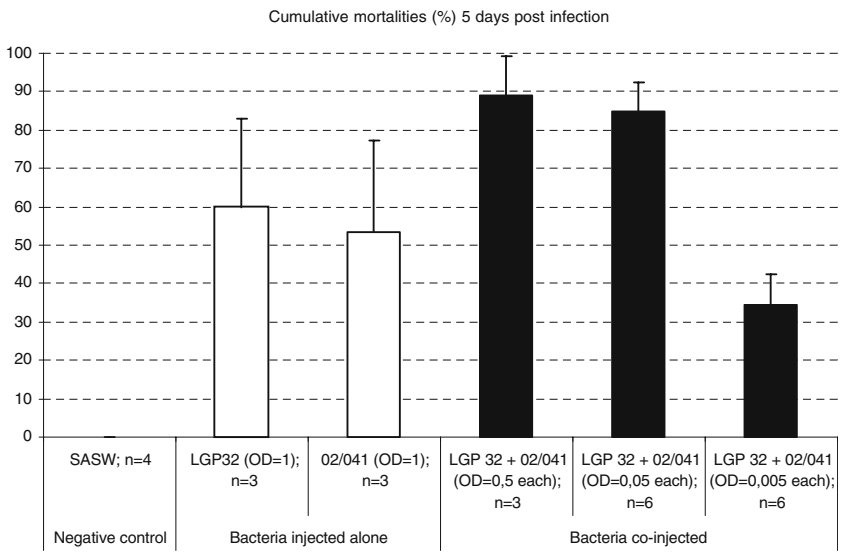

Figure 4 Cumulative mortality rates (%) at day 5 post-injection with bacterial strains. Strains LGP32 (*V. splendidus*) and 02/041 (*V. aestuarianus*) were injected individually using a bacterial suspension at OD=1. The same strains were injected as a mixture and at different doses. Sterile artificial seawater (*SASW*) was used as negative control. Fifteen to 20 injected oysters were placed in each aquarium. Symbol "*n*" indicates the number of aquaria or replicates; *bars* show standard deviations

Discussion

Previous geographically and temporally limited bacteriological studies performed during summer mortality phenomena in Pacific *C. gigas* oysters reared in France have shown that two *Vibrio* species, *V. aestuarianus* and *V. splendidus*, are frequently, but not systematically, associated with summer mortality phenomenon with several strains demonstrated to be virulent for this oyster species in experimental infections [3, 25]. In the present study we performed a large-scale epidemiological survey to identify the bacteria abundant in four mollusc species (mainly Pacific oyster) cultivated in France during 92 mortality episodes between 2003 and 2007 and evaluate experimentally their virulence. Throughout this survey, histological analyses were also performed on 30 animals for each sample batch received, but no other important pathogenic agent was detected (data not shown).

Strong seasonality was observed in mortality episodes, as 68 out of the 73 in *C. gigas* grown in open sea sites occurred between May and September, with an onset in June (39/68=57%) that coincided with the period of summer mortality phenomena. Incidence of *V. aestuarianus* and *V. splendidus* in oyster mortality outbreaks was confirmed, since a high frequency of detection (22% and 28%, respectively) was noted among the 115 identified strains. As observed in previous studies [33], isolated *V. splendidus* strains belong to a genetically complex polyphyletic group constituted by at least eight species [34]. Using *gyrB* as one of the more polymorphic housekeeping genes for phylogenetic analysis of this group, the majority of *V. splendidus*-related strains isolated (12 out of 15) were affiliated to the *V. splendidus* type strain. In contrast, isolated *V. aestuarianus* strains seemed to be genetically similar, with less than 1% nucleotide variation in their 16S rDNA gene. Both *V. splendidus*-related and, especially, *V. aestuarianus* species were more frequently detected in oysters reared in the open sea than in indoor facilities of private farms (33% and 23% compared with 27% and less than 5%, respectively) suggesting that coastal waters harbour these two organisms naturally. Indeed, *V. splendidus*-related strains were shown to be the most abundant planktonic species among the *Vibrionaceae* [35]. The fact that *Vibrio aestuarianus* was more frequently (25%) detected in oysters reared in IFREMER indoor experimental facilities suggests that infectiosity of these organisms may be favoured by zootechnical practices, such as the use of low and frequently thermo-regulated rearing water flow. However, we cannot exclude the possibility that these data arose from an improved reactivity in detection and bacteriological sampling during mortality episodes, due to the close proximity of oysters to the IFREMER laboratory. The observation of symptomatology (weakness of the adductor muscle) may also have been easier than in field-reared oysters.

Other more frequently detected bacteria species associated with *C. gigas* mortality events encompassed *V. harveyi* and *S. colwelliana*. Similar to the *V. splendidus* group, *V. harveyi* has been shown by a multilocus sequence typing approach to be constituted of at least seven species displaying a very high degree of both genetic and phenotypic similarity [36]. It seems relevant to note that in our study the number of *V. harveyi*-related strains associated with *C. gigas* mortality events rapidly increased in 2007, to 25 strains, whereas only two were isolated between 2003 and 2005. Further molecular typing studies are needed to assess the degree of similarity between these geographically distant isolates. In contrast, *Shewanella* spp. has never, to our knowledge, been associated with disease outbreaks in cultivated bivalve molluscs. In the present study, only one strain out of 5 was found to be moderately pathogenic for *C. gigas* oysters in experimental infections. This bacteria is reported to be a commensal in *O. edulis* [37] and *C. virginica* [38] oysters. Because *S. colwelliana* bacterial colonies isolated in this study display a similar macroscopic appearance to *V. splendidus*, and because cases of co-infection of samples by *S. colwelliana* and *V. splendidus*-related strains were frequently encountered (data not shown), we can hypothesize that we overestimated sample bacterial load due to *S. colwelliana* by erroneously considering that this bacterium could be dominant.

The association of microorganisms with animal tissue can be commensal, mutualistic, pathogenic or coincidental, due to the filter feeding behaviour of molluscs. As a consequence, many bacterial species and genera (mainly *Vibrio*) have been described as components of the bacterial flora naturally harboured by molluscs [39–43]. Nevertheless, they may act as opportunistic pathogens under adverse circumstances or in immuno-compromised animals. In our experience, the cultivable bacterial flora of healthy *C. gigas* oysters ranges from 10^2 to 10^4 CFU/mg tissue according to the origin of the oysters and the season, with a tendency towards higher bacterial load in oyster tissues when seawater temperature is higher (>19°C). In the present study, we considered that all bacterial isolates found dominant by the plate counting method were potentially pathogenic and not of environmental origin because they were isolated in a context of mortalities and found to be present at abnormal concentrations (>10^4 CFU/mg), mainly in tissue samples originating from diseased animals. Nevertheless, we cannot exclude the possibility that less abundant non-dominant bacteria could be also pathogenic and could be the primary etiological agent of disease. These bacteria may be also counter-selected by culture conditions or be uncultivable on the synthetic medium used.

Using standardized experimental challenges on healthy oysters, as described by Gay et al. [25] and Labreuche et al.

[44, 45], we evaluated the potential virulence status of some strains belonging to the more frequently occurring species in our collection. Most *V. splendidus* but all *V. harveyi*-related and *V. aestuarianus* strains tested were found virulent. Using two extensively studied pathogenic strains, LGP32 [25] and 01/042 [3], described by the same authors as one of the most virulent strains for *C. gigas* oyster and related to *V. splendidus* and *V. aestuarianus*, respectively, a synergistic effect was observed between these two strains when they were injected simultaneously. Such a phenomenon had already been observed in *C. gigas* oysters experimentally infected with two genetically close strains related to *V. splendidus* [5]. These results suggest that some not yet identified strain-specific virulence mechanisms are involved in pathogenicity expression. Because natural cases of co-infection by these two species-specific pathogens have been observed in our study, such synergistic virulence effects between two different *Vibrio* species would be expected to occur in some natural *C. gigas* disease outbreaks.

Although *V. splendidus* strains have already been found to be associated with mortality events in *P. maximus* [26, 46] and carpet shell clam *Ruditapes decussatus* [47] this is the first time, to our knowledge, that *V. splendidus* has been found to be associated with the mortality of clam *R. philippinarum* or abalone *H. tuberculata*. Even though further studies are needed to demonstrate their role in disease outbreaks of these other mollusc species, it is noticeable that several *V. splendidus* strains isolated from *R. philippinarum* were found to be virulent for *C. gigas* in experimental infection, suggesting that a common agent may infect different mollusc species. Similarly, considering *V. harveyi*, a well-known pathogen of cultivated *H. tuberculata* in France [48, 49], two strains isolated in this study exhibited a pathogenic effect on *C. gigas* when evaluated by experimental infection. In contrast to other *V. splendidus* or *V. harveyi* strains studied, which seemed to be ubiquitous, those identified as *V. aestuarianus* appear to only induce disease in *C. gigas*.

Finally, a high level of concordant results between azocaseinase activities and virulence of strains were obtained in this study. Azocaseinase activity was assumed to be due to a metalloprotease-like enzyme following the results of zinc-dependant chelators, such as phenanthrolein or EGTA inhibitory treatments (data not shown), with up to 90% inhibition for most strains and partial inhibition for a limited number of strains. Despite this correlation, metalloprotease appeared not to be the only virulence determinant of virulent strains, because 3 strains were found to be devoid of this enzyme, though they were in a minority compared with the 29 tested virulent strains. Conversely, three among nine avirulent strains exhibited metalloprotease activity, suggesting that these bacteria were unable to produce this enzyme in in vivo. In any case, the role of metalloprotease in pathogenesis of vibriosis in different host species has been explored by several in vitro approaches. Several host substrates and culture conditions for efficient metalloprotease production have been identified that could be involved in invasion process [19, 32, 50–54]. In contrast, few approaches have been conducted in vivo that aim to demonstrate the role of metalloproteases in virulence induced by *Vibrio* infection. This could be due to the lack of mutant strains, lack of tools to quantify metalloprotease production or its expression by pathogens during infection, or the absence of appropriate infection models. The small number of virulence studies that have been realized using mutants deficient in metalloprotease gave mitigated results and conclusions. Infecting fish with wild or metalloprotease-mutated pathogenic strains of *V. anguillarum*, Milton et al. [55] and Denkin and Nelson [51] noticed contrasted virulence profiles according to strains, infection route and infection model. For example, the NB10 metalloprotease-deficient mutant was found to be completely avirulent when injected intraperitoneally or by anal intubation into Atlantic salmon [51], whereas the same mutant exhibited attenuated virulence when administered to rainbow trout by intraperitoneal injection or immersion challenge [55]. Furthermore, metalloprotease mutant M99 derived from another virulent *Vibrio anguillarum* strain was found to be as virulent as the wild strain when injected intraperitoneally and to display attenuated virulence when administered by anal intubation [51]. In *Vibrio* sp. strains pathogenic to *C. gigas* oysters, high homology between amino acid sequences deduced from metalloprotease genes has been noticed, suggesting a similar role in pathogenesis. Those from *V. splendidus* LGP32 and *Vibrio tubiashii* RE22 strains (named *vsm* and *Vtpa* with GenBank accession numbers ACD87449 and CAV18407, respectively) displayed 75% identity and 88% similarity. Similarly, whole genome sequencing comparisons between LGP32 and two other geographically distant virulent strains related to *V. splendidus* (Med222 and 12B01), showed that genes encoding metalloprotease shared more than 98% identity and that *vsm* was localised on chromosome 1 in a region (position 1 673 221–1 365 498) devoid of genome plasticity [21, 56]. The predicted product of *vsm* gene product exhibits 67% identity and 81% similarity with *V. aestuarianus* strain 01/032 (AAU04777) or with *V. anguillarum* strain NB10 (AAA27517). The deletion of *vsm* and *Vtpa* genes from LGP32 and RE25 strains was shown to decrease the toxicity of extracellular products towards *C. gigas* spat and larvae [21, 24]. Furthermore, the metalloprotease activity and toxicity of *vsm* mutant were restored by ectopic complementation, suggesting that metalloprotease is important in its pathogenicity to oyster. Nevertheless, the fact that the *vsm* mutant displays an equal virulence to the parental strain when injected intramuscu-

larly to oyster [21] suggests that this metalloprotease may interact with other virulence factors that still need to be identified and/or that the experimental infection model used here, via an invasive route, is inappropriate.

To our knowledge, this is the first time that a correlation has been reported between *C. gigas* pathogenic bacterial strains related to *V. aestuarianus*, or polyphyletic groups of *V. splendidus* and *V. harveyi*, and the ability of these bacteria to produce metalloprotease in culture supernatants following large-scale strain screening from an epidemiological study. Because *V. harveyi* strains were recently isolated from *C. gigas* oyster mortality events occurring in France (mainly in 2007), further epidemiological studies and molecular typing methods are needed to gain an idea of whether a new *C. gigas* pathogen has emerged and to identify strains related to this complex polyphyletic group to the species level. Bacterial metalloprotease activities appear in this study as a common feature of pathogenic bacteria strains associated with mortality events of *C. gigas* reared in France, suggesting that this phenotypic test could be useful for the evaluation of virulence in bacterial strains associated with such mortality episodes. Indeed, experimental infection trials to evaluate virulence potential of bacterial strains are time consuming, as they require at least five consecutive working days, as well as dedicated experimental facilities, including a system for treating of seawater effluents. In contrast, measurement of metalloprotease activities requires less time (estimated at a total of one day of work for the treatment of approximately thirty bacterial strains). Few studies had been previously conducted in vivo to demonstrate the involvement of metalloproteases in virulence during *Vibrio* infection, compared with the number of in vitro studies. There is, therefore, a huge need to develop molecular and biochemical tools to study the encoding gene(s) at both the expression and translation level, and to identify host and environmental factors modulating the production of metalloprotease by both pathogenic and presumed commensal bacterial strains. For this purpose, the use of a non-invasive experimental model for infection of oysters with pathogenic *Vibrio* will be very useful and will allow future studying of the interactions between metalloprotease and other virulence factors that still need to be identified.

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