



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

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### ARTICLE INFO

#### Article history:

Received 8 April 2010

Received in revised form

16 June 2010

Accepted 2 July 2010

Available online 17 July 2010

#### Keywords:

*Vibrio aestuarianus*

Metalloprotease

*Crassostrea gigas*

Oyster

Hemocytes

Extracellular products

### 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<sub>BAD</sub> expression cassette was transferred to a *Vibrio splendidus* related strain, LMG20012<sup>T</sup>, previously characterized as non-pathogenic to oysters. Expression of Vam conferred a toxic phenotype to LMG20012<sup>T</sup> 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.

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### 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

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[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 *Escherichia coli* [12]. We recently characterized a close phylogenetic neighbor of *V. aestuarianus*, belonging to the *Vibrio splendidus* polyphyletic group [13]. This [13] strain, named LMG20012<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> 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. Materials 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<sup>T</sup> were routinely grown in Luria-Bertani (LB) broth 0.5 M NaCl or marine broth (MB), or on marine agar (MA) at 20 °C. *E. 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<sup>-1</sup> and 12.5 µg mL<sup>-1</sup>, respectively. Diaminopimelate (DAP) was supplemented when necessary to a final concentration of 0.3 mM. Gene expression under the control of the *P<sub>BAD</sub>* promoter was activated or repressed by addition to the growth medium of 1% L- 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<sup>-1</sup> in 50 mM Tris–HCl buffer pH 8.0) and to 245 µl of distilled water. The mixture was incubated

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

Strain or plasmid	Description	Reference or source
Bacterial strain		
01/32	<i>Vibrio aestuarianus</i>	[5]
LMG20012 <sup>T</sup>	<i>Vibrio splendidus</i> related strain	[35]
DH5α	(F <sup>-</sup> ) <i>supE44 ΔlacU169 (Φ80lacZΔM15)ΔargF</i>	[36]
β2163	<i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> (F <sup>-</sup> ) RP4-2-Tc::Mu <i>ΔdapA::(erm-pir)</i> [Km <sup>R</sup> Em <sup>R</sup> ]	[36]
Plasmid		
pSU18-oriT- <i>araC</i> - <i>P<sub>BAD</sub>gfp</i>	pSU18::oriT <sub>RP4</sub> ; <i>araC</i> - <i>P<sub>BAD</sub>gfp</i> ; [Cm <sup>R</sup> ]	[11]
pSU18-oriT- <i>araC</i> - <i>P<sub>BAD</sub>vam</i>	pSU18::oriT <sub>RP4</sub> ; <i>araC</i> - <i>P<sub>BAD</sub>vam</i> ; [Cm <sup>R</sup> ]	This study

**Table 2**  
Effects of protease inhibitors on ECP protease activity.

Reagent (mM concentration) <sup>a</sup>	Activity (%) <sup>b</sup>
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 <sub>2</sub> (0.1)	7.8 ± 0.8
1,10-Phenanthroline (1) + ZnCl <sub>2</sub> (0.5)	88 ± 0.6
1,10-Phenanthroline (1) + ZnCl <sub>2</sub> (1)	83 ± 0.7

<sup>a</sup> Final concentration of reagent in the assay mixture.

<sup>b</sup> 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<sup>2+</sup>, before the substrate was added. Values are described as mean ± standard deviation (*n* = 3).

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 Bionline 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 °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

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

oligonucleotide	Sequence
ClaIF	CAYGCRSANGCNRMNGNACYGGMC
ClaIR	TCYGCNGCTTCNCCYGCRAITRC
VamFor	TCTCANGGATTGAGAAATGAA
VamRev	ARTCMVANCNTAACGTTACACCA
OriT-XbaI	CGTCTAGACAGCGCTTTTCCGCTGCATAAC
OriT-PstI	AAAACCTGCAGCCGCCAGCCTCGCAGAGCA
<i>P<sub>BAD</sub>GFP</i> -Kpn	GCCCCGTACCAATTATGACAACCTTGACGGC
<i>P<sub>BAD</sub>GFP</i> -Xba	GCCCTCTAGACTATTGTATAGTTCATCCATGCC
Vam1C	GGAGTGAAACGATGGCGATTGCAATTGCAGGAACG
	AAAAATGAAAAACAACAACGTCAAAATAAAGTGG
Vam1	CCACTTTATTGACGTTGTTTTCATITTTTCGTT
	CCTGCAATTGCAATCGCCATCGTTTCACCTC
Vam2	GCCCTCGAGTTAGTCCAGGCTTAAACGTTACACC
Vam3	GCCCTCGAGTCTAGACAGCGCTTTTCCGCTGC

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 *Vibrio anguillarum* (GenBank accession no. P43147) and NprV of *Vibrio proteolyticus* (GenBank accession no. 00971) (expect [E] values,  $2 \times 10^{-97}$  and  $4 \times 10^{-88}$ , respectively). Based on EmpA and NprV protein sequence alignment, a new set of primers, Vam For 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<sub>BAD</sub>*vam* was constructed by introducing the *vam* gene in pSU18-oriT-*araC*-P<sub>BAD</sub>*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<sub>BAD</sub>*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 *Xho*I and self-ligated before DH5 $\alpha$  transformation. After sequence confirmation, the pSU18-oriT-*araC*-P<sub>BAD</sub>*vam* plasmid was transferred to  $\beta$ 2163 cells and conjugation with LMG20012<sup>T</sup> 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 transconjugant, 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  $\mu$ L of the resulting mixture (5  $\mu$ g protein g<sup>-1</sup> body weight). Positive and negative control groups were inoculated either with 200  $\mu$ L of untreated ECPs (5  $\mu$ g protein g<sup>-1</sup> body weight) or with 50 mM EGTA. Fifty percent lethal dose (LD<sub>50</sub>) was determined for ECPs prepared from *V. aestuarianus* strain 01/32 and *V. splendidus* related strain LMG20012<sup>T</sup> transconjugants, 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<sup>T</sup> carrying the pSU18-oriT-*araC*-P<sub>BAD</sub>*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  $\mu$ g mL<sup>-1</sup> for hemocyte phagocytosis and adhesion procedure 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  $\mu$ L sub-samples of each hemolymph pool into 5 mL polystyrene tubes (Falcon®) maintained on ice. Each sub-sample received a 150  $\mu$ L

volume of the ECPs to be tested. A negative control was included by adding a 150  $\mu$ L 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  $\mu$ m, 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  $\mu$ L volume of the ECPs to be tested or 100  $\mu$ L of FSSW as a control to 100  $\mu$ L sub-samples of each hemolymph pool. After 3 h 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<sup>2+</sup>. 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  $\pm$  2.5% cumulative mortality) compared to animals injected with crude ECPs (98  $\pm$  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 *V. 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<sup>25</sup> [18,19]. A putative prosequence of 174 aa (Ala<sup>26</sup> to His<sup>199</sup>) 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<sup>346</sup>EXXH<sup>350</sup>), as well as the thermolysin consensus sequence G<sup>366</sup>XXNEXXSD<sup>374</sup>, 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<sup>T</sup> 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 conjugant 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<sup>T</sup> carrying the *vam* plasmid upon addition of L-arabinose ( $140.4 \pm 10.6$  U mg<sup>-1</sup>), although this activity was 44% lower than that of ECPs from *V. aestuarianus* 01/32 ( $258.4 \pm 1.4$  U mg<sup>-1</sup>, 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<sup>T</sup> 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<sup>T</sup> 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 transconjugant (data not shown). Finally, *in vivo* LD<sub>50</sub> values were determined for ECPs of each transconjugant (Table 4). No mortality was observed for animals injected either with ECPs from LMG20012<sup>T</sup> harboring the *gfp* plasmid, or with ECPs from LMG20012<sup>T</sup> harboring the *vam* plasmid under repressing conditions. The LD<sub>50</sub> value of ECPs from the wild-type *V. aestuarianus* 01/32 was 3.2 µg protein g<sup>-1</sup> body weight, while the LD<sub>50</sub> of ECPs from LMG20012<sup>T</sup> carrying the *vam* plasmid grown with 1% L-arabinose was 6.2 µg protein g<sup>-1</sup> body weight. Oysters injected with either *V. aestuarianus* 01/32 ECPs or ECPs from LMG20012<sup>T</sup> expressing the *vam* gene died rapidly, all recorded deaths occurring within 24-h post inoculation.

**Table 4**

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

	Dose per oyster <sup>a</sup>	No of dead oysters <sup>b</sup>	Relative virulence <sup>c</sup>
ECPs from <i>V. aestuarianus</i> 01/32	1.25	0	0%
	2.5	8	33.3%
	5	16	85.7%
	10	20	100%
	LD <sub>50</sub> = 3.2 µg g <sup>-1</sup> body weight Specific protease activity = 258.4 ± 1.4 (U mg <sup>-1</sup> )		
ECPs from the <i>V. splendidus</i> related strain LMG20012 <sup>T</sup> carrying the pSU18-oriT-araC- $P_{BAD}$ <i>vam</i> plasmid and grown with L-arabinose	2.5	4	10%
	5	8	38%
	10	14	76%
	20	18	96%
	LD <sub>50</sub> = 6.2 µg g <sup>-1</sup> body weight Specific protease activity = 140.4 ± 10.6 (U mg <sup>-1</sup> )		

<sup>a</sup> In µg g<sup>-1</sup> body weight.

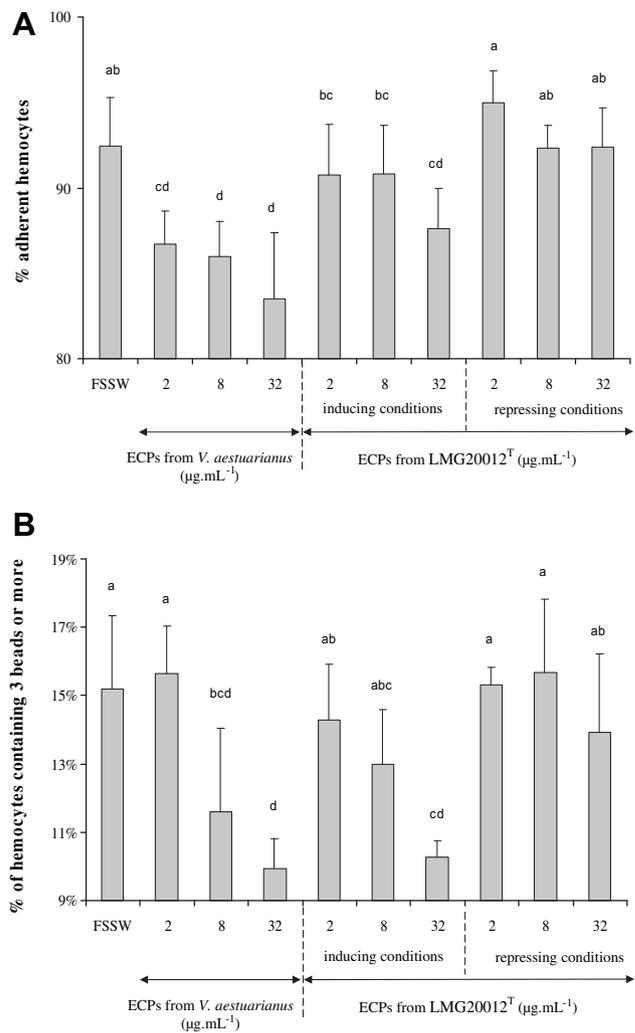
<sup>b</sup> Expressed as the number of dead oysters out of the total number of oysters in the treatment. Overall results of duplicate trial are presented.

<sup>c</sup> Expressed by dividing the cumulative number of dead oysters with the cumulative total number of oysters injected.

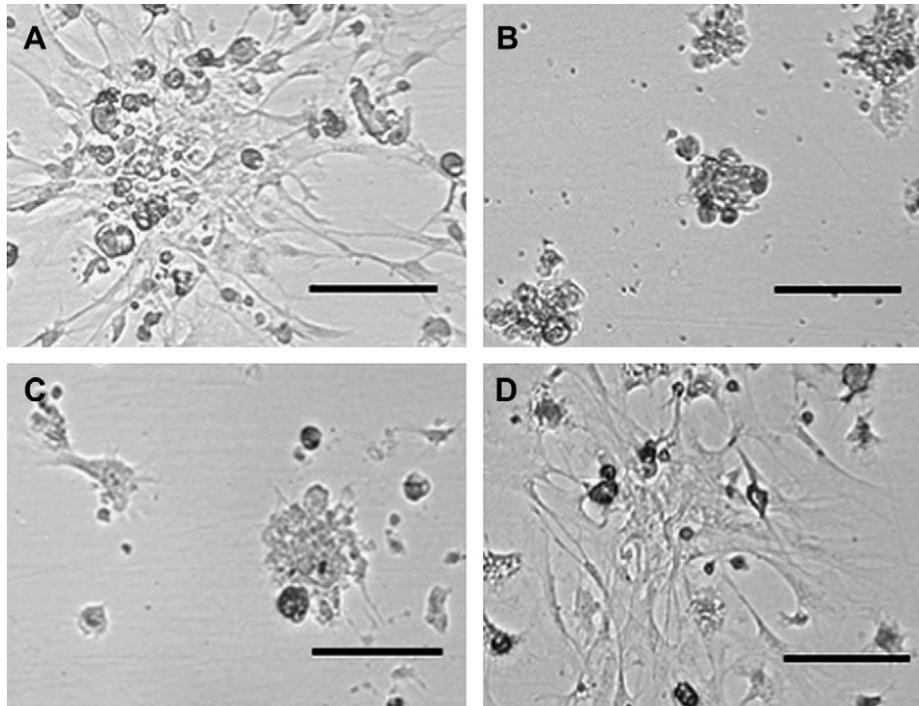
### 3.4. Recombinant expression of *Vam* metalloprotease confers cytotoxicity to the ECPs of the avirulent *V. splendidus* related strain LMG20012<sup>T</sup>

The ECPs produced by LMG20012<sup>T</sup> 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<sup>-1</sup> (ANOVA,  $P < 0.05$ ) (Fig. 1A). ECPs from LMG20012<sup>T</sup> 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<sup>-1</sup> (ANOVA,  $P < 0.05$ ) (Fig. 1B). Addition of ECPs from LMG20012<sup>T</sup> expressing the *Vam* metalloprotease induced a statistically significant decrease in phagocytic ability at 32 µg mL<sup>-1</sup>



**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<sup>T</sup> transconjugant expressing the *Vam* metalloprotease (inducing conditions) or not (repressing conditions) (µg mL<sup>-1</sup>). 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  $32 \mu\text{g mL}^{-1}$  of ECPs from *V. aestuarianus* 01/32 (B), from the *V. splendidus* related strain LMG20012<sup>T</sup> transconjugant grown under inducing conditions (expressing the Vam metalloprotease) (C), and from the *V. splendidus* related strain LMG20012<sup>T</sup> transconjugant carrying the pSU18-oriT-*araC*-P<sub>BAD</sub>*vam* plasmid and grown under repressing conditions (D). Scale bar = 50  $\mu\text{M}$ .

( $P < 0.05$ ). ECPs of LMG20012<sup>T</sup> 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  $32 \mu\text{g mL}^{-1}$  were observed to aggregate and lose their pseudopods, as did cells incubated with the same concentration of ECPs from LMG20012<sup>T</sup> expressing the Vam metalloprotease. Incubation of hemocytes with  $32 \mu\text{g mL}^{-1}$  of ECPs from LMG20012<sup>T</sup> carrying the *vam* plasmid and grown under repressing conditions did not induce any morphological modifications.

#### 4. Discussion

Since *V. aestuarianus* 01/32 was previously 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 *Vibrio 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<sup>T</sup>, using the arabinose inducible promoter P<sub>BAD</sub> [24–26]. This allowed us to demonstrate that addition of L-arabinose induced protease activity in ECPs prepared from strain LMG20012<sup>T</sup>

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<sub>50</sub> value of recombinant LMG20012<sup>T</sup> 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<sub>BAD</sub> promoter system can be modulated by several factors such as the inducer concentration or the ability of the strain to degrade arabinose [24]. LMG20012<sup>T</sup> transconjugant 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<sup>T</sup> 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 *Vibrio 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, *Crassostrea 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 impairment 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.

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