Fish & Shellfish Immunology
November 2010, Volume 29, Issue 5, Pages 753-758
http://dx.doi.org/ 10.1016/j.fsi.2010.07.007

Vibrio aestuarianus zinc metalloprotease causes lethality in the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses

Yannick Labreuche[a, b, *], Frédérique Le Roux[c, d], Joël Henry[e], Céline Zatylny[e], Arnaud Huvet[a], Christophe Lambert[f], Philippe Soudant[f], Didier Mazel[c] and Jean-Louis Nicolas[a]

[a] UMR 100 IFREMER Physiologie et Ecophysiologie des Mollusques Marins, Centre de Brest, B.P. 70, 29280 Plouzané, France
[b] Département Lagons, Ecosystèmes et Aquaculture Durable en Nouvelle-Calédonie, IFREMER, Station de St Vincent, B.P. 2059, 98846 Nouméa cedex, New Caledonia
[c] Institut Pasteur, Unité Plasticité du Génome Bactérien, Département Génomes et Génétique and CNRS, URA 2171, 75015 Paris, France
[d] Laboratoire de Génétique et Pathologie, IFREMER, 17390 La Tremblade, France
[e] UMR 100 IFREMER Physiologie et Ecophysiologie des Mollusques Marins, Laboratoire de Biologie et Biotechnologies Marines, Université de Caen/ Basse Normandie, Esplanade de la Paix, 14032 Caen cedex, France
[f] Laboratoire des Sciences de l'Environnement Marin, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, place Copernic, Technopôle Brest-Iroise, 29280 Plouzané, France

*: Corresponding author : Yannick Labreuche, Tel.: +687 35 25 88; fax: +687 35 11 77, email address : yannick.labreuche@ifremer.fr

Abstract:

Extracellular products (ECPs) of the pathogenic *Vibrio aestuarianus* 01/32 were previously reported to display lethality in *Crassostrea gigas* oysters and to cause morphological changes and immunosuppression in oyster hemocytes. To identify the source of this toxicity, biochemical and genetic approaches were developed. ECP protease activity and lethality were shown to be significantly reduced following incubation with metal chelators, suggesting the involvement of a zinc metalloprotease. An open reading frame of 1836 bp encoding a 611-aa metalloprotease (designated Vam) was identified. The deduced protein sequence showed high homology to other *Vibrio* metalloproteases reported to be involved in pathogenicity. To further confirm the role of this enzyme in ECP toxicity, a plasmid carrying the *vam* gene under the control of an *araC*-P_{BAD} expression cassette was transferred to a *Vibrio splendidus* related strain, LMG20012^T, previously characterized as non-pathogenic to oysters. Expression of Vam conferred a toxic phenotype to LMG20012^T ECPs *in vivo* and cytotoxicity to oyster hemocytes *in vitro*. Collectively, these data suggest that the Vam metalloprotease is a major contributor to the toxicity induced by *V. aestuarianus* ECPs and is involved in the impairment of oyster hemocyte functions.

Keywords: *Vibrio aestuarianus*; Metalloprotease; *Crassostrea gigas*; Oyster; Hemocytes; Extracellular products

1. Introduction

Vibrio aestuarianus is a naturally occurring gram-negative bacterium, widely spread in marine ecosystems [1]. Recent epidemiological studies conducted during recurrent summer mortality events of *Crassostrea gigas* oysters along the French Atlantic coast have also documented the predominance of this bacterial species in the hemolymph of diseased animals, and have demonstrated its pathogenicity to *C. gigas* by experimental challenge [2-4]. Previous studies designed to understand *V. aestuarianus* pathogenicity mechanisms have shown that one of the isolated strains, named 01/32, secretes extracellular products (ECPs) which induce immunosuppressant activities on *C. gigas* hemocyte functions *in vitro* and display lethality to oysters *in vivo* [5]. During the time course of infection, this bacterial isolate was also reported to circumvent the host cellular immune defenses [6]. However, the mechanisms and bacterial effector(s) responsible for these immunomodulatory and toxic effects remain poorly understood. Since we previously established that *V. aestuarianus* 01/32 releases bacterial proteases into the host hemolymph during infection, we hypothesized that these proteases may be responsible, either directly or indirectly, for some of the observed pathological signs. Indeed, the pathogenesis of *Vibrionaceae* associated with marine invertebrate infections has frequently been linked with the production of extracellular proteases [7-9].
To date, only two studies have genetically demonstrated the causal relationship between proteases and virulence in these *Vibrio* agents, thus fulfilling the molecular version of Koch's postulates [10, 11]. The most common procedure to prove cause-effect relationships for suspected bacterial virulence factors relies on loss-of-function studies using reverse genetic methods. However, genetic tools are sometimes not available in bacterial species of environmental origin because of either inoperative or inefficient DNA transformation, poor DNA delivery or inefficient allelic exchange. For such cases, gene expression in a heterologous system constitutes a useful alternative. A potential difficulty with this approach is that genes from heterologous systems may have adverse effects on cell growth and viability when expressed in *E. coli* [12]. We recently characterized a close phylogenetic neighbor of *V. aestuarianus*, belonging to the *V. splendidus* polyphyletic group [13]. This strain, named LMG20012^T and previously reported to be non pathogenic to oysters, can be easily manipulated genetically and is devoid of any protease activity [14]. Considering all these features, the *V. splendidus* related strain LMG20012^T constitutes an excellent candidate for heterologous expression of *V. aestuarianus* proteases. In the present study, a biochemical approach allowed us to associate *V. aestuarianus* ECP protease activity and lethality to oysters with the involvement of a metalloprotease-like enzyme. After identification of this factor, we successfully used LMG20012^T to heterologously express the *V. aestuarianus* zinc metalloprotease and genetically demonstrate its role in toxicity to *C. gigas* and impairment of oyster immune cells.

2. Material and methods

2.1 Bacterial strains and media.

Bacterial strains and plasmids used in this study are listed in Table 1. *V. aestuarianus* and *V. splendidus* related strain LMG20012^T were routinely grown in Luria-Bertani (LB) broth 0.5 M NaCl or marine broth (MB), or on marine agar (MA) at 20°C. *Escherichia coli* strains were grown in LB at 37°C. Ampicillin (Ap) and chloramphenicol (Cm) (Difco Antibiotics) were used at a final concentration of 100 µg.mL^{-1} and 12,5 µg.mL^{-1}, respectively. Diaminopimelate (DAP) was supplemented when necessary to a final concentration of 0.3 mM. Gene expression under the control of the P_{BAD} promoter was activated or repressed by addition to the growth medium of 1% L-arabinose or 1% D-glucose, respectively.

2.2 Preparation of extracellular products and protease activity assays.

Extracellular products (ECPs) were produced by the cellophane overlay method and their protease activity determined using azocasein, as previously described [6]. Briefly, crude ECPs (5 µl) were added to 250 µl of azocasein (5 mg.mL^{-1} in 50 mM Tris–HCl buffer pH 8.0) and to 245 µl of distilled water. The mixture was incubated at 20°C for 10 min. The undigested substrate was precipitated by adding 500 µl of 10 % trichloroacetic acid to the reaction mixture, followed by centrifugation at 12 000 g and 4°C for 5 min. The supernatant (500 µl) was neutralized by the addition of an equal volume of 1 M NaOH. After mixing, the absorbance was measured at 440 nm for triplicate samples. One unit of protease activity was defined as the amount of enzyme that caused an increase of one absorbance unit under the conditions of the assay. ECP protease activity of *V. aestuarianus* 01/32 was evaluated in the presence of commercially available protease inhibitors (Sigma) and compared to that of the control (Table 2).

2.3 Polymerase chain reaction (PCR).

Genomic DNA from *V. aestuarianus* 01/32 was purified using the standard phenol-chloroform extraction method [15]. PCR was done in 50-µL volumes using the Pfu DNA polymerase (Promega) following the manufacturer's instructions. Other PCRs were performed in 50-µL volumes using Bioline Taq polymerase according to the manufacturer's instructions. Primers used in PCR reactions are listed in Table 3. Conditions for amplification were as follows: 94^{o}C for 3 min, followed by 30 cycles of 94°C for 30 s, (Tm-10°C) for 30 s, and 72°C for 60 s per kb.

2.4 Cloning of the *V. aestuarianus* 01/32 metalloprotease gene.

ClaIF and ClaIR degenerate primers, designed from an alignment of the conserved regions surrounding the zinc-binding site characterized in other *Vibrio* metalloproteases (HAQADGTG and SDIAGEAA, respectively), were successfully used to amplify by PCR, from *V. aestuarianus* 01/32 genomic DNA, a 548-bp amplicon which was sequenced by Genome Express SA (Meylan, France). A BlastX match of this fragment revealed high sequence homologies with two bacterial metalloproteases : EmpA of *V. anguillarum* (GenBank accession no. P43147) and NprV of *V. proteolyticus* (GenBank accession no. 00971) (expect [E] values, 2 x 10^{-97} and 4 x 10^{-88}, respectively). Based on EmpA and NprV protein sequence alignment, a new set of primers, VamFor and VamRev, were designed in the 5'- and 3'-regions and used to obtain the full-length sequence of the *vam* gene. The nucleotide sequence of the *vam* gene and the deduced amino acid sequence have been deposited in the GenBank database under the accession number AY605667.

2.5 Plasmid construction.

Plasmid pSU18-oriT-*araC*-P_{BAD}*vam* was constructed by introducing the *vam* gene in pSU18-oriT-*araC*-P_{BAD}*gfp* plasmid through a two-step PCR method [16]. The *vam* gene was amplified using primers Vam1C and Vam2 from genomic DNA of *V. aestuarianus* strain 01/32, while the plasmid pSU18-oriT-*araC*-P_{BAD}*gfp* was amplified using primers Vam1 and Vam3. After gel purification, a PCR assembly was set up by mixing together 100 ng of the two fragments and the most external primers, Vam2 and Vam3. The final product was digested with *XhoI* and self-ligated before DH5α transformation. After sequence confirmation, the pSU18-oriT-*araC*-P_{BAD}*vam* plasmid was transferred to β2163 cells and conjugation with LMG20012^{T} was performed by the filter mating procedure as previously described [14].

2.6 Protein identification by peptide mass fingerprinting.

To evaluate Vam metalloprotease production of the resulting transconjuguant, ECPs were subjected to MS and MS/MS analyses, as previously described [17].

2.7 *In vivo* assays.

In vivo assays were performed on one-year-old oysters *C. gigas* following previously described procedures [5, 6]. Briefly, toxicity inhibition was assayed by incubating ECPs with 50 mM EGTA for 1 h and then injecting oysters (3 replicate tanks, 10 oysters per tank) with 200 µL of the resulting mixture (5 µg protein.g^{-1} body weight). Positive and negative control groups were inoculated either with 200 µL of untreated ECPs (5 µg protein.g^{-1} body weight) or with 50 mM EGTA. Fifty percent lethal dose (LD_{50}) was determined for ECPs prepared from *V. aestuarianus* strain 01/32 and *V. splendidus* related strain LMG20012^T transconjuguants, as previously described [5]. Observations were made twice a day, and mortalities were recorded for 2-days after inoculation.

2.8 *In vitro* assays : hemocyte cellular parameters.

ECPs of *V. splendidus* related strain LMG20012^T carrying the pSU18-oriT-*araC*-P_{BAD}*vam* plasmid and grown under repressing (by addition of 1% D-glucose) or inducing (by addition of 1% L-arabinose) conditions were tested at 2, 8 and 32 µg.mL^{-1} for hemocyte phagocytosis and adhesion assays. ECPs from *V. aestuarianus* 01/32 in the same concentration range were also included in the experimental design. Briefly, 4 pools comprised of 4-5 individual hemolymph samples were prepared for each treatment. Hemocyte phagocytic activity was assayed by distributing 150 µL sub-samples of each hemolymph pool into 5 mL polystyrene tubes (Falcon®) maintained on ice. Each sub-sample received a 150 µL volume of the ECPs to be tested. A negative control was included by adding a 150 µL volume of filtered sterile seawater (FSSW) to one sub-sample of each pool. Each sub-sample was subsequently incubated at 18°C for 60 min with fluorescent beads (Fluoresbrite, YG Microspheres, 2 µm, Polysciences) at a final concentration of 0.2 % of the commercial solution. Results of phagocytosis were expressed as the percentage of hemocytes containing three beads or more [5]. Hemocyte adhesive capacities were assessed by adding a 100 µL volume of the ECPs to be tested or 100 µL of FSSW as a control to 100 µL sub-samples of each hemolymph pool. After three hours of incubation at 18°C, the percentage of adhering hemocytes was calculated relatively to the initial total hemocyte count of the tested pool [5].

2.9 Statistical analyses

Significant differences between treatments were tested by one-way analysis of variance (ANOVA) using Statgraphics Plus 5.0 software. Data collected as percentages were transformed (arcsine of the square root) before analysis. The method used to discriminate among the means was Fisher's least significant difference (LSD) procedure. Results were deemed significant at $P<0.05$

3. Results

3.1 *V. aestuarianus* 01/32 ECPs display a metalloenzyme-like activity associated with toxicity.

Experiments were first performed to characterize the protease activity of *V. aestuarianus* 01/32 ECPs in the presence of protease inhibitors. ECP protease activity was sensitive to EDTA, EGTA and the zinc specific metal chelator 1,10-phenanthroline (Table 2). In contrast, these ECPs were resistant to the serine protease inhibitor PMSF. Inactivation of the ECPs by 1,10-phenanthroline could be reversed to near-control levels by titration with 0.5 and 1 mM Zn^{2+}. Taken together, these results suggest that the protease activity of ECPs was due to one or more zinc-dependent metalloenzyme(s). To investigate the role of this protease(s) in oyster lethality, toxicity inhibition tests were performed by inactivating the ECP metalloprotease activity with 50 mM EGTA. Incubation of ECPs with EGTA prior to injection significantly reduced their toxicity (33 ± 2.5 % cumulative mortality) compared to animals injected with crude ECPs (98 ± 3.5 % cumulative mortality). Cumulative mortality of control oysters did not exceed 3% throughout the experiment.

3.2 The *vam* gene encodes a zinc-dependent metalloprotease.

The structural gene coding for the metalloprotease (designated Vam for *Vibrio aestuarianus* metalloprotease) was characterized. The *vam* gene consists of a 1836-bp open reading frame encoding a putative 611-amino-acid protein, with a calculated molecular mass of 66,3 kDa. A cleavage site is predicted to take place at the C-terminus of the Ala^{25} [18, 19]. A putative prosequence of 174 aa (Ala^{26} to His^{199}) was identified between the end of the putative signal peptide sequence and the beginning of the mature protein, in agreement with the long propeptides (172 and 174 aa) proposed for the metalloprotease precursors of *V. proteolyticus* [20] and *V. anguillarum* [21], respectively. Thus, the mature protein is predicted to consist of 412 aa, with a calculated molecular mass of 44,4 kDa. A zinc-binding motif ($H^{346}EXXH^{350}$), as well as the thermolysin consensus sequence $G^{366}XXNEXXSD^{374}$, were also identified within the deduced amino acid sequence [22]. Homology searches using the Blast algorithm revealed that the Vam sequence exhibited a high degree of homology to other *Vibrio* metalloproteases, including *V. anguillarum* EmpA (85% identity).

3.3 Vam expression in a heterologous host potentiates the lethal effect of its ECPs.

To investigate the involvement of the *vam* metalloprotease gene in the toxicity to oysters, *vam* was expressed under the control of a P_{BAD} promoter in the avirulent $LMG20012^T$ strain. The same plasmid, carrying the *gfp* gene instead of the *vam* gene was used as a negative control. The protease activity of ECPs from each conjuguant cultivated in the presence of 1% D-glucose (P_{BAD} promoter repression) or 1% L-arabinose (P_{BAD} promoter induction) was evaluated. Protease activity was only detected in the ECPs of *V. splendidus* related strain $LMG20012^T$ carrying the *vam* plasmid upon addition of L-arabinose (140.4 ± 10.6 $U.mg^{-1}$), although this activity was 44% lower than that of ECPs from *V. aestuarianus* 01/32 (258.4 ± 1.4 $U.mg^{-1}$, Table 4). Growth in 1% D-glucose very efficiently repressed Vam expression, as no enzymatic activity was quantified. Protease activity was not detected in the ECPs produced by *V. splendidus* related strain $LMG20012^T$ carrying the *gfp* plasmid following growth in D-glucose or L-arabinose, confirming previously obtained results [14]. A comparison of tryptic peptide masses from ECPs produced by $LMG20012^T$ carrying the *vam* plasmid grown under inducing conditions with the deduced aa sequence of the *vam* gene allowed the identification of four peptides. Molecular masses of all four peptides showed mass matches to the predicted Vam protein sequence, confirming that Vam was readily produced and secreted in the ECPs of the transconjuguant (data not shown). Finally, *in vivo* LD_{50} values were determined for ECPs of each transconjuguant (Table 4). No mortality was observed for animals injected either with ECPs from $LMG20012^T$ harboring the *gfp* plasmid, or with ECPs from $LMG20012^T$ harboring the *vam* plasmid under repressing conditions. The LD_{50} value of ECPs from the wild-type *V. aestuarianus* 01/32 was 3.2 µg protein.g^{-1} body weight, while the LD_{50} of ECPs from $LMG20012^T$ carrying the *vam* plasmid grown with 1% L-arabinose was 6.2 µg protein.g^{-1} body weight. Oysters injected with either *V. aestuarianus* 01/32 ECPs or

ECPs from LMG20012^T expressing the *vam* gene died rapidly, all recorded deaths occurring within 24-h post inoculation.

3.4 Recombinant expression of Vam metalloprotease confers cytotoxicity to the ECPs of the avirulent *V. splendidus* related strain LMG20012^T.

The ECPs produced by LMG20012^T expressing the *vam* gene were examined for effects on hemocyte adhesion and phagocytosis. Percentages of adherent hemocytes significantly differed from that of the control following hemocyte exposure to these ECPs at 32 µg.mL^{-1} (ANOVA, P <0.05) (Fig. 1A). ECPs from LMG20012^T carrying the *vam* plasmid and grown under repressing conditions did not affect hemocyte adhesion over the tested range (P >0.05). Compared to the control, *V. aestuarianus* ECPs displayed a significant inhibition of hemocyte adhesion at all tested concentrations, (P <0.05).
Hemocyte phagocytic activity, evaluated using fluorescent beads, was significantly affected by *V. aestuarianus* ECPs at 8 µg.mL^{-1} (ANOVA, P <0.05) (Fig. 1B). Addition of ECPs from LMG20012^T expressing the Vam metalloprotease induced a statistically significant decrease in phagocytic ability at 32 µg.mL^{-1} (P<0.05). ECPs of LMG20012^T carrying the *vam* plasmid and grown under repressing conditions did not induce any change, whatever the concentration tested (P < 0.05).
After 3 h, oyster hemocytes incubated with filtered sterile seawater (FSSW) appeared healthy, with cytoplasmic extensions (Fig. 2). Hemocytes exposed to *V. aestuarianus* ECPs at 32 µg.mL^{-1} were observed to aggregate and lose their pseudopods, as did cells incubated with the same concentration of ECPs from LMG20012^T expressing the Vam metalloprotease. Incubation of hemocytes with 32 µg.mL^{-1} of ECPs from LMG20012^T carrying the *vam* plasmid and grown under repressing conditions did not induce any morphological modifications.

4. Discussion

Since *V. aestuarianus* 01/32 was previoulsy demonstrated to release extracellular proteases during infection, we hypothesized that protease secretion might be associated to virulence mechanisms [6]. In this study we showed that ECP toxicity to oysters and metalloprotease activity were correlated. We identified a 1836-bp gene (named *vam*) encoding a putative protein sharing all the features of the thermolysin family [23]. To demonstrate a cause-effect relationship for the *vam* gene in pathogenicity, a genetic approach was implemented. For bacterial model organisms, such as *V. cholerae*, this is usually done by specific inactivation of the candidate gene. We sought to develop a similar strategy here, but conjugation experiments with broad-host range plasmids failed when using *V. aestuarianus* 01/32 as a recipient, and attempts to transfer plasmids by electroporation were ineffective (data not shown). The *vam* gene was therefore expressed in a non-pathogenic phylogenetic neighbor, *V. splendidus* related strain LMG20012^T, using the arabinose inductible promoter P_{BAD} [24-26]. This allowed us to demonstrate that addition of L-arabinose induced protease activity in ECPs prepared from strain LMG20012^T carrying the *vam* plasmid, although this activity was 1.8-fold less than that of *V. aestuarianus*. This lowered activity correlated well with toxicity and hemocyte assay results, as i) the LD_{50} value of recombinant LMG20012^T ECPs was 2 times higher than that calculated for *V. aestuarianus* ECPs and ii) these ECPs induced the same immunosuppressant effects on cells as those observed for *V. aestuarianus* ECPs except only at higher concentrations. The nature of this discrepancy in toxicity remains to be clarified : the *araC*-P_{BAD} promoter system can be modulated by several factors such as the inducer concentration or the ability of the strain to degrade arabinose [24]. LMG20012^T transconjuguant culture conditions may therefore explain this discrepancy. Another hypothesis relies on an incomplete proteolytic processing of the heterologously expressed enzyme. Metalloproteases are indeed synthesized as inactive precursors inside the cell and

undergo several stages of proteolytic processing [27, 28]. Several *Vibrio* metalloproteases have been shown to be proteolytically activated by additional proteases [29, 30]. Full activity of Vam protease may similarly require cleavage by an accessory protease that could be lacking in the *V. splendidus* related strain LMG20012^T genetic background. Together, the results presented here clearly indicate the functionality of this expression system, which provides an efficient and inexpensive method for producing an active recombinant protease in the culture medium without the time-consuming need to solubilize the inclusion bodies that may form in *E. coli* and thus to refold the recombinant protein into its native structure.

The data obtained in this study also demonstrate the role of Vam metalloprotease in oyster toxicity and impairment of hemocyte functions. Metalloprotease involvement in bacterial virulence is being extensively studied because of their wide variety of pathological actions [31]. Recently, the role of various *Vibrio* metalloproteases has been investigated in *C. gigas*. For instance, the *V. tubiashii* VtpA metalloprotease is believed to contribute to pathogenicity by degrading oyster larvae tissues, thus providing the pathogen with nutrients [10]. The Vsm metalloenzyme from *V. splendidus* LGP32 has been shown to display toxicity following ECP injection into oysters and to induce cytotoxicity in snail and mouse fibroblast cell lines [14]. The protozoan parasite *Perkinsus marinus*, the agent of Dermo disease of the eastern oyster, *C. virginica*, is known to release during infection several extracellular proteases with serine and metalloproteinase-like activity [32], that were found to degrade oyster proteins and to decrease hemocyte motility, lysozyme activity and haemagglutinin titres [33, 34]. These proteases are believed to be part of the mechanisms developed by this obligatory intracellular parasite to evade the host immune defense system. The results obtained in this work show that the *V. aestuarianus* metalloprotease Vam is similarly implicated in avoidance of *C. gigas* immune responses and reinforce our previous observations indicating that this pathogen may have evolved a so-called “outsider strategy”, to promote its own extracellular life cycle within the oyster.

The findings of the present work describe a new mode of action for a *Vibrio* metalloprotease and provide thus new insights to explain how *V. aestuarianus* 01/32 succeeds in avoiding phagocytic engulfment in order to successfully replicate within the host, ultimately leading to death. To date, the biochemical mechanisms behind Vam impairement of oyster hemocyte functions remain unknown. In light of obtained data, it is likely that Vam affects protein structures required for hemocytes to keep their normal morphology and develop a phagocytic activity. Indeed, a number of bacterial cytotoxins have been shown to act on the eukaryotic cell cytoskeleton as part of their virulence mechanisms by targeting GTPases from the Rho protein subfamily [35]. GTPases play crucial roles in several cellular processes, including morphogenesis, migration, cytokinesis, and phagocytosis [36]. As compared to vertebrate and invertebrate model organisms, much less is known regarding the cellular and molecular mechanisms involved in phagocytosis in bivalve molluscs. Additional investigations are therefore needed to determine how the Vam metalloprotease modulates hemocyte cell physiology.

Acknowledgments

This work was supported by the MOREST national project funded by IFREMER and by the Régions Basse-Normandie, Bretagne, Pays de la Loire and Poitou-Charentes and the Conseil Général du Calvados. We thank Patricia Mirella Da Silva and Philippe Miner for technical assistance and helpful discussions during the course of this work. The authors are indebted to Matt Gerding for the critical review of the manuscript and for his help in editing the English language.

References

1. Tison DL., Seidler RJ. *Vibrio aestuarianus* : a new species from estuarine waters and shellfish. Int J Syst Bacteriol. 1983; 33:699-702.
2. Garnier M., Labreuche Y., Garcia C., Robert M., Nicolas JL. Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. Microb Ecol. 2007; 53:187-96.
3. Garnier M., Labreuche Y., Nicolas JL. Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp. *francensis* subsp. nov., a pathogen of the oyster *Crassostrea gigas*. Syst Appl Microbiol. 2008; 31:358-65.
4. Saulnier D., De Decker S., Haffner P., Cobret L., Robert M., Garcia C. A Large-Scale Epidemiological Study to Identify Bacteria Pathogenic to Pacific Oyster *Crassostrea gigas* and Correlation Between Virulence and Metalloprotease-like Activity. Microb Ecol. 2009; In Press.
5. Labreuche Y., Soudant P., Goncalves M., Lambert C., Nicolas JL. Effects of extracellular products from the pathogenic *Vibrio aestuarianus* strain 01/32 on lethality and cellular immune responses of the oyster *Crassostrea gigas*. Dev Comp Immunol. 2006; 30: 367-79.
6. Labreuche Y., Lambert C., Soudant P., Boulo V., Huvet A., Nicolas JL. Cellular and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32. Microbes Infect. 2006; 8:2715-24.
7. Sussman M., Mieog JC., Doyle J., Victor S., Willis BL., Bourne DG. *Vibrio* zinc metalloprotease causes photoinactivation of coral endosymbionts and coral tissue lesions. PloS One. 2009; 4:e4511.
8. Hasegawa H., Gharaibeh DN., Lind EJ., Häse CC. Virulence of metalloproteases produced by *Vibrio* species on Pacific oyster *Crassostrea gigas* larvae. Dis Aquat Organ. 2009; 85:123-31.
9. Nottage AS., Birkbeck TH. Purification of a proteinase produced by the bivalve pathogen *vibrio alginolyticus* ncmb 1339. J Fish Dis. 1987; **10**.
10. Hasegawa H., Lind EJ., Boin MA., Häse CC. The extracellular metalloprotease of *Vibrio tubiashii* is a major virulence factor for Pacific oyster (*Crassostrea gigas*) larvae. Appl Environ Microbiol. 2008; 74:4101-10.
11. Le Roux F., Binesse J., Saulnier D., Mazel D. Construction of a *Vibrio splendidus* Mutant Lacking the Metalloprotease Gene vsm by Use of a Novel Counterselectable Suicide Vector. Appl Environ Microbiol. 2007; 73:777-84.
12. Studier FW. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. J Mol Biol. 1991; 219:37-44.
13. Thompson FL., Iida T., Swings J. Biodiversity of Vibrios. Microbiol Mol Biol Rev. 2004; 68:403-31.
14. Binesse J., Delsert C., Saulnier D., Champomier-Verges M-C., Zagorec M., Munier-Lehmann H., et al. Metalloprotease Vsm Is the Major Determinant of Toxicity for Extracellular Products of *Vibrio splendidus*. Appl Environ Microbiol. 2008; 74:7108-17.
15. Sambrook J., Fritsch EF., Maniatis T. Molecular cloning : a laboratory manual. Cold Spring Harbor, NY 2nd ed. 1989.
16. Matsumoto-Mashimo C., Guerout A-M., Mazel D. A new family of conditional replicating plasmids and their cognate *Escherichia coli* host strains. Res Microbiol. 2004; 155:455-61.
17. Bernay B., Baudy-Floc'h M., Zanuttini B., Zatylny C., Pouvreau S., Henry J. Ovarian and sperm regulatory peptides regulate ovulation in the oyster *Crassostrea gigas*. Mol Reprod Dev. 2006; 73:607-16.
18. Perlman D., Halvorson H. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J Mol Biol. 1983; 167:391-409.
19. von Heijne G. Patterns of amino acids near signal-sequence cleavage sites. Eur J Biochem. 1983; 133:17-21.
20. David VA., Deutch AH., Sloma A., Pawlyk D., Ally A., Durham DR. Cloning, sequencing and expression of the gene encoding the extracellular neutral protease, vibriolysin, of *Vibrio proteolyticus*. Gene. 1992; 112:107-12.

21. Milton DL., Norqvist A., Wolf-Watz H. Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum*. J Bacteriol. 1992 174:7235-44.
22. Vallee BL., Auld DS. Zinc coordination, function, and structure of zinc enzymes and other proteins. Biochemistry. 1990; 29:5647-59.
23. Rawlings ND., Barrett AJ. Evolutionary families of metallopeptidases. Meth Enzymol. 1995; 248:183-228.
24. Guzman LM., Belin D., Carson MJ., Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol. 1995; 177:4121-30.
25. Johnson CM., Schleif RF. *In vivo* induction kinetics of the arabinose promoters in *Escherichia coli*. J Bacteriol. 1995; 177:3438-42.
26. Miyada CG., Stoltzfus L., Wilcox G. Regulation of the *araC* gene of *Escherichia coli*: catabolite repression, autoregulation, and effect on *araBAD* expression. Proc Nat Aca Sci USA. 1984; 81:4120-4.
27. Häse CC., Finkelstein RA. Bacterial extracellular zinc-containing metalloproteases. Microbiol Rev. 1993; 57:823-37.
28. Miyoshi S-i., Shinoda S. Microbial metalloproteases and pathogenesis. Microbes Infect. 2000; 2:91-8.
29. Varina M., Denkin SM., Staroscik AM., Nelson DR. Identification and characterization of Epp, the secreted processing protease for the *Vibrio anguillarum* EmpA metalloprotease. J Bacteriol. 2008; 190:6589-97.
30. Sonoda H., Daimon K., Yamaji H., Sugimura A. Efficient production of active *Vibrio proteolyticus* aminopeptidase in *Escherichia coli* by co-expression with engineered vibriolysin. Appl Microbiol Biotechnol. 2009; 84:191-8.
31. Shinoda S., Miyoshi S-i., Wakae H., Rahman M., Tomochika K-i. Bacterial proteases as pathogenic factors, with special emphasis on vibrio proteases. J Toxicol Tox Rev. 1996; 15:327-39.
32. Muñoz P., Vance K., Gómez-Chiarri M. Protease activity in the plasma of American oysters, *Crassostrea virginica*, experimentally infected with the protozoan parasite, *Perkinsus marinus*. J Parasitol. 2003; 89:941-51.
33. Tall B., La Peyre J., Bier J., Miliotis M., Hanes D., Kothary M., et al. *Perkinsus marinus* extracellular protease modulates survival of *Vibrio vulnificus* in Eastern oyster (*Crassostrea virginica*) hemocytes. Appl Environ Microbiol. 1999; 65:4261-3.
34. Garreis K., La Peyre J., Faisal M. The effects of *Perkinsus marinus* extracellular products and purified proteases on oyster defense parameters *in vitro*. Fish Shellfish Immunol. 1996; 6:581-97.
35. Aktories K., Barbieri JT. Bacterial cytotoxins: targeting eukaryotic switches. Nat Rev Microbiol. 2005; 3:397-410.
36. Raftopoulou M., Hall A. Cell migration: Rho GTPases lead the way. Dev Biol. 2004; 265:23-32.

Tables

Strain or plasmid	Description	Reference or source
Bacterial strain		
01/32	*Vibrio aestuarianus*	[5]
LMG20012^T	*Vibrio spendidus related strain*	[35]
DH5α	(F $^-$) *supE44* Δ*lacU169 (*Φ*80lacZ*Δ*M15)*Δ*argF hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	[36]
β2163	(F $^-$) RP4-2-Tc::Mu Δ*dapA* ::(*erm-pir*) [Km ^{R} Em ^{R}]	[36]
plasmid		
pSU18-oriT-*araC*-P$_{BAD}$*gfp*	pSU18::*oriT*$_{RP4}$; *araC* P$_{BAD}$ *gfp;* [Cm^R]	[11]
pSU18-oriT-*araC*-P$_{BAD}$*vam*	pSU18::*oriT*$_{RP4}$; *araC* P$_{BAD}$ *vam;* [Cm^R]	This study

Table 1. Bacterial strains and plasmids used in this study.

Reagent (mM concentration) [a]	Activity (%) [b]
PMSF (50)	96 ± 3.3
PMSF (5)	108 ± 3.7
EDTA (50)	48 ± 1.8
EDTA (5)	81 ± 1.7
EGTA (50)	0
EGTA (5)	55.2 ± 0.8
1,10-Phenanthroline (10)	1.1 ± 0.2

1,10-Phenanthroline (1)	1.7 ± 0.3
1,10-Phenanthroline (1) + $ZnCl_2$ (0.1)	7.8 ± 0.8
1,10-Phenanthroline (1) + $ZnCl_2$ (0.5)	88 ± 0.6
1,10-Phenanthroline (1) + $ZnCl_2$ (1)	83 ± 0.7

Table 2. Effects of protease inhibitors on ECP protease activity. [a] Final concentration of reagent in the assay mixture, [b] Caseinolytic activity is expressed as a percentage of the control sample (with no addition). Inhibitors were incubated with ECPs for 30 min before the substrate was added. ECPs were incubated for 30 min with 1,10-phenanthroline and supplemented with increasing concentrations of Zn^{2+}, before the substrate was added. Values are described as mean ± standard deviation (n=3).

oligonucleotide	**Sequence**
ClaIF	CAYGCRSANGCNRMNGGNACYGGMC
ClaIR	TCYGCNGCTTCNCCYGCRATRTC
VamFor	TCTCANGGATTGAGAAATGAA
VamRev	ARTCMAVNCKTAACGTTACACCA
OriT-XbaI	CGCTCTAGACAGCGCTTTTCCGCTGCATAAC
OriT-PstI	AAAACTGCAGCCGGCCAGCCTCGCAGAGCA
P_{BAD}GFP-Kpn	GCCCGGTACCAATTATGACAACTTGACGGC
P_{BAD}GFP-Xba	GCCCTCTAGACTATTTGTATAGTTCATCCATGCC
Vam1C	GGAGTGAAACGATGGCGATTGCAATTGCAGGAACGAAAAAT
	GAAAAACAAACAACGTCAAATAAAGTGG
Vam1	CCACTTTATTTGACGTTGTTTGTTTTTCATTTTTCGTTCCTGCA
	ATTGCAATCGCCATCGTTTCACTCC
Vam2	GCCCCTCGAGTTAGTCCAGGCTTAACGTTACACC
Vam3	GCCCCTCGAGTCTAGACAGCGCTTTTCCGCTGC

Table 3. List of the oligonucleotide primers used in this study.

	Dose per oyster [a]	N° of dead oysters [b]	Relative virulence [c]
ECPs from *V. aestuarianus* 01/32	1.25	0	0 %
	2.5	8	33.3 %
	5	16	85.7 %
	10	20	100 %
	LD_{50} = 3.2 µg. g^{-1} body weight		
	Specific protease activity = 258.4 ± 1.4 **(**$U.mg^{-1}$)		
ECPs from the *V. splendidus* related strain LMG20012^T carrying the pSU18-oriT-*araC*-P_{BAD}*vam* plasmid and grown with L-arabinose	2.5	4	10 %
	5	8	38 %
	10	14	76 %
	20	18	96 %
	LD_{50} = 6.2 µg. g^{-1} body weight		
	Specific protease activity = 140.4 ± 10.6 **(**$U.mg^{-1}$)		

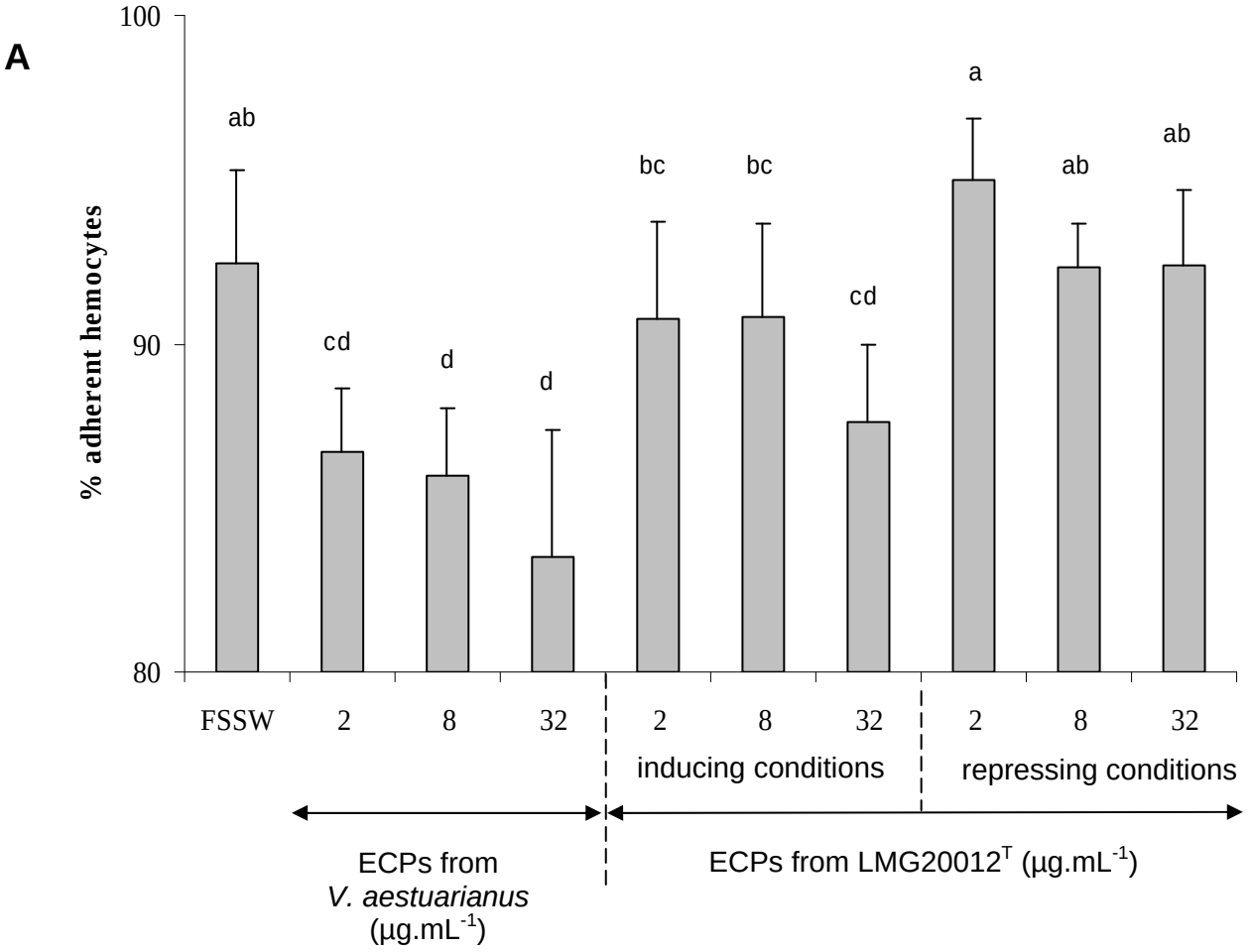
[a] in $\mu g.g^{-1}$ body weight

[b] expressed as the number of dead oysters out of the total number of oysters in the treatment. Overall results of duplicate trial are presented.

[c] expressed by dividing the cumulative number of dead oysters with the cumulative total number of oysters injected.

Table 4. Toxicity and specific protease activity of ECPs from *V. aestuarianus* strain 01/32 and *V. splendidus* related strain LMG20012^T transconjuguants carrying either the pSU18-oriT-*araC*-P_{BAD}*gfp* or pSU18-oriT-*araC*-P_{BAD}*vam* plasmid, and grown under 1% L-arabinose.

Figures


A
100
90
80
% adherent hemocytes
ab
cd
d
d
bc
bc
cd
a
ab
ab
FSSW
2
8
32
2
8
32
2
8
32
inducing conditions
repressing conditions
ECPs from
V. aestuarianus
(µg.mL-1)
ECPs from LMG20012T (µg.mL-1)

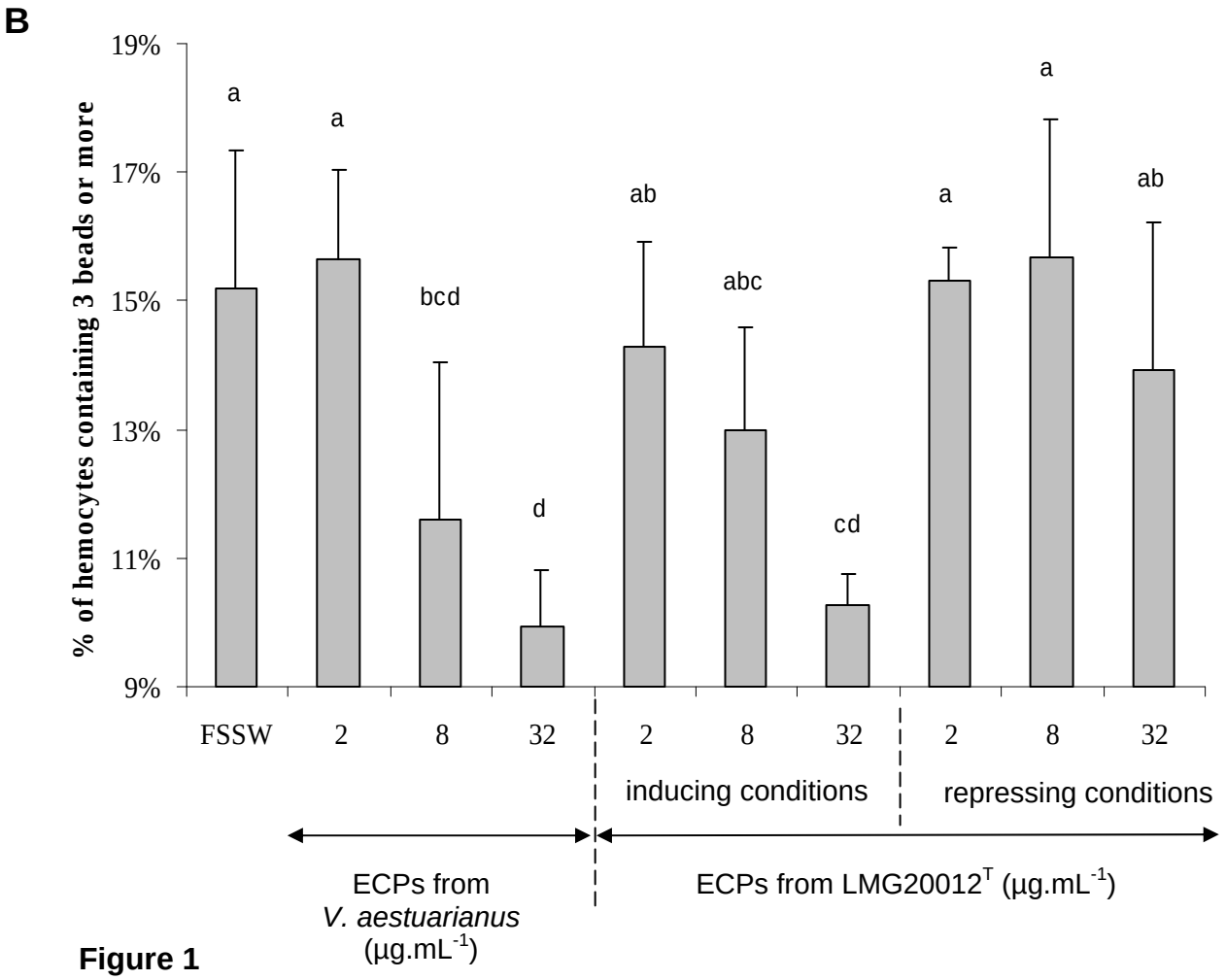

B
19%
17%
15%
13%
11%
9%
% of hemocytes containing 3 beads or more
a
a
bcd
d
ab
abc
cd
a
a
ab
FSSW
2
8
32
2
8
32
2
8
32
inducing conditions
repressing conditions
ECPs from
V. aestuarianus
(µg.mL-1)
ECPs from LMG20012T (µg.mL-1)

Figure 1

Fig. 1 *Crassostrea gigas* oyster hemocyte functions following exposure to increasing concentrations of ECPs from *V. aestuarianus* 01/32 and from the *V. splendidus* related strain LMG20012^T transconjuguant expressing the Vam metalloprotease (inducing conditions) or not (repressing conditions) (µg.mL^{-1}). Different lower-case letters indicate significant difference between treatments. **A*/*** Percentage of adherent hemocytes (mean ± S.D., N = 4, ANOVA, P < 0.05). **B*/*** Phagocytic activity (mean ± S.D., N = 4, ANOVA, P<0.05)

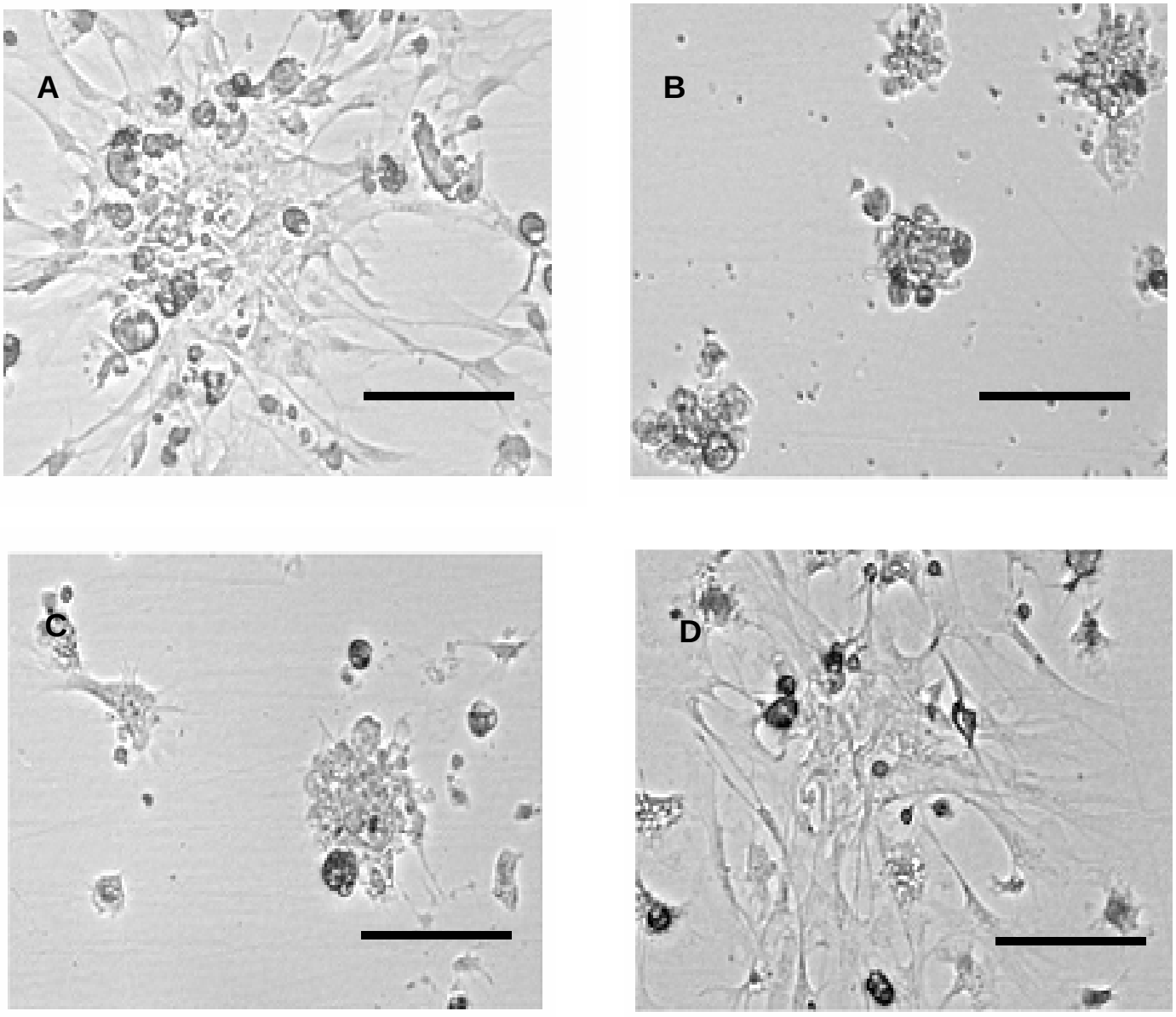


Fig. 2 *Crassostreas gigas* hemocytes after incubation for 3 h either with FSSW (**A**) or with 32 µg.mL^{-1} of ECPs from *V. aestuarianus* 01/32 (**B**), from the *V. splendidus* related strain LMG20012^T transconjuguant grown under inducing conditions (expressing the Vam metalloprotease) (**C**), and from the *V. splendidus* related strain LMG20012^T transconjuguant carrying the pSU18-oriT-*araC*-P_{BAD}*vam* plasmid and grown under repressing conditions (**D**). Scale bar = 50 µM.