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

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Abstract :

Vibrio tubiashii is a marine pathogen isolated from larval and juvenile bivalve molluscs causing 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 *V. tubiashii* is virulent to larvae and can cause bacillary necrosis symptoms with a LD50 about 2.3 × 103 cfu ml-1 after 24 h. Moreover, complete or GP- HPLC fractionated extracellular products (ECPs) of this strain appeared toxic to larvae. MS-MS analysis of the different ECPs 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 *V. tubiashii* in *C. gigas* oysters is caused by a group of toxic factors and not only the metalloprotease

Keywords : *Crassostrea gigas*, ECPs, Pathogenicity, Toxins, Mass spectrometry

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, *V. 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), 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 done to characterize toxic molecules (metalloproteinases, ciliostatic toxin, cytolysin…) and mechanisms associated with oysters mortalities (juveniles and larvae) (Kothary *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 or with live bacteria initially all classified as *V. tubiashii*. However, recent studies demonstrated that strains RE22, RE98, LMG 1095 and ATCC19105 thought to be *V. tubiashii* are finally strains of *V. corallilyticus* (Ben-Haim *et al*., 2003; Wilson *et al*., 2013; Richards *et al*., 2014b).

This reclassification of some *V. tubiashii* strains does not change the fact that this bacterium is considered as one of the potential pathogen of bivalves usually associated with major mortality events in shellfish hatcheries but reveals that *V. coralliilyticus* should be also treated as a serious threat contributing to a big loss in bivalve hatcheries (Richard *et al.*2014a).

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

As few knowledge is available about true *V. tubiashii* strains and their mechanisms of pathogenicity, the present study aims at (i) reproducing the pathogenicity of the French *V. tubiashii* and the toxicity of its ECPs on Pacific oyster larvae, and (ii) characterizing 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.

MATERIAL AND 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 *V. nigripulchritudo* LMG3896^T (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 glycerol 15% (v/v).

Larval production

Larval production was achieved according to the protocol described by Dégremont *et al.* (2005) at the Ifremer hatchery in La Tremblade. Briefly, fifteen *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 analyzed under 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-liter tanks at 26 °C in filtered sea water, which was changed 3 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 length were between 200 to 300 µm.

Preparation of *V. tubiashii* ECPs, fractionation and biochemical assays

Extracellular products were produced using the cellophane overlay method as described previously by Travers *et al.* (2014). Briefly, bacteria were grown in 5 ml of LBS at 22 °C for 18 h. A volume of 1 ml of exponential phase culture (OD_{600} =1) was spread on a sterile cellophane film overlying LBS Agar plates. After 48 h of incubation at 22 °C, the cells were washed off the cellophane using 10 ml of cold sterile artificial sea water and removed by centrifugation at 3,000 × g (45 min at 4°C). The supernatant was filtered at 0.22 µm and then concentrated by lyophilisation, resuspended in Tris-HCl buffer (50 mM Tris-HCl, pH 8.0) and dialyzed 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-high performance liquid chromatography (GP-HPLC). This purification step was performed using a Biosuite TM 250 column (5 µm, 300 × 7.8 mm, Waters) and conducted with a Waters system (600 Controller, 2996 Photodiode Array detector and 2707 Autosampler). 10 µg per run of crude ECP fractions were 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 lyophilisation, 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 Weights 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 (Bradford, 1976) using Bradford reagent (Sigma-Aldrich) and bovine serum albumin 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) of *Vibrio* strains grown in LBS at 22°C were centrifuged at 3,000 × g for 20 min, washed twice and resuspended in sterile sea water. In a 6-well flat-bottomed plate (Falcon), 1.5 ml of ten-fold dilutions of the cell suspensions between 10 and 10^6 cfu 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 (Ethylenediaminetetraacetic acid) (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 of protein per ml) and GP-HPLC fractions of ECPs (f.c. 5 µg of proteins per ml) were tested on model larvae in the presence and absence of EDTA (preincubated with EDTA 5 mM, f.c., 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 of larvae (about 50 larvae per ml) with 25 ml of bacteria (10^5 cfu 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 3,000 × g for 15 min was carried out and the pellet (larvae + bacteria) was conserved with RNAprotect Bacteria Reagent (Qiagen, UK) to stabilize/fix the bacterial RNA for Quantitative polymerase chain reaction (QPCR) assays. Pellets were stored at -80°C until use. Three biological replicates were performed for each assay and assays were replicate.

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 cold isopropanol (0.5:1 isopropanol:Trizol). RNA pellets were washed with 1 ml of 75% ethanol and resuspended in ultrapure water. 17 µl RNA suspensions were treated with 2 µl of DNase (2U for 10 µg of RNA) (Ambion) for 45 min at 37°C. Total RNA concentration was analyzed by spectrophotometry (Nanodrop/Thermo Scientific) and a negative RT-QPCR with diluted RNA was performed to check RNA purity.

The RT was carried out following the protocol of SuperScript™ III Reverse Transcriptase (Invitrogen). For the first-strand cDNA Synthesis, in a total volume of 13µl, 800 ng of total RNA was well mixed with 200 ng of random primers, 1 µl of dNTP (10 mM) and nuclease free water. The mix was incubated at 60 °C for 5 min and then chilled on ice. 1 µl of SuperScript III RT (200 U μl^{-1}) was added to 1 µl of RNase OUT (40 U μl^{-1}), 1 µl of DTT (dithiothreitol) and 4 µl of 5X buffer. The reaction mix was incubated for 5 min at 25 C, followed by an activation step of 60 min at 50 °C and ending with a deactivation step of 15 min at 70 °C. cDNA was conserved at -20°C until use.

Quantitative polymerase chain reaction

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 of each primer. Primer 3 software was used to design QPCR primers (Table 1).

Reactions were initiated with an initial 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 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^{-8}) of linearized plasmid (pCR2.1-TOPO, Invitrogen) containing an insert of gyrase, metalloprotease, aerobactin and thermolabile hemolysin 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 $(1+\text{efficiency})^{-\Delta\Delta Ct}$ formula (Pfaffl, 2001) with reference to the point of infection at 30 min.

Two Dimensional electrophoresis (2-DE) analyses and mass spectrometric analyses

Before isoelectric focusing, a volume corresponding to 50 µg of proteins for each ECP fraction was prepared and concentrated for 2-DE analysis using the Ready Prep 2-D Cleanup kit (Biorad). The final pellet was suspended in 250 µl of Destreak Rehydration (GE Healthcare) containing 1% IPG buffer pH 3–10 (GE Healthcare) for protein solubilization. Strips were hydrated with each ECPs fraction for 15 h prior to IEF (isoelectric focusing). 2-DE 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 IPGphor III isoelectric focusing 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 8, 000 V, and finally step 5: 1.5h at 8, 000 V.
Before the second dimension, strips were equilibrated for 15 min in an equilibration solution (2% SDS, 6 M Urea, 30% glycerol (v/v), 50 mM Tris–HCl pH 8.8 and bromophenol blue containing 1% of DTT, followed by a 15-min incubation in the same solution containing 260 mM of 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: v/v/v) solution. Gels were scanned with SFLauncher software and compared using the Prodigy SameSpot software (Nonlinear Dynamics).

Selected spots were manually excised from the gels and mass spectrometric analysis was performed by the "Structural and Functional Mass Spectrometry Facility" in the JACQUES MONOD Institute (CNRS and Paris-Diderot University). After trypsin digestion, the spots were analyzed by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight tandem mass spectrometry (MALDI TOF–TOF). The system and peptide mass fingerprint obtained was examined using MASCOT (Matrix Science Ltd, London, UK). MS-MS data were searched against NCBInr 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 MicroScope Microbial Genome Annotation & Analysis Platform (https://www.genoscope.cns.fr/agc/microscope/)

Statistical analysis

Significant differences between analyses were tested using the "Student t" test in 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-days 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 cfu ml^{-1} and the LD_{50} (lethal dose 50%) values were approximately 2.3×10^3 cfu ml^{-1} (Fig. 1a). Moreover, 100% of mortality was noticed with heat-killed *V. tubiashii* 07/118 T2 at 10^6 cfu ml^{-1} and with live bacteria preincubated with EDTA (ion metal chelator). However, no mortality was observed with the boiled 07/118 T2 strain, preincubated with EDTA, at 10^6 cfu ml^{-1}. In the positive controls (Fig. 1b), *V. coralliilyticus* caused significant mortality even at 10^3 cfu 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 cfu ml^{-1}.

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

Extracellular products of *V. tubiashii* 07/118 T2 induced a dose dependent toxicity on 13-days old oyster larvae (Fig. 2), with an LD_{50} estimated around 7.16 µg of 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, eluted fraction 1 (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. 100% mortality was observed after the reconstitution of ECPs (mixing F1 + F2). The preincubation of F1 and F2 with EDTA (5 mM) prevented toxicity (Fig. 3c).

Two dimensional electrophoresis and MALDI-TOF TOF analyses of GP-HPLC fractions

The two GP-HPLC fractions obtained from crude *V. tubiashii* 07/ 118 T2 ECPs were separated on large 2-DE gels in a p*I* 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 localised in the acidic part of the pH range. For F1, visual inspection revealed different proteins which differed in their molecular weights. However, for F2 the detected proteins were in single horizontal line which differed only by their isoelectric point (Fig. 4).

All the visualised proteins, ie 19 spots for F1 and 12 spots for F2, were analysed by using mass spectrometry. Only 12 proteins for F1 and 5 proteins for F2 were identified with no ambiguity using MALDI-TOF TOF analyses (Fig. 4, Supplementary table1). 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" (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 (10^5 cfu 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 microorganisms 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 factor 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 h, 6 h, 16 h and 18 h) (Fig.6). The chosen genes corresponded to previously described factors implicated in bacterial virulence: thermolabile hemolysin (TLH) (Wong *et al.*, 2012), metalloprotease (Mersni *et al*. 2014) and exochitinase (Wang *et al.*, 2001).
Metalloprotease gene expression underwent a small down regulation 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 over expression remained stable even 18 h post infection (Fig. 6a). At the same post-infection time-points (16 h and 18 h), azocaseinase activity was detected in the supernatant-of mixtures of larvae and bacterial cells (Fig. 6a).
By contrast, tlh gene expression was significantly up-regulated during the first few hours of larval infection (3 h and 6 h, $p < 0.05$) (Fig. 6b). Exochitinase gene expression showed few variations: a little down-regulation 6 hours post-infection, followed by a significant up-regulation by 16 h ($p< 0.05$) (Fig. 6c).

DISCUSSION

It has previously been demonstrated that the French *V. tubiashii* 07/118 T2 and its extracellular products cause pathogenesis to juvenile Pacific oyster (Travers *et al.*, 2014). Moreover, we previously showed the proteolytic fraction of those toxic ECPs contained (1) active and thermostable extracellular zinc metalloprotease(s) and (2) a protease belonging to the thermolysin family (Mersni *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 larvae immersion model, we demonstrated that this metalloprotease is expressed *in vivo*. Moreover, an enriched fraction of extracellular products 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 are 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-days old larvae with an LD50 value of approximately 2.3×10^3 cfu ml^{-1} 24 h post-infection (Fig.1). These results were comparable to those found with some *V. coralliilyticus* strains (initially described as *V. tubiashii* (Wilson *et al.*,2013*;* Richards *et al.*,2014b)) by Estes *et al.* (2004), who observed an LD_{50} between 1.6 and 3.6×10^4 cfu ml^{-1} 24 h post infection in 10- to 12-days 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 to explore the molecular diversity of these toxic ECPs, a gel permeation-HPLC separation was conducted. Both collected fractions (2 fractions) showed a partial toxicity to oyster larvae with the greatest toxicity for 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 formation 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 *V. tubiashii* strain, the 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.*,2014b). This means that some isolates from *V. coralliilyticus* could also cause a disease with similar symptoms as the one caused by 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 previously reported (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, hemolysin 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 killed 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 *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 kind 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 2-D separation of GP-HPLC fractions coupled with MALDI TOF- TOF analyses was performed. Mass spectrometry 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 (Supplementary Table 1). 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.

Unike fraction 1, only one protein was detected in fraction 2 by mass spectrometry analyses and identified with no ambiguity as an extracellular zinc metalloprotease (accession no. EGU55378) (Supplementary Table 1). 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 & Häse, 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 *V. vulnificus* in the eel is not only caused by bacterial growth in the blood and internal organs, but also by the effect of potent toxic factors other than metalloprotease. 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 (OMP) OmpU is required for *V. 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 (Mersni *et al.*, 2014) and one exochitinase gene are up regulated 16 h post infection, while, the thermolabile hemolysin gene was up regulated at all the infection times examined (Fig. 6). 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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Table

Table 1: Primers used in QPCR expression analysis and cloning

Amplicon	Accession Number	Primers	Melting temperature (Tm)	Sequence	Application
Gyrase B	490881616	gyrB-94F gyrB-296R	60°C	TTGGTGAATCTGAGCAAACG CGCTTGGATACCACCTTCAT	**QPCR**
Metalloprotease	574609375	Vtmp-F2 Vtmp-R2a	60°C	TCATCGATGCAATGACAGGT AGCATTTTCCAGCTTGCACT	
Thermolabile hemolysin	342822783	TL856-QF TL996-QR	60°C	AAGGCTCCGCAGTTCAAGTA GTTTGCATCGTAGAGCACCA	
Exochitinase	342818655	exochi2018-QF exochi2152-QR	60°C	AAGGTGCGTCAGCTTCAGAT AGGCATCAACCACAACAACA	
Gyrase B	490881616	VgyrB-274F VgyrB-1171R	60°C	GAAGTTATCATGACGGTACTTC CCTTTACGACGAGTCATTTC	**Cloning** **PCR**
Metalloproteae	574609375	Metallo-DF1 Metallo-Nc R1	60°C	CAACAACGTCATTTAAGCTGGA TACTCGTCGTTGGTTTGCTG	
Thermolabile hemolysin	342822783	TL151F TL1140R	60°C	CCTGCTCAAACTCATGACGA TGAACCGTGATGAGCACAAT	
Exochitinase	342818655	exochi1092F exochi2399R	60°C	TGGTGTGGGTTACGACAAGA ACCGCTGTATCAACCCAAAG	

Figure legends

Figure 1: (a) Dose-response effect of *V. tubiashii* 07/118 T2 (10-10^6 cfu/ml) on 13-day old *C.gigas* larvae estimated 24 h post infection. The virulence of *V. tubiashii* 07/118 T2 was also assayed with a heat killed strain and in the presence or absence of EDTA. **(b)** *V. nigripulchritudo* (*V. nigri*) and *V. coralliilyticus* (*V. coralli*) were used as controls at 10^3 and 10^6 cfu/ml. The error bars indicate the standard deviation of the three biological replicates.

Figure 2: Dose-response effect of *V. tubiashii* 07/118 T2 ECPs (2.5 µg, 5 µg and 10 µg of proteins per well) on 13-day old *C. gigas* larvae in the absence (black bars) and presence (grey bars) of EDTA (f.c 5mM) 24 h post infection. The error bars indicate the standard deviation of the three biological replicates.

Figure 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 of protein per well) on 13-day old oyster (*C. gigas*) larvae in absence (black bars) and presence (grey bars) of 5 mM EDTA 24 h post infection. The error bars indicate the standard deviation of the three biological replicates.

Figure 4: 2-DE patterns of 07/118 T2 ECP GP-HPLC fractions (F1 and F2). Proteins (50 µg of protein loaded) were separated over pH range 3–10 (13cm strips) and gradient (10-15%) SDS-polyacrylamide gel. The gel was stained with Coomassie blue. Numbered spots correspond to those showing qualitative differences and associated letters designate the same protein but with different protein/ion score. The identified proteins using MS or MS/MS are labeled with spot numbers. F1: GP-HPLC fraction 1, F2: GP-HPLC fraction 2

Figure 5: Morphological evolution of 13-day old *C. gigas* larvae during infection by **(a)** *V. tubiashii* 07/118 T2 strain at 10^5 cfu ml^{-1} and **(b)** *V. tubiashii* 07/118 T2 ECPs and GP-HPLC fractions. Pictures were taken and treated using a BX51 System Microscope (Olympus®). Arrows indicate bacterial likes. SSW: sterile sea water, *V. cora*: *V. coralliilyticus* 06/210, F1: GP-HPLC fraction 1 and F2: GP-HPLC fraction 2.

Figure 6: The relative expression of **(a)** metalloprotease **(b)** thermolabile hemolysin and **(c)** exochitinase genes from *V. tubiashii* 07/118 T2 strain at different times of larval infection (3 h, 6 h, 16 h, and 18 h). **(a')** Azocaseinase activity (UP: unity of protease) assayed in culture supernatant during larval infection. The level of expression of the target genes was normalized to the *gyrB* housekeeping gene and was calculated using the (1+efficiency) $^{-\Delta\Delta Ct}$ formula with reference relative expression at 30 min. The error bars indicate the standard deviation of the three biological replicates per time of infection. The line corresponds to the threshold of expression (relative expression >1 means up-regulated). An asterisk indicates a significant ECP effect (Student's t, $p < 0.05$).

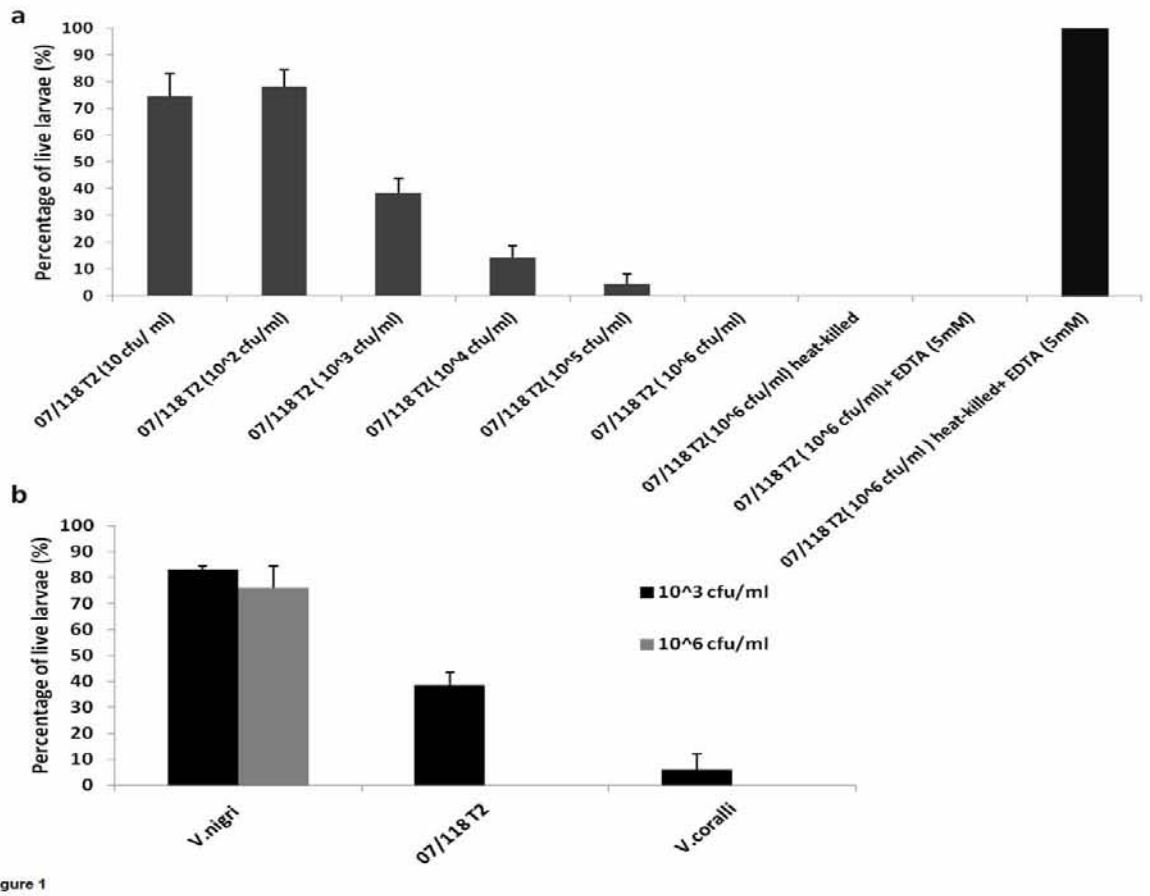


Figure 1

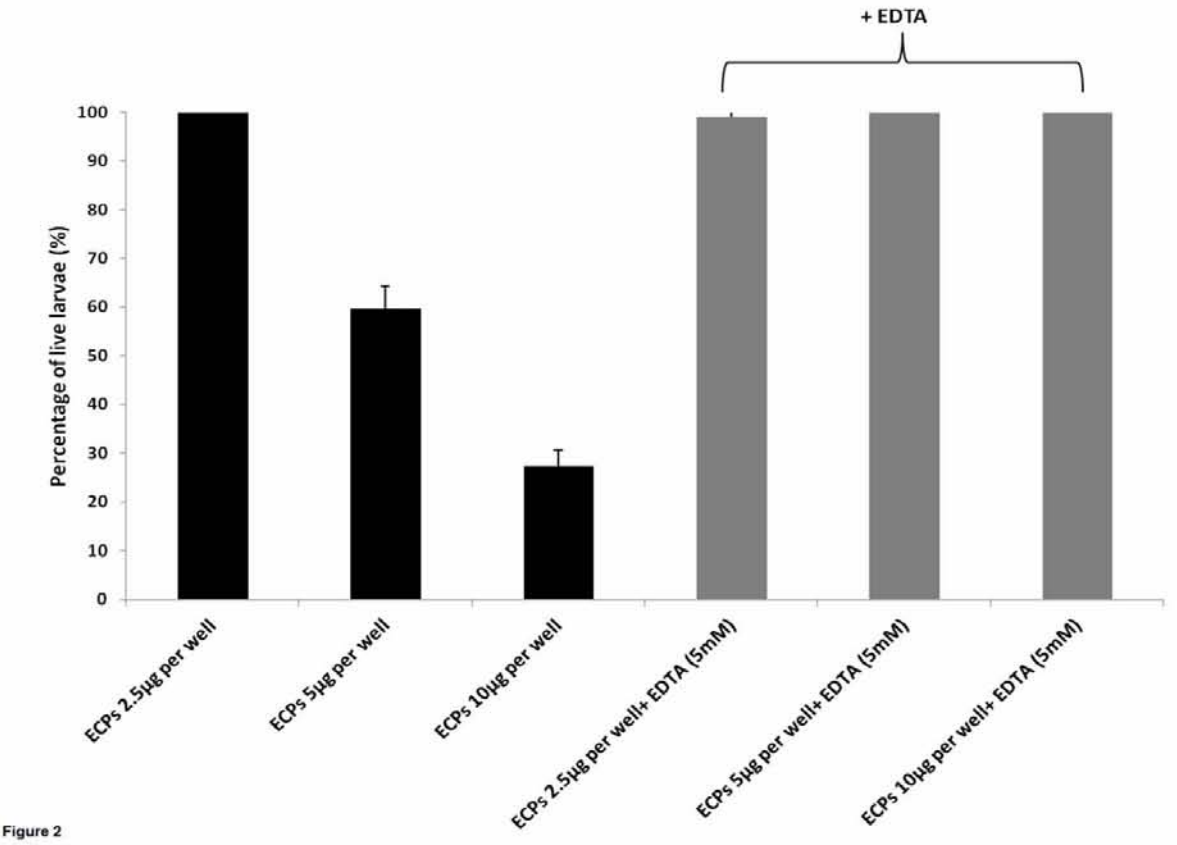
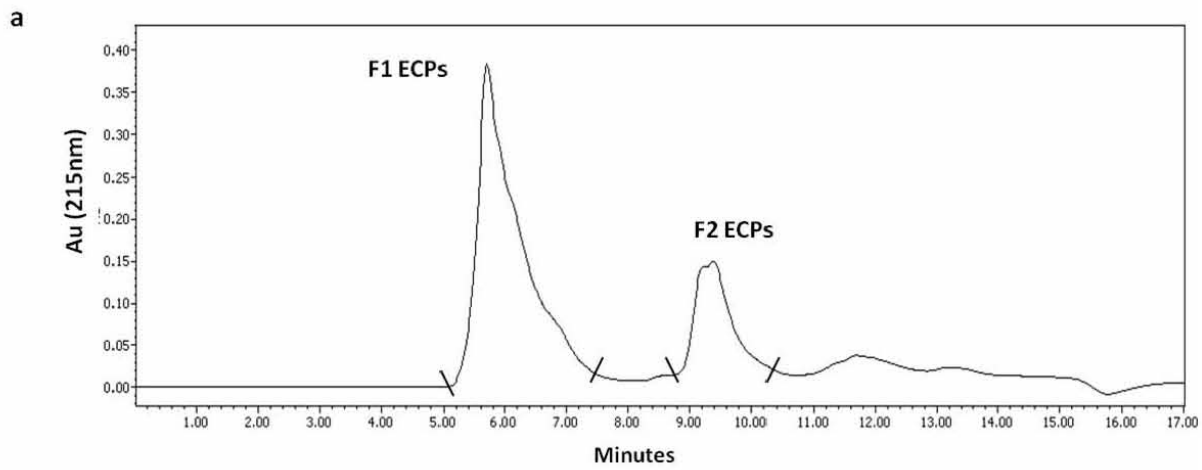


Figure 2



b

Azocaseinase activity	-	++

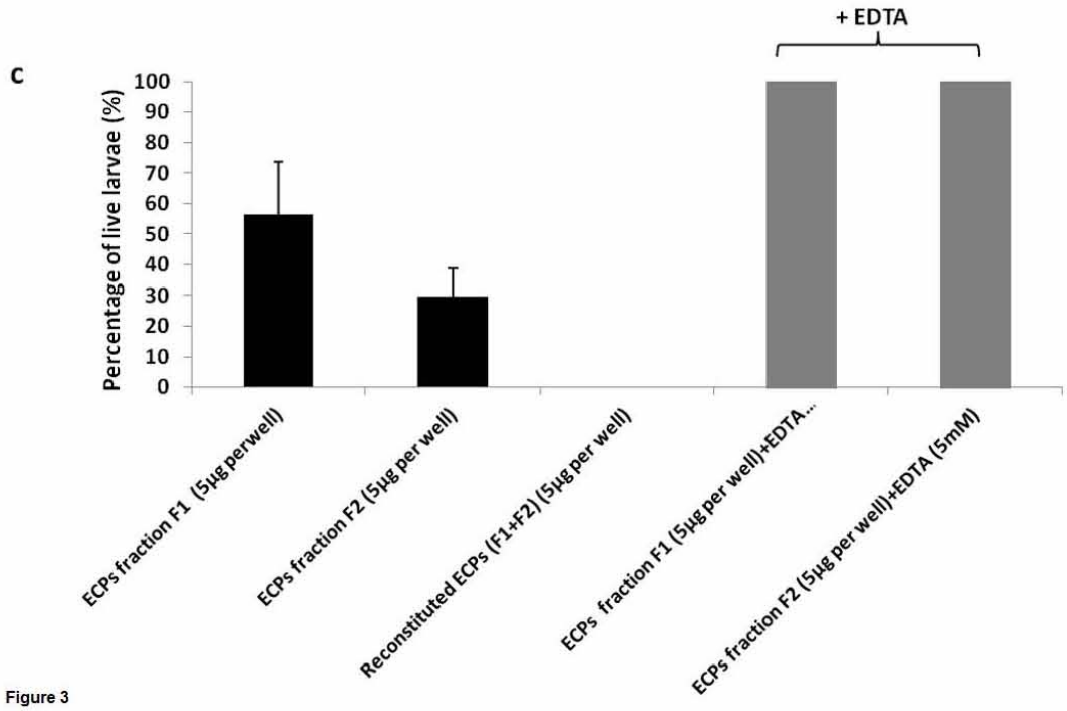


Figure 3

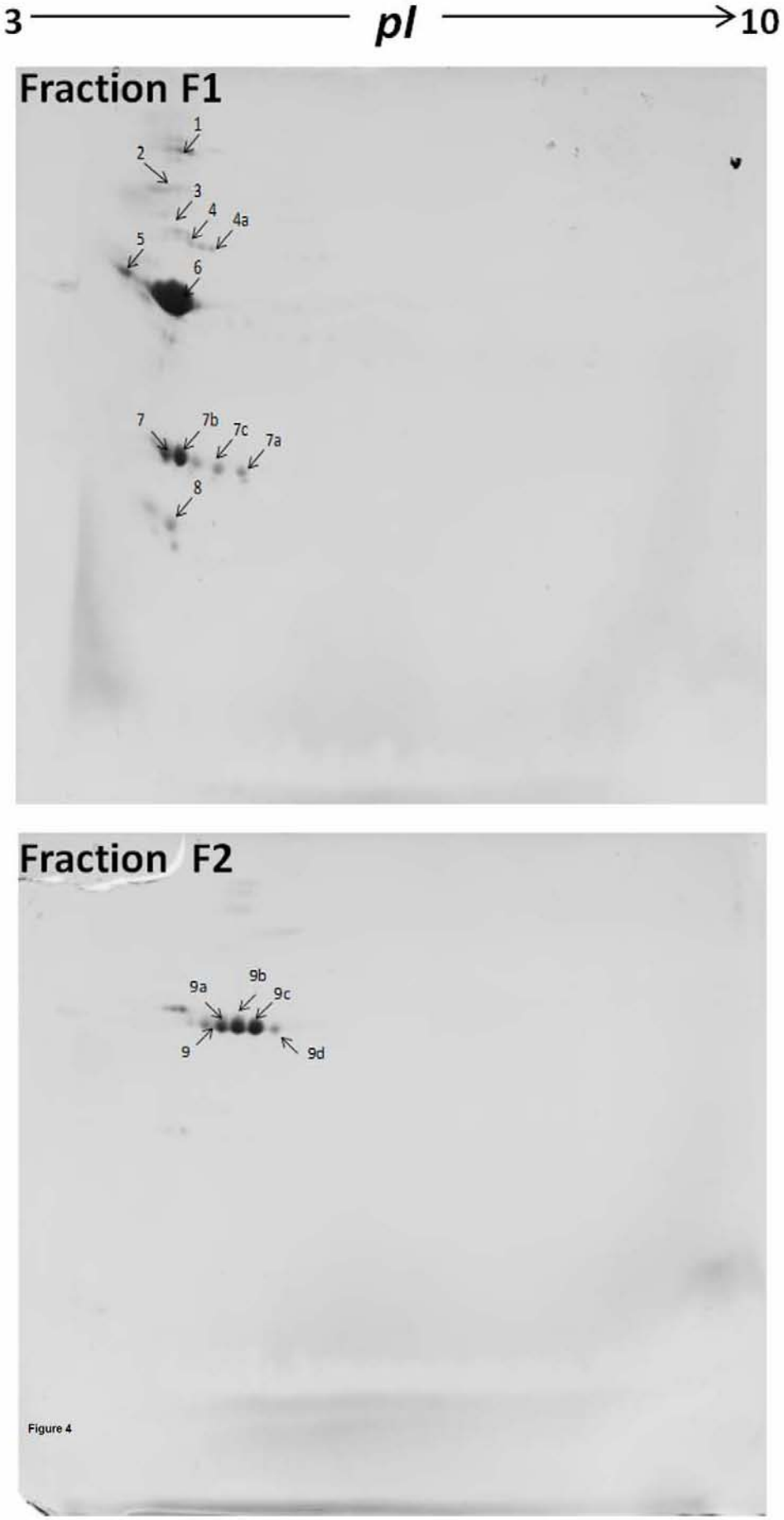

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Figure 4

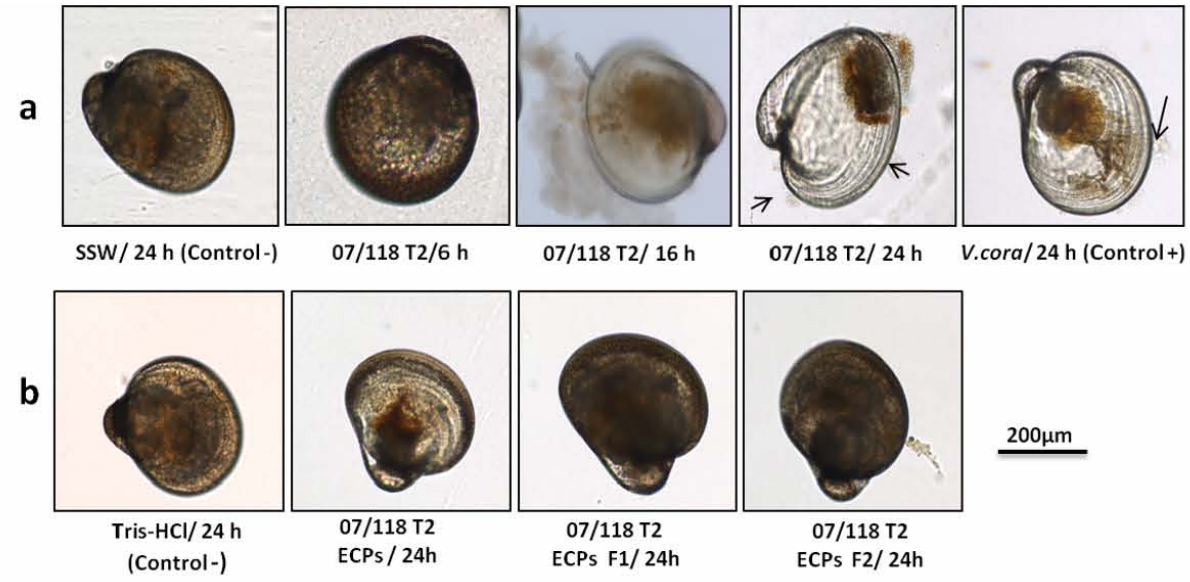

Figure 5

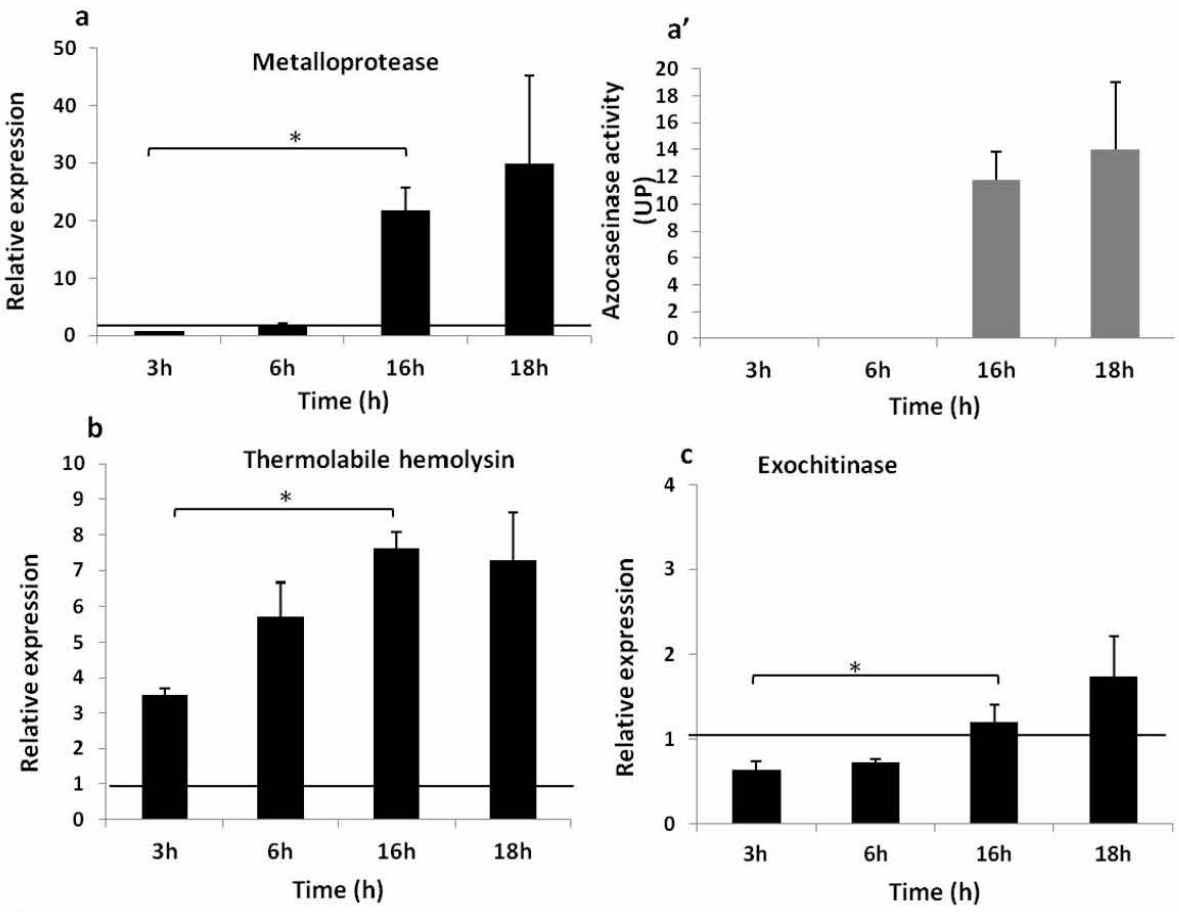

Figure 6

Supplementary table

Table 1: MALDI TOF–TOF mass spectrometry identification of proteins in *V. tubiashii* 07/118 T2 ECPs GP-HPLC fractions. Only proteins with CI% (Confidence Interval) > 92% and peptides with ions score were presented. Protein localization were determined using MicroScope Microbial Genome Annotation & Analysis Platform (https://www.genoscope.cns.fr/agc/microscope/). F1: GP-HPLC fraction 1 , F2: GP-HPLC fraction 2

F1 spots identification

Spot (F1)	Protein identification/Localization	Accession Number	Mascot scores		Peptide sequences with ions score	Coverage (%)	Total Matched peptides	Theoretical	
			Protein score/protein score CI%	Total ion score/ total ion CI%				MW (kDa)	p*I*
1	Ferrichrome-iron receptor/Outer membrane	EGU49312	491/100	491/100	EINEDVAFR YDDTSNEQR TDLAIHETPR INDWVVQAGVR TPQQVADTLASAWAK VSAYTLYDATVSYR YQFFDGVLNGLSVGGGVR GPASVLYGQNPPGGLINTVSK	42	26	82.97	4.76
2	Bifunctional UDP-sugar hydrolase/5'nucleotidase periplasmic precursor/periplasmic	EGU50117	204/100	204/100	GVNAPGDVALAR ADFAVMNSGGVR LTVLHTNDHHGR IGNPEFIGGIDFR FTVPSFNASGGDGYPK	32	18	61.07	4.99
3	Outer membrane channel protein/Outer membrane	EGU54889	69/99.93	69/99.93	VSTAYFDVLR	31	11	47.59	4.6
4	Long-chain fatty acid transport protein/Outer membrane	EGU47320	86 /99.999	86 /100	NAAAMSLFDR FGLSYHYSPEK	26	10	47.61	4.76
4a	Long-chain fatty acid transport protein/Outer membrane	EGU47320	112/100	112/100	NAAAMSLFDR FGLSYHYSPEK	23	8	47.61	4.76

5	Porin-like protein H precursor/ outer membrane	EGU50462	491/100	491/100	NTDVGYGVGIK QEIDDADFGFDTR VADFDFTAFYGAAELK YDYDNGSFYAGLGLIQDK YAVNDDLQVGAFLEFSG DNSDR AIGNDQTTKQEIDDADFG FDTR	49	12	36.94	4.4
6	Outer membrane protein N/Outer membrane	EGU58844	49/92.919	49/99.75	LGYVGLSHDSYGR	16	6	38.92	4.68
7	Hypothetical protein/ Outer membrane	EGU50715	58/99.129	58/ 99.966	FNYFTGNEHK	30	6	24.06	5.81
7a	Hypothetical protein/ Outer membrane	EGU50715	327/100	327 /100	GGSYLTENVR FNYFTGNEHK SSNQGYINATYK TVVDKLDNAGSFAIK FQLDGYQFGAGSDYVYHIDK	30	6	24.06	5.81
7b	Hypothetical protein/ Outer membrane	EGU50715	209/100	209/100	GGSYLTENVR FNYFTGNEHK SSNQGYINATYK TVVDKLDNAGSFAIK	26	6	24.06	5.81
7c	Hypothetical protein/Outer membrane	EGU50715	599/100	599/100	GGSYLTENVR FNYFTGNEHK SSNQGYINATYK SSNQGYINATYKF TVVDKLDNAGSFAIK SFYVLAGGSLGYYNSELETK FQLDGYQFGAGSDYVYHIDK VYGYLQYNGESELSIIDTEENA QVK	51	8	24.06	5.81
8	Hypothetical protein/Uknown	EGU58130	108/100	108/100	AGLHSYELTGTNFK	8	1	17.43	4.87

7

8

9 **F2 spot identification**

Spot (F2)	Protein identification/Localization	Accession Number	Mascot scores		Peptide sequences with ions score	Coverage (%)	Total Matched peptides	Theoretical	
			Protein score/protein score CI%	Total ion score/total ion CI%				MW (kDa)	p*I*
9	Extracellular zinc metalloprotease/ Extracellular	EGU55378	285/100	285/100	DFPAFAIDK YFDQPSKDGR GNVDWIVGADIFK YDYGKDFPAFAIDK SIDHASQYYDGLNVHYSSGVFNR	24	14	66.08	5.22
9a	Extracellular zinc metalloprotease/Extracellular	EGU55378	414/100	414/100	DFPAFAIDK YFDQPSKDGR AAADMGYSVDDVK GNVDWIVGADIFK YDYGKDFPAFAIDK SIDHASQYYDGLNVHYSSGVFNR	24	16	66.08	5.22
9b	Extracellular zinc metalloprotease/ Extracellular	EGU55378	462/100	462/100	DFPAFAIDK YFDQPSKDGR GNVDWIVGADIFK YDYGKDFPAFAIDK SIDHASQYYDGLNVHYSSGVFNR	24	15	66.08	5.22
9c	Extracellular zinc metalloprotease/ Extracellular	EGU55378	498/100	498/100	DFPAFAIDK YFDQPSKDGR GNVDWIVGADIFK YDYGKDFPAFAIDK SIDHASQYYDGLNVHYSSGVFNR	30	18	66.08	5.22
9d	Extracellular zinc metalloprotease/ Extracellualr	EGU55378	202/100	202/100	DFPAFAIDK YFDQPSKDGR GNVDWIVGADIFK YDYGKDFPAFAIDK SIDHASQYYDGLNVHYSSGVFNR	18	11	66.08	5.22