Factors other than metalloprotease are required for full virulence of French Vibrio tubiashii isolates in oyster larvae

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Vibrio tubiashii is a marine pathogen isolated from larval and juvenile bivalve molluscs that causes bacillary necrosis. Recent studies demonstrated the isolation of this species in a French experimental hatchery/nursery affecting Crassostrea gigas spat in 2007. Here, using larvae of C. gigas as an interaction model, we showed that the French Vibrio tubiashii is virulent to larvae and can cause bacillary necrosis symptoms with an LD₅₀ of about 2.3×10³ c.f.u. ml⁻¹ after 24 h. Moreover, complete or gel permeation HPLC fractionated extracellular products (ECPs) of this strain appeared toxic to larvae. MS-MS analysis of the different ECP fractions revealed the existence of an extracellular metalloprotease and other suspected virulence factors. This observation is also supported by the expression level of some potential virulence factors. The overall results suggest that the pathology caused by the French Vibrio tubiashii in C. gigas oysters is caused by a group of toxic factors and not only the metalloprotease.

INTRODUCTION

Marine Vibrio species are considered as the most serious problem affecting hatchery-reared oyster larvae (Tubiash et al., 1970; Sugumar et al., 1998; Estes et al., 2004; Elston et al., 2008). Among these species, Vibrio tubiashii is one of the main causative agents of larval and juvenile bivalve mollusc mortalities in hatcheries. Originally found on the east coast of America, isolated from diseased hard-shell clams (Tubiash et al., 1965, 1970; Hada et al., 1984), and in England and Spain isolated from Pacific and flat oysters (Jeffries, 1982; Lodeiros et al., 1987), V. tubiashii has been detected in recent decades in North America infecting new hosts, the Pacific and Kumamoto oysters and Geoduck clams (Elston et al., 2008), and very recently in France in diseased Pacific oysters and abalone (Travers et al., 2014). Strains of this species are known to cause bacillary necrosis in mollusc larvae. This disease is clinically characterized by a reduction in larval motility and an increase in soft-tissue necrosis (Tubiash et al., 1965). Considerable efforts were made to characterize the toxic molecules (e.g. metalloproteinases, ciliostatic toxin, cytolsin) and mechanisms associated with oyster mortalities (juveniles and larvae) (Kothyary et al., 2001; Delston et al., 2003; Estes et al., 2004; Elston et al., 2008; Hasegawa et al., 2008, 2009; Hasegawa & Häse, 2009a). All of these works were conducted either with bacterial extracellular products (ECPs) or with live bacteria initially all classified as Vibrio tubiashii. However, recent studies demonstrated that strains RE22, RE98, LMG 1095 and ATCC 19105 thought to be V. tubiashii are

Abbreviations: 2DE, 2D electrophoresis; ECP, extracellular product; f.c., final concentration; GP-HPLC, gel permeation HPLC; OPCR, quantitative PCR; RT, reverse transcription.

A supplementary table is available with the online Supplementary Material.

This reclassification of some *V. tubiashii* strains does not change the fact that this bacterium is considered as one of the potential pathogens of bivalves usually associated with major mortality events in shellfish hatcheries, but reveals that *V. coralliilyticus* also should be treated as a serious threat contributing to a big loss in bivalve hatcheries (Richards *et al.*, 2015).

Consequently, in regard to this recent update in the classification of *V. tubiashii* strains, few results are available today about true *V. tubiashii* strains and their pathogenicity. Work conducted by us (Travers *et al.*, 2014) on a group of isolates classified them phylogenetically and biochemically close to the American *V. tubiashii*, and as French *V. tubiashii*. They were found in an experimental hatchery/nursery of *Crassostrea gigas* spat during mortality events in France in 2007. Demonstration of their virulence was carried out and toxicity of their ECPs was confirmed by experimental challenges with juvenile Pacific oysters. Moreover, Mersni-Achour *et al.* (2014) demonstrated that the ECPs of *07/118 T2*, a representative strain of the group, inhibited the adhesion capacity and phagocytosis activity of *C. gigas* haemocytes. Complementary biochemical analyses showed that the proteolytic fraction of ECPs contained active and thermostable extracellular zinc metalloprotease(s).

As little knowledge is available about true *V. tubiashii* strains and their mechanisms of pathogenicity, the aims of the present study were: (i) to reproduce the pathogenicity of the French *V. tubiashii* and the toxicity of its ECPs on Pacific oyster larvae; and (ii) to characterize the potential virulence factors supporting the pathogenicity of the French *V. tubiashii* *07/118 T2*. Therefore, experimental infections coupled with microscopic observations were performed, and the expression of some potential virulence factors during infection was monitored. Finally, the fractionation of ECPs and proteomic analysis of the purified material were conducted to access the molecular diversity of this bacteria secretome.

**METHODS**

**Bacteria growth conditions.** *V. tubiashii 07/118 T2* (LMG 27884=CECT 8426) (Travers *et al.*, 2014), *V. coralliilyticus* 06/210 (Genard *et al.*, 2013) and *Vibrio nigripulchritudo* LMG 3896⁵ (Le Roux *et al.*, 2011) were used in this study. Bacteria were grown in Luria–Bertani medium (Difco) supplemented with 1% sodium chloride (LBS) and stock cultures were stored at −80°C in LBS containing 15% (v/v) glycerol.

**Larval production.** Larval production was achieved according to the protocol described by Déregremet *et al.* (2005) at the IFREMER hatchery in La Tremblade, France. Briefly, 15 *C. gigas* oysters were randomly sampled from a wild population of the Marennes-Oléron Bay. Oysters were opened, and a sample of the gonad was analysed under the microscope in order to separate the oysters by sex. Eggs were collected by stripping of the gonad, as well as the sperm, and after cleaning the gametes by appropriate sieving, mating was carried out. Larvae were reared in 30 l tanks at 26°C in filtered sea water, which was changed three times per week. The larvae were fed daily with a mixed diet of cultured phytoplankton (*Isochrysis galbana, Chaetoceros calcitrans* and *Skeletonema costatum*). When larvae were retained on a 150 μm screen, they were transferred into the laboratory and then tested with different *Vibrio* strains. At this step, larvae were 13 days old and their shell lengths were between 200 and 300 μm.

**Preparation of *V. tubiashii* ECPs, fractionation and biochemical assays.** ECPs were produced using the cellophane overlay method as described previously (Travers *et al.*, 2014). Briefly, bacteria were grown in 5 ml LBS at 22°C for 18 h. A volume of 1 ml of exponential phase culture (OD600 1) was spread on a sterile cellophane film overlaying LBS agar plates. After 48 h incubation at 22°C, the cells were washed off the cellophane using 10 ml cold sterile artificial sea water and removed by centrifugation at 3000 g (45 min at 4°C). The supernatant was filtered at 0.22 μm and then concentrated by lyophilization, resuspended in Tris/HCl buffer (50 mM Tris/HCl, pH 8.0) and dialysed against distilled water for 12 h and Tris/HCl buffer for 12 h. Finally, crude ECP samples were stored at −80°C until use.

Crude ECPs were purified using a gel permeation HPLC (GP-HPLC). This purification step was performed using a Biosuite TM 230 column (5 μm, 300 x 7.8 mm; Waters) and conducted with a Waters system (600 controller, 2996 photodiode array detector and 2707 autosampler). A total of 10 μg per run of crude ECP fractions was eluted with 50 mM Tris/HCl buffer, pH 8, at a rate of 1 ml min⁻¹ and collected according to the chromatographic profile obtained at 215 nm. Each eluted fraction obtained was concentrated by lyophilization, resuspended in Tris/HCl buffer, assayed for protein concentration and stored at −20°C until further utilization. The column was calibrated using gel filtration markers kit for protein molecular masses 29 000–700 000 Da (Sigma-Aldrich) following the same analysis conditions applied to the samples.

Proteins were quantified using the method described by Bradford (1976) using Bradford reagent (Sigma-Aldrich) and BSA as the standard protein. Azocaseinase activity was determined as previously described (Travers *et al.*, 2014).

**In vivo larval experiments.** Cultures of early stationary phase (18 h) *Vibrio* strains grown in LBS at 22°C were centrifuged at 3000 g for 20 min, washed twice and resuspended in sterile sea water. In a 6-well flat-bottomed plate (Falcon), 1.5 ml tenfold dilutions of the cell suspensions between 10 and 10² c.f.u. ml⁻¹ were added to 1.5 ml of the larval suspension at a density of about 50 larvae per well and incubated at 22°C with a gentle stirring. *V. nigripulchritudo* was used as a non-virulent control strain and *V. coralliilyticus* as a virulent one for *C. gigas* larvae. Boiled bacteria (boiled 15 min at 100°C, centrifuged, washed twice and resuspended in sterile sea water), some incubated with EDTA [at a final concentration (f.c.) of 5 mM], and others not incubated with EDTA, were also tested. Further, the toxicity of different crude ECP concentrations (f.c. 10 μg, 5 μg and 2.5 μg protein ml⁻¹) and GP-HPLC fractions of ECPs (f.c. 5 μg proteins ml⁻¹) were tested on model larvae in the presence and absence of EDTA (preincubated with 5 mM f.c. EDTA for 30 min).

The counting of the live larvae was carried out by visualization using a binocular microscope and a Sedgewick Rafter cell. Oyster larvae were considered dead based on microscopic examination and phenotypic observations described by Hasegawa *et al.* (2008). The percentage of live larvae was calculated with reference to live larvae incubated in sterile sea water for the live-bacteria experiments, and with reference to the live larvae incubated with Tris/HCl buffer for ECP and GP-HPLC fractions. Larval survival was recorded after 24 h, three biological replicates were performed for each assay and assays were replicated independently.
Following the same protocol described above, a kinetic study was performed by bathing 25 ml larvae (about 50 larvae ml\(^{-1}\)) with 25 ml bacteria (10\(^{10}\) c.f.u. ml\(^{-1}\)) at different times (30 min, 3 h, 6 h, 16 h and 18 h) in aerated bottles with gentle stirring. Azocaseinase activity in the bath was performed as previously described (Travers et al., 2014) for each kinetic point. At the end of each time point, a centrifugation at 3000 g for 15 min was carried out and the pellet (larvae + bacteria) was conserved with RNAProtect bacteria reagent (Qiagen) to stabilize/fix the bacterial RNA for quantitative PCR (QPCR) assays. Pellets were stored at –80 °C until use. Three biological replicates were performed for each assay.

**Microscopic observation.** For microscopic observations, larvae from each well were filtered and fixed in a 1 ml glutaraldehyde (0.25 %)/paraformaldehyde (4 %) fixation solution (Sigma-Aldrich) overnight. Photographs were taken and treated using a BX51 system microscope (Olympus).

**RNA extraction and reverse transcription (RT) cDNA synthesis.**

Total RNA was extracted from each bacterial pellet using TRIzol reagent (Invitrogen), at a ratio of 1 ml TRIzol per pellet according to the manufacturer’s protocol. Bacterial RNA was separated using chloroform (0.2:1 chloroform: TRIzol, v/v) and precipitated using reagent (Invitrogen), at a ratio of 1 ml TRIzol per pellet according to RNA extraction and reverse transcription (RT) cDNA synthesis. The RT was carried out following the manufacturer’s protocol for RNA extraction and reverse transcription (RT) cDNA synthesis, in a total volume of 13 ml dNTP (10 mM) and 200 nM each primer. Primer 3 software was used to design QPCR primers (Table 1).

Reactions were initiated with a denaturation for 3 min at 95 °C, followed by 40 cycles at 95 °C for 10 s and at 60 °C for 20 s. Each run included blank controls (water) and dissociation curves for each primer. The threshold was set using an amplification-based algorithm from the MX 3000-3005 software (Stratagene) for the initial plate.

For the QPCR efficiencies of each primer pair used, standard curves were generated using eight serial dilutions (2.10\(^{-1}\) to 2.10\(^{-6}\)) of linearized plasmid (pCR2.1-TOPO; Invitrogen) containing an insert of gyrase-, metalloprotease-, aerobactin- and thermolabile haemolysin-encoding gene obtained by PCR (Table 1), and the efficiency of exochitinase primers was estimated directly with genomic 07/118 T2 DNA as the template. The level of expression of the target genes at different kinetic times, normalized to the gyrB housekeeping gene, was then calculated using the (1–efficiency)\(^{-DD_{t}}\) formula (Pfaffl, 2001) with reference to the point of infection at 30 min.

**QPCR.** QPCR analysis was conducted on an MX3000 and MX3005 thermocycler (Agilent) using Brilliant III Ultra-Fast SYBR Green QPCR master mix (Stratagene). Each reaction was run in triplicate with a final volume of 20 µl containing 5 µl cDNA (1/30 dilution) and 200 nM each primer. Primer 3 software was used to design QPCR primers (Table 1).

Reactions were initiated with a denaturation for 3 min at 95 °C, followed by 40 cycles at 95 °C for 10 s and at 60 °C for 20 s. Each run included blank controls (water) and dissociation curves for each primer. The threshold was set using an amplification-based algorithm from the MX 3000-3005 software (Stratagene) for the initial plate.

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**2D electrophoresis (2DE) and MS analyses.** Before IEF, a volume corresponding to 50 µg proteins for each ECP fraction was prepared and concentrated for 2DE analysis using the Ready Prep 2-D Cleanup kit (Bio-Rad). The final pellet was suspended in 250 µl DeStreak Rehydration (GE Healthcare) containing 1 % IPG buffer pH 3–10 (GE Healthcare) for protein solubilization. Strips were hydrated with each ECP fraction for 15 h prior to IEF.

2DE was carried out using the protocol described by Galland et al. (2013). Briefly, the first dimension was performed on linear pH 3–10 gradient IPG strips (13 cm; GE Healthcare) in an Ettan IEF system (GE Healthcare), using the following protocol: step 1: 15 min at 250 V, step 2: 2 h at 500 V, step 3: 1 h gradient to 1000 V, step 4: 2.5 h gradient to 8000 V, and finally step 5: 1.5 h at 8000 V.

Before the second dimension, strips were equilibrated for 15 min in an equilibration solution (2 % SDS, 6 M urea, 30 % (v/v) glycerol,

### Table 1. Primers used in QPCR expression analysis and cloning

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Accession no.</th>
<th>Primer</th>
<th>Melting temperature ((T_{m})) (°C)</th>
<th>Sequence ((5’\rightarrow3’))</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gyrase B</td>
<td>490881616</td>
<td>gyrB-94F, gyrB-296R</td>
<td>60</td>
<td>TTGGTGAATCTGAGCAAACG</td>
<td>QPCR</td>
</tr>
<tr>
<td></td>
<td>574609375</td>
<td>Vtmp-F2, Vtmp-R2a</td>
<td>60</td>
<td>CGCAGTATGACCAAATCATGACG</td>
<td>Cloning PCR</td>
</tr>
<tr>
<td>Thermolabile</td>
<td>342822783</td>
<td>TL856-QF, TL996-QR</td>
<td>60</td>
<td>TCATCGATGCAATGACAGGT</td>
<td>QPCR</td>
</tr>
<tr>
<td>Exochitinase</td>
<td>342818655</td>
<td>exochi2018-QF, exochi2152-QR</td>
<td>60</td>
<td>ACGATTTCCTAGCTGACGATG</td>
<td>QPCR</td>
</tr>
<tr>
<td>Metalloprotease</td>
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<td>60</td>
<td>ACGTTTGGATACCCCGCTCATG</td>
<td>QPCR</td>
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<tr>
<td>Thermolabile</td>
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<td>Metallo-DF1, Metallo-Nc R1</td>
<td>60</td>
<td>TCACTCGATGACCAAATCATGACG</td>
<td>QPCR</td>
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<tr>
<td>Exochitinase</td>
<td>342822783</td>
<td>TL151F, TL1140R, exochi1092F, exochi2399R</td>
<td>60</td>
<td>TGAACCGTGATGACGAACTGAACT</td>
<td>QPCR</td>
</tr>
</tbody>
</table>
50 mM Tris/HCl pH 8.8 and bromophenol blue containing 1% DTT, followed by a 15 min incubation in the same solution containing 260 mM iodoacetamide. The strips were then transferred onto vertical 10–15% gradient SDS-polyacrylamide gels (12.5 cm × 12.5 cm) and the separation was performed at 50 mA per gel. Gels were stained with Coomassie blue solution (overnight) and destained in a methanol/acetic acid/water (30/7/63, by vol.) solution. Gels were scanned with SFLauncher software and compared using the Prodigy SameSpot software (non-linear dynamics).

Selected spots were manually excised from the gels and MS analysis was performed by the Structural and Functional Mass Spectrometry Facility in the Jacques Monod Institute (CNRS and Paris Diderot University, Paris, France). After trypsin digestion, the spots were analysed by MALDI-TOF-MS-MS. The system and peptide mass fingerprint obtained was examined using MASCOT (Matrix Science). MS-MS data were searched against the National Center for Biotechnology Information (NCBI) non-redundant database and V. tubiashii ATCC 19109 genome NCBI database. Peptide validator node was used for false discovery rate determination and a 1% threshold was used for the obtaining was examined using MASCOT (Matrix Science). MS-MS data were searched against the National Center for Biotechnology Information (NCBI) non-redundant database and V. tubiashii ATCC 19109 genome NCBI database. Peptide validator node was used for false discovery rate determination and a 1% threshold was used for the validation of peptide identification. Protein localization were predicted using the MicroScope microbial genome annotation and analysis platform (https://www.genoscope.cns.fr/agc/microscope/).

**Statistical analysis.** Significant differences between analyses were tested using Student’s t-test in BiostaTGV (http://marne.u707.jussieu.fr/biostatgv/). Results were considered significant at P<0.05.

**RESULTS**

**Virulence of the French V. tubiashii 07/118 T2 on oyster larvae**

The virulence of the 07/118 T2 strain on 13-day-old oyster larvae was estimated 24 h post-infection (Fig. 1). V. tubiashii 07/118 T2 caused important dose-dependent mortality: no live larvae were observed at 10^6 c.f.u. ml^-1 and the LD_{50} values were approximately 2.3 × 10^5 c.f.u. ml^-1 (Fig. 1a). Moreover, 100% of mortality was observed with heat-killed V. tubiashii 07/118 T2 at 10^6 c.f.u. ml^-1 and with live bacteria preincubated with EDTA (metal ion chelator). However, no mortality was observed with the boiled 07/118 T2 strain, preincubated with EDTA, at 10^6 c.f.u. ml^-1. In the positive controls (Fig. 1b), V. coralliilyticus caused significant mortality even at 10^5 c.f.u. ml^-1 (only 6% live larvae) compared with 07/118 T2. With the negative control, V. nigripulchritudo, no significant mortality was detected even at 10^6 c.f.u. ml^-1.

**Toxicity of total and fractionated V. tubiashii 07/118 T2 ECPs on oyster larvae**

ECPs of V. tubiashii 07/118 T2 induced a dose-dependent toxicity on 13-day-old oyster larvae (Fig. 2), with an LD_{50} estimated at around 7.16 µg protein per well (for 50 larvae). No larval mortality was observed after preincubating the ECPs with 5 mM EDTA, regardless of the concentration of ECPs tested.

Using GP-HPLC, two major fractions were separated from V. tubiashii 07/118 T2 ECPs (Fig. 3a). Unlike the second eluted fraction (F2), which had azocaseinase activity, the first eluted fraction (F1) did not show any activity (Fig. 3b). However, both fractions were toxic to larvae. Indeed, we observed 43% mortality in the presence of the F1 fraction, and 70% in the presence of the F2 fraction. Mortality of 100% was observed after the reconstitution of ECPs (mixing F1 and F2). The preincubation of F1 and F2 with EDTA (5 mM) prevented toxicity (Fig. 3c).

**2DE and MALDI-TOF MS-MS analyses of GP-HPLC fractions**

The two GP-HPLC fractions obtained from crude V. tubiashii 07/118 T2 ECPs were separated on large 2DE gels in a pH range of 3–10 (Fig. 4). More proteins were detected in the first fraction of ECPs (F1) than in the second one (F2). For both, most of the proteins detected were localized in the acidic part of the pH range. For F1, visual inspection revealed different proteins that differed in their molecular masses. However, for F2 the detected proteins were in a...
single horizontal line and differed only by their isoelectric point (Fig. 4).

All the visualized proteins, i.e. 19 spots for F1 and 12 spots for F2, were analysed by using MS. Only 12 proteins for F1 and 5 proteins for F2 were identified with no ambiguity using MALDI-TOF MS-MS analyses (Fig. 4, Table S1, available in the online Supplementary Material). F1 showed a diversity of outer-membrane proteins (porin-like protein H precursor, outer-membrane channel protein, long-chain fatty acid transport protein, outer-membrane protein N and hypothetical proteins). By contrast, the second fraction of ECPs (F2) appeared as an enrichment of a unique protein identified as an extracellular zinc metalloprotease (GenBank/EMBL/DDBJ accession no. EGU55378) potentially secreted in different isoforms.

**Phenotypic description of C. gigas larvae after incubation with V. tubiashii 07/118 T2 and with total and fractionated ECPs**

After a 24 h incubation period in sterile sea water, control larvae were observed to be swimming normally, to have a velum with cilia, two closed shells and regular soft-tissue pigmentation. The same phenotypic profile was observed in fixed larvae (Fig. 5a) apart from the absence of the velum, which is typically in a retracted state when larvae are fixed (Elston, 1999).

The first morphological changes started to appear 6 h post-infection with 07/118 T2 (10⁶ c.f.u. ml⁻¹). Larvae that had a damaged velum, that were immobile or exhibiting a circular swimming movement were observed (Fig. 5a). The majority of larvae were considered as dead (no motion) with damaged tissues as early as 16 h post-infection. Twenty-four hours post-infection, an almost complete decomposition of velar epithelial cells was observed (a clear shell) with micro-organisms swarming around cellular debris (Fig. 5a). Larvae infected with V. coralliilyticus, the positive control, displayed a similar phenotype (Fig. 5a).

After a 24 h period of incubation with 07/118 T2 ECPs, larvae stopped moving (a sign of death) and fixed larvae showed soft-tissue decomposition (Fig. 5b). With GP-HPLC fractions 1 (F1) and 2 (F2), the same phenotype was observed as with the ECPs (swimming stopped) and fixed larvae showed signs of the start of decomposition after incubation with F2 (Fig. 5b).

**Expression of genes encoding potential virulence factors during infection with V. tubiashii 07/118 T2**

The relative expression of some potential virulence genes from V. tubiashii 07/118 T2 was determined using RT-QPCR at different larval infection times (3, 6, 16 and 18 h) (Fig. 6). The chosen genes corresponded to previously described factors implicated in bacterial virulence: thermolysin family (Mersni-Achour et al., 2014), metalloprotease (Mersni-Achour et al., 2014) and exochitinase (Wang et al., 2001).

Metalloprotease-encoding gene expression underwent a small downregulation during the first few hours of infection (3 h), a significant (20-fold) increase in expression was observed 16 h post-infection (P<0.05). This overexpression remained stable even at 18 h post-infection (Fig. 6a). At the same post-infection time points (16 and 18 h), azocaseinase activity was detected in the supernatant of mixtures of larvae and bacterial cells (Fig. 6b).

By contrast, tlh gene expression was significantly upregulated during the first few hours of larval infection (3 and 6 h, P<0.05) (Fig. 6c). Exochitinase-encoding gene expression showed few variations: a little downregulation 6 h post-infection, followed by a significant upregulation by 16 h (P<0.05) (Fig. 6d).

**DISCUSSION**

It has previously been demonstrated that the French V. tubiashii 07/118 T2 and its ECPs cause pathogenesis to juvenile Pacific oyster (Travers et al., 2014). Moreover, we previously showed the proteolytic fraction of those toxic ECPs contained (i) active and thermostable extracellular zinc metalloprotease(s) and (ii) a protease belonging to the thermolysin family (Mersni-Achour et al., 2014). However, the implication of this protease and its expression during an infection in vivo was still unknown. In this paper, taking advantage of the larvae immersion model, we demonstrated...
that this metalloprotease is expressed in vivo. Moreover, an enriched fraction of ECPs containing this protein appeared toxic to larvae, suggesting its implication into the toxicity. However, we also demonstrated that other GC-HPLC fractions that did not contain this protein were also toxic to larvae, revealing that *V. tubiashii* virulence cannot be attributed to one unique factor.

Using experimental infection of larvae, we showed that the French *V. tubiashii* (represented by the model strain 07/118 T2) is virulent to 13-day-old larvae with an LD50 value of approximately 2.3 × 10^3 c.f.u. ml^{-1} at 24 h post-infection (Fig. 1). These results were comparable to those found with some *V. corallilyticus* strains [initially described as *V. tubiashii* (Wilson et al., 2013; Richards et al., 2014)] by Estes et al. (2004), who observed an LD50 of 1.6–3.6 × 10^4 c.f.u. ml^{-1} at 24 h post-infection in 10–12-day-old larvae. Secondly, consistent with previous studies (Takahashi et al., 2000; Hasegawa et al., 2008), the 07/118 T2 culture supernatants showed a high toxicity to larvae (Fig. 2). Furthermore, with the aim of exploring the molecular diversity of these toxic ECPs, a gel permeation-HPLC separation was conducted. Both collected fractions showed a partial toxicity to oyster larvae, with the greatest toxicity for

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**Fig. 3.** (a) Fractionation of *V. tubiashii* 07/118 T2 ECPs by GP-HPLC into two major fractions, F1 and F2. (b) Each eluted fraction was assayed for azocaseinase activity. (c) Toxicity of fractions (5 μg protein per well) on 13-day-old oyster (*C. gigas*) larvae in the absence (black bars) and presence (grey bars) of 5 mM EDTA at 24 h post-infection. The error bars indicate the SD of three biological replicates. AU, Absorbance units.
the second fraction (F2), the only one exhibiting protease activity. It is interesting also to note that the reconstitution of ECPs with the two GP-HPLC fractions caused total toxicity to the larvae, although the loss of some of the toxins may have occurred during the purification step (Fig. 3).

During experimental infections, larval pathological manifestations were monitored (Fig. 5). It was reported that *V. tubiashii* caused bacillary necrosis, a larval and juvenile bivalve disease characterized by a reduction in larval motility and the appearance of bacterial cells (Tubiash *et al.*, 1965). Histological sections showed an extensive destruction of larval epithelial cells with a massive bacterial invasion and proliferation (Tubiash *et al.*, 1965; Estes *et al.*, 2004; Elston *et al.*, 2008). It is important to note that some of the *V. tubiashii* strains, RE 22, RE 98 and ATCC 19105 [which were described by Tubiash *et al.* (1965), Estes *et al.* (2004), Elston *et al.* (2008) and Hasegawa *et al.* (2008)], were recently reclassified as *V. coralliilyticus* (Wilson *et al.*, 2013; Richards *et al.*, 2014). This means that some isolates of *V. coralliilyticus* could also cause a disease with similar symptoms to the bacillary necrosis. Herein, with the French *V. tubiashii*, approximately the same signs of bacillary necrosis were observed. However, infection with 07/118 T2 ECPs showed a less pronounced effect (without complete decomposition) and GP-HPLC fractions caused larval mortality without reproducing the same phenotype observed with live bacteria or with crude ECPs.

Results obtained, both by experimental infections and by microscope observations, raise the question of whether proteases, specifically metalloproteases as reported elsewhere (Binesse *et al.*, 2008; Hasegawa *et al.*, 2008; Labreuche *et al.*, 2010), are the sole primary virulence factor or whether the association of several factors is required to induce a complete infection phenotype. The toxicity of the GP-HPLC fraction 1, which doesn’t present protease activity, is an important finding to support this second assumption.

It has been described that pathogenic *Vibrio* is capable of producing various pathogenic factors. Of these, enterotoxins, haemolysin and cytotoxins are the direct toxic factors causing the symptoms, whereas siderophores and adhesive factors are indirect factors involved in the establishment of the infection, and proteases are recognized as playing pathogenic roles in the subsequent infection (Shinoda & Miyoshi, 2011).

In order to explore the involvement of several factors in the pathogenicity of the French *V. tubiashii*, other characterization tests were applied using EDTA as a cation chelating agent, to further characterize metalloprotease activity (Teo *et al.*, 2003; Miyoshi *et al.*, 2002; Sousa *et al.*, 2007; Hasegawa *et al.*, 2009; Labreuche *et al.*, 2010), and heat treatment to kill the bacteria via the restriction of adhesion capacity, proliferation ability and the denaturation of a group of thermolabile factors. Interestingly, neither the use of EDTA nor the heat-killing of the bacteria were sufficient to limit the toxicity of the French *V. tubiashii* (Fig. 1).

Indeed, heat treatment could destroy the bacterial cell and make it non-culturable but couldn’t abolish all pathogenic effectors (Fontana, 1988; Kudryashova *et al.*, 1998; Lin *et al.*, 2010; Shinoda & Miyoshi, 2011). Moreover, the addition of EDTA into the infection bath could partially affect protease activity (Mersni-Achour *et al.*, 2014; Labreuche *et al.*, 2010; Teo *et al.*, 2003) and some bacterial properties (Kavitha *et al.*, 2013), but not all
bacterial virulence factors. However, when we combined the chemical and physical treatments, toxicity to larvae was removed (Fig. 1). In addition, using EDTA, the importance of the secreted metalloprotease in the toxicity of the French 
V. tubiashii supernatants was confirmed; more clearly and completely with the GP-HPLC fraction 2. Nevertheless, a total inhibition of the fraction 1 toxicity was observed despite the absence of protease activity. This could be explained by an interaction of EDTA with other kinds of secreted toxins (Marvin et al., 1989).

These results expand previous observations on the implication of multiple factors on the toxicity of the French 
V. tubiashii.

In order to examine the composition of culture supernatants in more details, a 2D separation of GP-HPLC fractions coupled with MALDI-TOF MS-MS analyses was performed. MS revealed the presence of some potential toxic factors in fraction 1, such as the UDP-sugar hydrolase [cleaves uridine nucleotides (Glaser et al., 1967)], the outer-membrane protein N, the porin-like protein H precursor, the long-chain fatty acid transport protein and some hypothetical proteins (Table S1). It should be noted that some receptor, transport and channel proteins were also detected in fraction 1. Their presence may have come from bacterial cell lysis during the experimental steps. Unlike fraction 1, only one protein was detected in fraction 2 by MS analyses and was identified with no ambiguity as an extracellular zinc metalloprotease (GenBank/EMBL/DDBJ accession no. EGU55378) (Table S1). Extracellular metalloproteases have been well studied as the main pathogenic factors in several species of Vibrio pathogen for the Pacific oyster (Binesse et al., 2008; Hasegawa & Hase, 2009b; Hasegawa et al., 2008; Labreuche et al., 2010). Nevertheless, other virulence factors have also been found to play an important role in the pathogenicity of the genus Vibrio. Indeed, Valiente et al. (2008) suggested that the pathogenicity of Vibrio vulnificus in the eel is caused not only by bacterial growth in the blood and internal organs, but also by the effect of potent toxic factors other than metalloproteinase. They explain that the abundance of proteases in ECPs could mask the effect produced by other cytotoxins like the RtxA toxin. Duperthuy et al. (2010, 2011) showed that the outer-membrane protein OmpU is required for Vibrio splendidus LGP32 virulence by acting on the adhesion and on the destruction of the host-cell actin cytoskeleton during the invasion step. Lee et al. (2008) proved that V. vulnificus RtxA toxin (a pore-forming protein toxin) induced apoptotic death in human intestinal epithelia. Moreover, long-chain fatty acid transport proteins have the ability to uptake lipids used for bacterial growth (Black, 1988), which were also shown to be essential for larval development (Chu & Webb, 1984).

The monitoring of gene expression of some potential virulence factors during infection was also carried out in this study. Results showed that one metalloprotease-encoding gene (Mersni-Achour et al., 2014) and one exochitinase-encoding gene are upregulated 16 h post-infection, while the thermolabile haemolysin-encoding gene was upregulated at all the infection times examined.

![Fig. 5. Morphological evolution of 13-day-old C. gigas larvae (a) during infection by V. tubiashii 07/118 T2 strain at 10^5 c.f.u. ml⁻¹, and (b) during incubation with V. tubiashii 07/118 T2 ECPs and GP-HPLC fractions. Pictures were taken using a BX51 system microscope (Olympus). Arrows indicate bacteria. F1, GP-HPLC fraction 1; F2, GP-HPLC fraction 2; SSW, sterile sea water.](image-url)
This difference in the gene expression of some virulence factors was in accordance with the morphological evolution of oyster larvae observed during infection (Hasegawa & Häse, 2009a; Krukonis & DiRita, 2003).

In summary, in order to understand the processes leading to larval disease, *C. gigas* larvae were used as an interaction model. Results showed that the virulence of the French *V. tubiashii* seems to be related to multiple toxic factors (including a metalloprotease). Further studies will be required, using gene mutation or recombinant proteins of the potent virulence factors, to further understand the involvement of these toxins in the virulence of the French strain.

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