Fish & Shellfish Immunology 35 (2013) 1906–1915

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Interactions between *Mytilus galloprovincialis* hemocytes and the bivalve pathogens *Vibrio aestuarianus* 01/032 and *Vibrio splendidus* LGP32

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ARTICLE INFO

Article history:
Received 25 July 2013
Received in revised form
16 September 2013
Accepted 19 September 2013
Available online 27 September 2013

Keywords:
Mytilus
Vibrio splendidus LGP32
Vibrio aestuarianus 01/032
Hemocytes
Lysosomal membrane stability

ABSTRACT

Marine bivalves can accumulate large numbers of bacteria, in particular *Vibrio* species, whose persistence in bivalve tissues largely depends on their sensitivity to the bactericidal activity of circulating hemocytes and hemolymph soluble factors. The interactions between vibrios and hemolymph have been investigated, in particular in bivalve species susceptible to infection by certain *Vibrio* spp. and strains.

In this work, the effects of two bivalve pathogens, *Vibrio splendidus* LGP32 (*V.s.*) and *Vibrio aestuarianus* 01/032 (*V.a.*), isolated from oyster mortality outbreaks, on the hemocytes of *Mytilus galloprovincialis* were investigated. *In vitro*, *V.s.*, but not *V.a.*, induced a dramatic decrease in lysosomal membrane stability-LMS in the hemocytes; both vibrios induced a moderate lysozyme release, with *V.s.* > *V.a.*. The *V.s.*-induced decrease in LMS was mediated by activation of PI-3Kinase, as shown by use of different kinase inhibitors. TEM analysis showed rapid internalization of both vibrios; however, *V.s.* lead to cellular and lysosomal damage and was able to survive within the hemocytes, whereas significant killing of *V.a.* was observed. *In vivo*, in mussels challenged with either vibrio and sampled at 6, 24 and 96 h post-injection, transient decreases in hemocyte LMS and progressive increases in serum lysozyme activity were observed, with *V.s.* > *V.a.*. Moreover, whereas *V.a.* was efficiently cleared from hemolymph, *V.s.* showed significant growth, that was maximal at 24 h p.i. when lowest LMS values were recorded in the hemocytes. Both vibrios also induced significant decreases in LMS in the digestive gland, again with *V.s.* > *V.a.*.

The results indicate distinct interactions between mussel hemocytes and the two vibrio strains tested. The effects of *V.s.* may be due to the capacity of this strain to interfere with the signaling pathways involved in hemocyte function, thus escaping the bactericidal activity of the host cell, as observed for certain mammalian pathogens. Although *V.s.* is considered not pathogenic to *Mytilus*, this vibrio strain can affect the lysosomal function at the cellular and tissue level, thus leading to stressful conditions.

1. Introduction

Marine bivalves (mussels, clams, oysters…) can accumulate large numbers of bacteria as a consequence of their filter-feeding habit. In particular, *Vibrio* species are very abundant in coastal waters and are commonly isolated from edible bivalves [1] where they can persist even after depuration processes [2]. Persistence of different bacteria, including vibrios, in the bivalve host largely depends on their sensitivity to the bactericidal activity of the hemolymph, resulting from complex interactions between bacteria, bacterial components and circulating hemocytes and hemolymph soluble factors [2–4].

Host-pathogen interactions have been increasingly investigated in different bivalves, with the aim of understanding the pathogenesis of diseases in cultured and wild populations of species susceptible to infection by certain *Vibrio* spp. and strains [5–15]. These studies demonstrated that different *Vibrios* can elicit distinct responses in bivalve hemocytes both *in vitro* and *in vivo*. Some strains of *Vibrio splendidus* and *Vibrio aestuarianus* have been associated with the summer mortalities affecting the production of *Crassostrea gigas* oysters worldwide [7,16]. *V. splendidus* strain LGP32 is a well recognized pathogen of oyster juveniles; the

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http://dx.doi.org/10.1016/j.fsi.2013.09.027

secreted Vsm metalloprotease was identified as a major determinant of toxicity of this strain [17]. Moreover, an outer membrane protein (OmpU) is required for virulence of *V. splendidus* LGP32 in oyster experimental infections, affording protection from the host immune defense [18,19].

On the other hand, the mussel *Mytilus* spp. is considered to be particularly resistant to *Vibrio* infection. In *Mytilus galloprovincialis* the effects of *V. splendidus* LGP32 on functional and molecular immune parameters have been previously investigated [10–14,20,21]. In particular, *in vitro* studies carried out with heat-killed LGP32 indicated that components other than secretion of metalloproteases may be involved in determining the responses of mussel hemocytes, and that this strain might affect the hemocyte function through disregulation of components of immune signaling pathways [20].

V. aestuarianus 01/032, a strain originally isolated from a mortality outbreak, was also shown to be pathogenic to *C. gigas* juveniles [16]; this strain is able to impair the functional response of the hemocytes and secrete extracellular products including a metalloprotease [9,22]. On the other hand, no data are so far available on the interactions between *Mytilus* immune system and *V. aestuarianus* 01/032.

In this work, the effects of live *V. aestuarianus* 01/032 (*V.a.*) and *V. splendidus* LGP32 (*V.s.*) on *M. galloprovincialis* hemocytes were compared. *In vitro*, hemocyte responses in the presence of serum were evaluated in terms of lysosomal membrane stability (LMS) and extracellular lysozyme activity as markers of cellular stress and of lysosomal enzyme release, respectively; the role of signaling components in mediating the interactions between *V.s.* and hemocytes was investigated by using specific kinase inhibitors. *Vibrio* internalization was observed by transmission electron microscopy (TEM), and the bactericidal activity of hemocyte monolayers towards both strains was evaluated. *In vivo*, hemocyte LMS, serum lysozyme activity and bactericidal activity of whole hemolymph were evaluated in mussels challenged with live *V.a.* or *V.s.* at different times post-injection (6, 24 and 96 h). Moreover, LMS was evaluated in the digestive gland, as a marker of general stress.

2. Methods

2.1. Bacterial cultures

Vibrio aestuarianus 01/032 (*V.a.*) and *Vibrio splendidus* LGP32 (*V.s.*) were kindly provided by IFREMER Institute (La Tremblade, France). Both strains were cultured in Zobell medium at 20 °C under static conditions; after overnight growth, cells were harvested by centrifugation (5000 × *g*, 15 min), washed three times with phosphate-buffered saline (PBS–NaCl; 0.1 M KH_2PO_4, 0.1 MK_2HPO_4, 0.15 M NaCl, pH 7.2–7.4) and resuspended to an $A_{600} = 1$ (about 10^9 CFU/ml). Luria–Bertani agar supplemented with NaCl 3% (LB agar 3% NaCl) and Marine Agar (Conda Lab, Spain) were used for culturing *V.a.* or *V.s.*, respectively.

2.2. Animals and hemolymph collection

Mussels (*M. galloprovincialis* Lam), 4–5 cm long, were purchased from an aquaculture farm (Arborea-OR, Italy) in October 2012 and kept for 3 days in static tanks containing aerated artificial sea water (ASW), salinity 36 ppt (1 L mussel^{-1}) at 18 °C.

Hemolymph was extracted from the posterior adductor muscle using a sterile 1 ml syringe with an 18 G1/2″ needle. With the needle removed, hemolymph was filtered through a sterile gauze and pooled in 50 ml Falcon tubes at 18 °C. Hemolymph serum was obtained by centrifugation of whole hemolymph at 100 × *g* for 10 min, and the supernatant was sterilized through a 0.22 μm-pore filter. Hemocyte monolayers were prepared as previously described [23].

For experiments with oyster hemocytes, specimens of *C. gigas*, 8–10 cm long, were purchased from an aquaculture farm (La Rochelle, France) at the same time of the year and acclimated in ASW 23 ppt salinity (1 L oyster^{-1}) at 16 °C. Hemolymph sampling and preparation of hemocyte monolayers were performed as described above.

2.3. In vitro challenge of Mytilus hemocytes with V. aestuarianus and V. splendidus

Hemocyte monolayers were incubated at 18 °C with suspensions of *V.a.* or *V.s.*, suitably diluted in hemolymph serum, unless otherwise indicated, to obtain a bacteria:hemocyte ratio of about 50:1, for different periods of times, depending on the endpoint measured. Untreated hemocyte samples in serum were run in parallel. All experiments were performed in triplicate.

2.3.1. Determination of hemocyte lysosomal membrane stability and extracellular lysozyme release

Hemocyte monolayers on glass slides were pre-incubated for 30 min with different live bacteria in hemolymph serum and lysosomal membrane stability-LMS was evaluated by the NRRT assay as previously described [13,20]. The endpoint of the assay was defined as the time at which 50% of the cells showed sign of lysosomal leaking (the cytosol becoming red and the cells rounded).

In experiments with kinase inhibitors, before addition of bacteria hemocyte monolayers were pretreated for 20 min with 20 μM SB203580 (for p38 MAPK), 0.1 μM Wortmannin (for PI3-kinase) or 30 min with 2.5 μM GF109203X (for PKC) suitably diluted in serum from 10 mM stock solutions in DMSO as previously described [24,25]. After pre-incubation, serum was discarded, hemocytes were incubated with bacteria resuspended in serum for 30 min and then tested for the NRRT assay. Control hemocytes were maintained for 20 min in serum in the presence of DMSO (0.1% final concentration), as pre-treated cells, then washed and incubated with serum for 30 min. In experiments with the dynamin inhibitor, cells were incubated with bacteria for 30 min in the presence of 5 μM Dynasore [26,27] and then processed for the NRRT assay as described above.

Lysozyme activity was determined as the ability to lyse a standard suspension of *Micrococcus lysodeikticus* (15 mg/100 ml in 66 mM phosphate buffer, pH 6.4) and measured as decrease in absorbance at 450 nm [2]. Lysozyme activity in the extracellular medium was determined in samples incubated with or without live bacteria for different periods of time (from 30 to 120 min). Hen egg-white (HEW) lysozyme was used as a concentration reference and lysozyme activity was expressed as HEW lysozyme equivalents (U mg protein^{-1} ml^{-1}). Protein content was determined according to the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard.

The same experimental procedures were applied to oyster hemocytes.

2.3.2. Transmission electron microscopy

Hemocyte monolayers were seeded on glass chamber slides for 20 min at 18° C (Lab-Tek, Nunc, 177380), and incubated with different vibrios for 5, 15 and 30 min. Samples were washed out with 0.1 M cacodylate buffer in ASW and fixed in 0.1 M cacodylate buffer in ASW containing 2.5% glutaraldehyde in ASW, for 1 h at room temperature. The cells were postfixed in 1% osmium tetroxide in ASW for 10 min and 1% uranyl acetate in ASW for 1 h. Subsequently, samples were dehydrated through a graded ethanol series and embedded in epoxy resin (Poly-Bed; Polysciences, Inc.,

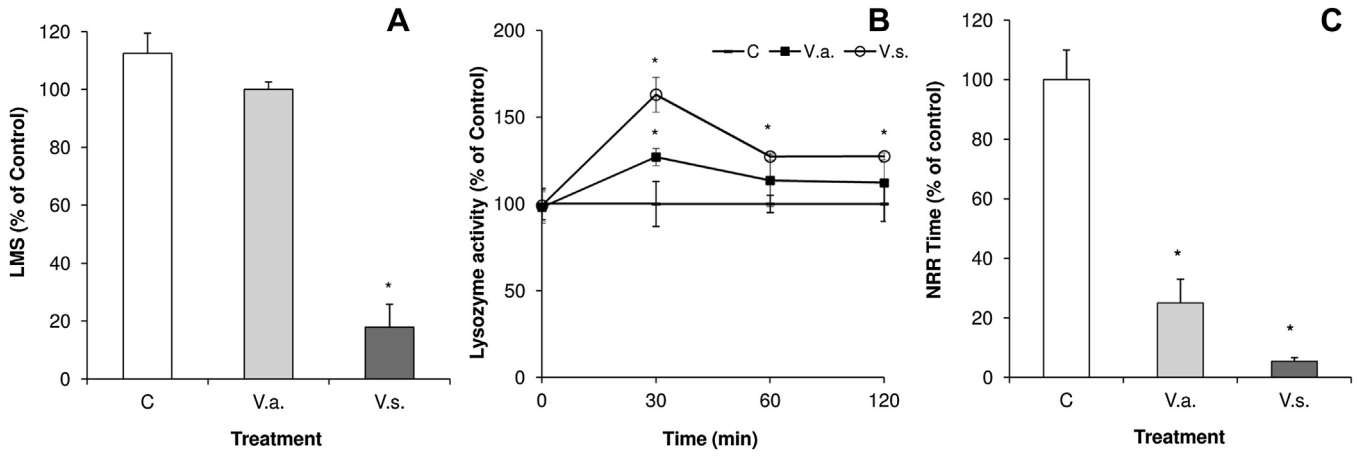

Fig. 1. *In vitro* effects of *V. aestuarianus* 01/032 (*V.a.*) and *V. splendidus* LGP32 (*V.s.*) on the hemocytes of *M. galloprovincialis*. A) lysosomal membrane stability-LMS; B) extracellular lysozyme release. For comparison, the effects of *V.a.* and *V.s.* on LMS of the hemocytes of *C. gigas* are reported (C). LMS and lysozyme activity were analyzed as described in Methods. Data, expressed as percent values with respect to controls and representing the mean ± SD of 4 experiments in triplicate, were analysed by ANOVA followed by Tukey's post hoc test. For A and C: * = $p < 0.01$, all treatments vs control; for B: * = $p < 0.05$, all treatments vs control.

Warrington, PA) overnight at 60 °C. About 50 cells per sample were observed and representative images were taken with Eagle CCD camera and iTEM software and processed with Adobe Photoshop CS2. 3.2.

2.3.3. Evaluation of bactericidal activity of hemocyte monolayers

The sensitivity of different vibrios to the bactericidal activity of mussel hemolymph in *vitro* was evaluated as previously described [23] in hemocyte monolayers incubated with *V.a.* or *V.s.* at 18 °C for different periods of time. Immediately after the inoculum ($T = 0$) and at different times of incubation at 18 °C supernatants were collected from monolayers and hemocytes were lysed by adding 0.5 ml of filter sterilized ASW supplemented with 0.05% Triton x-100 and by 10 s agitation. The collected monolayer supernatants and hemocyte lysates were pooled and tenfold serial diluted in ASW. Aliquots (100 μl) of the diluted samples were plated onto LB agar 3% NaCl for *V.s.* and Marine Agar for *V.a.* After overnight incubation at 20 °C the number of CFU per hemocyte monolayer (representing culturable bacteria survived to hemocyte bactericidal activity) was evaluated. Percentages of killing at 30, 60 and 90 min were then determined relative to values obtained at $T = 0$. To evaluate the presence of endogenous bacteria in hemocytes, control samples of hemocyte monolayers without added bacteria were run in parallel. The number of CFU in controls never exceeded 0.1% of those enumerated in experimental samples. To detect and correct for bacterial growth in hemolymph serum, separate samples were seeded with bacteria and 1.5 ml of sterile hemolymph serum. No appreciable bacterial growth was observed at the same time intervals used in killing experiments.

2.4. In vivo challenge of mussels with V. aestuarianus and V. splendidus

Mussels were kept for 4 days in static tanks containing aerated artificial sea water (ASW) (1 L mussel^{-1}) at 18 °C. Sea water was changed daily. Animals were not fed during the experiments. Mussels were *in vivo* challenged by one injection of live *V.s.* or *V.a.* into the posterior adductor muscle, as previously described [13], with 50 μl of a bacterial suspension containing 10^9 CFU/ml in PBS–NaCl (5×10^7 CFU/mussel, to obtain a bacteria:hemocyte ratio of about 20:1). Control mussels were injected with PBS–NaCl. After challenge, mussels were returned to sea water. At 6, 24 and 96 h post injection (p.i.), hemolymph was collected from the posterior adductor muscle of 4 pools of 4 mussels each. No mortality was observed during the experiments.

2.4.1. Determination of hemocyte lysosomal membrane stability (LMS) and serum lysozyme activity

In hemolymph samples from control and vibrio-injected mussels, hemocyte NRRT and soluble lysozyme activity were evaluated as described above.

2.4.2. Determination of in vivo bactericidal activity

Mussels were injected with suspensions of *V. aestuarianus* and *V. splendidus* as described above. Before the first hemolymph sample was removed, the injected bacterial suspension was allowed to equilibrate for 30 min in the whole hemolymph volume, as previously described [23]. At this time (arbitrarily considered zero time) and at different times p.i. (6, 24 and 96 h) hemolymph was collected from the posterior adductor muscle of 4 pools of 4 mussels each. Aliquots (0.1 ml) of the pooled hemolymph samples were placed in a tube containing 9.9 ml ASW supplemented with 0.05% Triton x-100 and vortexed for 10 to lyse the hemocytes. Tenfold serial dilutions in ASW of this lysate were plated onto LB agar 3% NaCl for *V. splendidus* and Marine Agar for *V. aestuarianus*. Control samples obtained from a parallel set of non injected animals were plated and incubated under the conditions described above. The hemolymph of these samples was virtually free of bacteria.

2.4.3. Evaluation of lysosomal membrane stability (LMS) in the digestive gland

LMS was evaluated *in vitro* in cryostat sections of 5 digestive glands in duplicate. Sections (10 μm) were cut with a cryostat (Bright CM3050), flash-dried by transferring them to room temperature, and then stained for *N*-acetyl-β-hexosaminidase activity [28]. Digital images were acquired by an Olympus BX60 light microscope equipped with a scientific grade ColourViewII CCD Camera (Olympus Italy, Segrate, Milan, Italy). Staining intensity of lysosomes was determined at 400× magnification using the Scion Image software package (Scion Corporation, Frederick, MD, USA).

2.5. Data analysis

The results are the mean ± SD of at least 4 experiments and analyses, unless otherwise indicated, performed in triplicate.

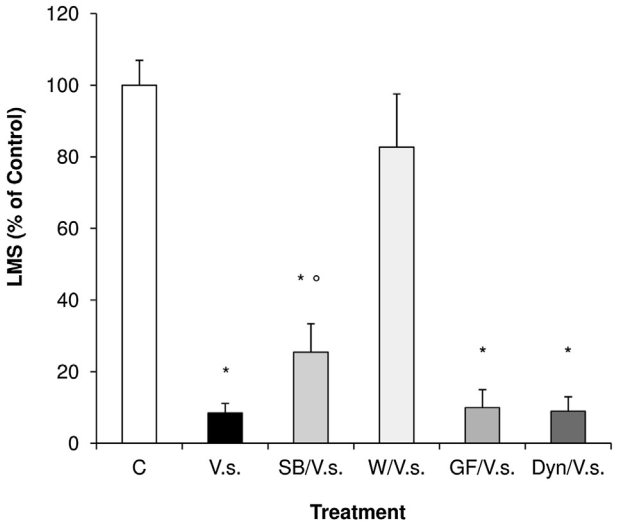

Fig. 2. Effects of pretreatment with different inhibitors on *V. splendidus*-induced decrease in LMS in *Mytilus* hemocytes. Before addition of bacteria, hemocytes were pre-treated for 20 min with 20 μM SB203580 (SB), or 0.1 μM Wortmannin (W) or for 30 min with 2.5 μM GF109203X (GF). In experiments with Dynasore, cells were incubated with bacteria for 30 min in the presence of the inhibitor (5 μM). After incubations, cells were analyzed by the NRRT assay as described in Methods. Data presented and statistic calculations as in Fig. 1. $^{*}p < 0.01$ = treatment vs. controls; $^{\circ} = p < 0.05$ = hemocytes pre-treated with SB vs. *V. splendidus* alone.

Statistical analysis was performed by ANOVA followed by Tukey's post hoc test using the GraphPad Instat software.

3. Results

3.1. In vitro effects of bacterial challenge on hemocyte LMS and lysozyme release

The effects of either *V.a.* or *V.s.* on lysosomal membrane stability (LMS) of *Mytilus* hemocytes were first evaluated by the NRRT assay, and the results are reported in Fig. 1A. Pre-incubation of hemocyte monolayers for 30 min with *V.a.* did not significantly affect LMS, whereas *V.s.* induced a dramatic decrease with respect to controls (−82%, $p < 0.01$). Similar results were obtained in the absence of hemolymph serum (not shown). *V.a.* induced a small stimulation of lysosomal enzyme release, evaluated as lysozyme activity in the extracellular medium, only at 30 min incubation (+27% with respect to controls, $p < 0.05$) (Fig. 1B). A stronger effect was observed with *V.s.*, with lysozyme release peaking at 30 min (about +60% with respect to controls, $p < 0.05$) and persisting until at least 120 min.

For comparison, the effects of both vibrios were evaluated in the hemocytes of the oyster *C. gigas* in the same experimental conditions. In control oyster hemocytes, NRR time was generally lower than that observed in *Mytilus* hemocytes (80 ± 8 min *C. gigas* vs 112 ± 7 min *M. galloprovincialis*) at the same time of the year. In *C. gigas* hemocytes *V.a.* induced a large lysosomal destabilization (−75% with respect to controls; $p < 0.01$) (Fig. 1C); complete destabilization was induced by *V.s.* (−95% with respect to controls; $p < 0.01$). Moreover, neither vibrio induced significant stimulation of lysozyme release by *C. gigas* hemocytes (not shown).

3.2. Role of signaling components in mediating the effects of *V. splendidus* in mussel hemocytes

The possible role of different kinases, in particular of p38 MAPK, PI-3K and PKC, in mediating *V.s.*-induced destabilization of lysosomal membranes in mussel hemocytes was investigated in cells pre-incubated with specific inhibitors and the results are reported in Fig. 2. SB203580, the specific inhibitor of p38 MAPK activation, significantly reduced the decrease in LMS induced by *V.s.*, although to a limited extent (from −85 to −68%, $p < 0.05$). On the other hand, the PI-3K inhibitor Wortmannin completely prevented lysosomal membrane destabilization induced by this strain. The specific PKC inhibitor GF109203X was ineffective. Similarly, the effect of *V.s.* was not affected in the presence of Dynasore, a specific blocker of the dynamin GTPase activity.

3.3. Transmission electron microscopy-TEM

Hemocyte monolayers incubated with *V.a.* or *V.s.* for different times (5, 15 and 30 min) were observed by TEM and representative images are reported Figs. 3 and 4. In experiments with *V.a.* (Fig. 3) bacterial uptake could be observed from 5 min of incubation (Fig. 3A); several bacteria were observed within phagosomes (P) at 15 min (Fig. 3B). At 30 min, several vibrios were present within phagosomes and lysosomes (lys) (Fig. 3C), some of them apparently undergoing degradation (Fig. 3D). However, at all times of incubation, mussel hemocytes showed intact intracellular structures, as well as intact membrane extensions.

On the other hand, in hemocytes incubated with *V.s.* (Fig. 4) signs of cellular damage were observed as early as from 5 min (Fig. 4A): loss of integrity of plasma membranes and absence of, or broken membrane extensions, alterations of cytoplasm, enlarged intracellular vacuoles. Moreover, several intact bacteria were observed within both phagosomes (P) and lysosomes (lys) at all times post addition (Fig. 4A, C, D). In particular, early and later fusion events between lysosomes could be observed (Fig. 4A and D).

Similar results were obtained in absence of hemolymph serum (not shown).

3.4. In vitro bactericidal activity of mussel hemocytes

The capacity of mussel hemocyte monolayers to kill the two *Vibrio* strains was investigated in a bactericidal assay that evaluates the number of live, cultivable bacteria in the hemocyte monolayer at different times of incubation (Fig. 5). The results show a significant and time dependent increase in bactericidal activity towards *V.a.*, from 20% at 30 min to a maximum of 50% at 90 min ($p < 0.05$). In contrast, no bactericidal activity was observed towards *V.s.* at any time of incubation (data not shown). No changes in the number of CFU/monolayer were observed when either *Vibrio* was incubated in hemolymph serum alone (data not shown).

3.5. Effects of in vivo challenge of *M. galloprovincialis* with different vibrios

3.5.1. Hemolymph parameters

Mussels were injected with either *V.a.* and *V.s.* and hemolymph samples collected at 6, 24 and 96 h p.i. Hemocyte LMS, serum lysozyme activity and bactericidal activity of whole hemolymph samples were evaluated.

LMS: In the hemocytes of control mussels, average NRR time was 125 ± 10 min, and no changes were observed in PBS−NaCl-injected mussels (not shown). Challenge with both vibrios lead to a significant decrease in LMS as soon as 6 h p.i. (Fig. 6A). However, the extent of LMS at this time was different according to the bacteria: −30% with respect to controls ($p < 0.05$) in *V.a.*-injected mussels, and −82% ($p < 0.05$) in *V.s.*-injected mussels (Fig. 6A). A further decrease was observed at 24 h p.i., when a dramatic decrease was observed also with *V.a.* (−80%) and complete

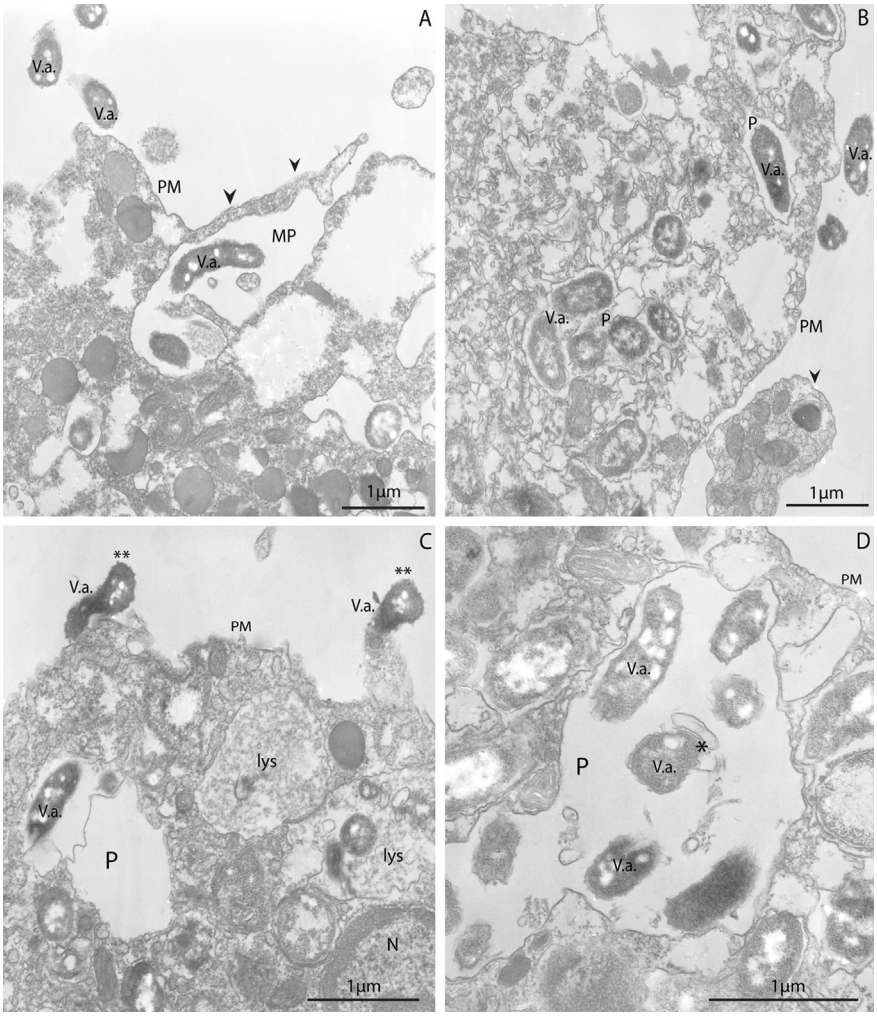

Fig. 3. Representative TEM micrographs of hemocytes of *Mytilus galloprovincialis* incubated with *V. aestuarianus* 01/032 for 5 (A), 15 (B) and 30 min (C and D) at 16 °C. *V. aestuarianus* (*V.a.*) is readily internalized by hemocytes through macropinocytosis-MP (A) and found within electron-lucent phagosomes (P) at 15 and 30 min after incubation (B–D). Intact filopodia (A) and tips of pseudopodia (B) can be observed (arrowheads). In C) note VA adhesion to the plasma membrane prior to internalization (**). At 30 min vibrios are found within intact lysosomal structures (Lys) filled with electron-dense material (C); in D) several bacteria showing signs of degradation (*) are also observed within phagosomal vacuoles (P). Plasma membrane (PM) and nucleus (N) are indicated. Scale bars: 1 µm.

lysosomal destabilization with *V.s.* However, at longer times p.i. (96 h), LMS was fully restored to control values in hemocytes of mussels injected with either vibrio.

Lysozyme: in control mussel, background serum lysozyme activity was recorded (60 ± 8 U/mg protein), and no changes were observed following by injection of PBS–NaCl whatever the time p.i. Both vibrios induced increases in lysozyme activity at all times p.i.; at 96 h, the effect of *V.s.* was stronger than that of *V.a.* (+120% and +50%, respectively, with respect to PBS–NaCl-injected mussels; $p < 0.05$) (Fig. 6B).

Bactericidal activity: in the hemolymph of mussels injected with *V.a.*, a rapid decrease in the number of viable bacteria (CFU/ml) was observed from 6 h p.i. (−88% with respect to T_0; $p < 0.05$) (Fig. 7A). No bactericidal activity was observed in mussels injected with *V.s.*: on the contrary, the number of viable bacteria was significantly increased with respect to T_0 at all times p.i. ($p < 0.05$); in particular, growth of *V.s.* peaked at 24 p.i., when a ten-fold increase in CFU/ml was recorded, followed by a decrease at longer times p.i. (Fig. 7B).

3.5.2. Effects on digestive gland LMS

LMS was evaluated as a general biomarker of stress induced in the digestive gland by bacterial challenge at different times p.i. and the results are reported in Fig. 8. In untreated mussels, lysosomal latency was of 35 ± 8 min (data not shown). In PBS/NaCl-injected mussels, lower values were generally found at 6 h p.i. with respect to un-injected mussels considered as controls, followed by progressive recovery at longer times p.i. as previously described [29]. In mussels challenged with *V.a.*, a significant decrease in LMS was observed at all times p.i. with respect to PBS/NaCl-injected mussels ($p < 0.05$), with stronger effects at shorter times p.i. (−65% at 6 h p.i.). On the other hand, *V.s.* did not affect digestive gland LMS at 6 h p.i., but a large and progressive decrease was observed at longer times p.i.; complete lysosomal destabilization was observed at 96 h p.i. (−95% with respect to controls).

4. Discussion

The results obtained in this work show differential *in vitro* and *in vivo* responses of the mussel *M. galloprovincialis* hemolymph to live *V. aestuarianus* 01/032 (*V.a.*) and *V. splendidus* LGP32 (*V.s.*), two vibrio strains associated with oyster mortalities, in terms of hemocyte LMS, serum lysozyme activity and bactericidal activity. These data extend previous studies carried out in mussels with heat killed *V. splendidus* LGP32 [13,20], and represent the first

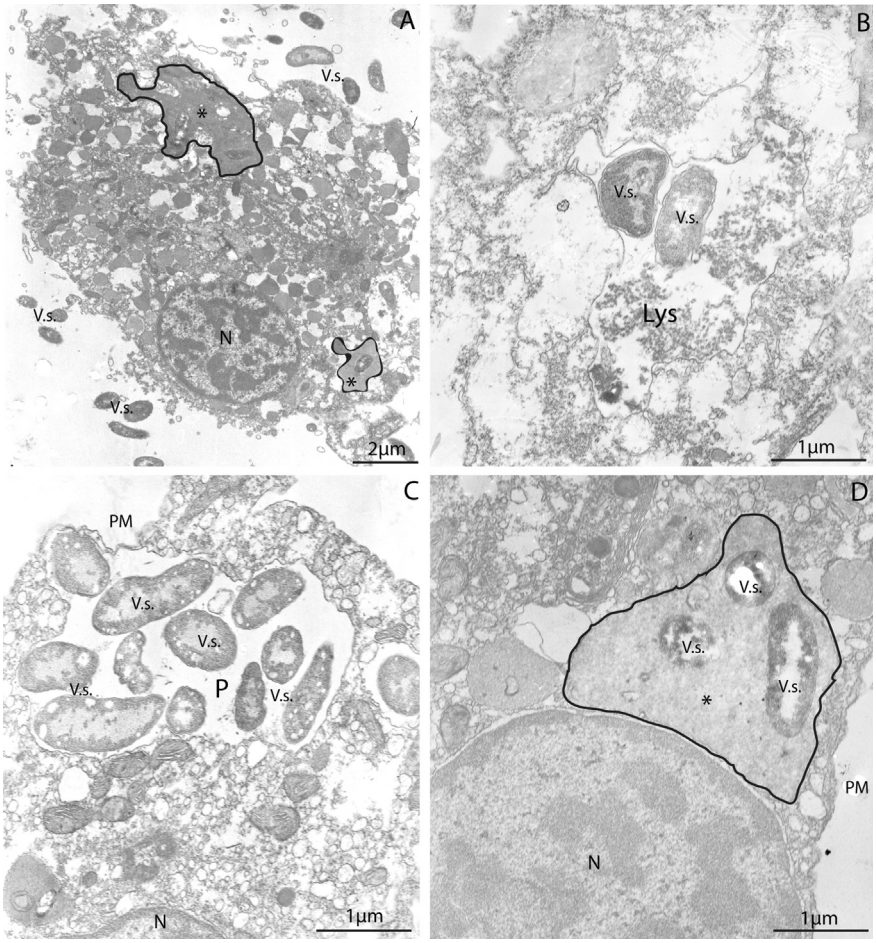

Fig. 4. Representative TEM micrographs of hemocytes of *Mytilus galloprovincialis* incubated with *Vibrio splendidus* LGP32 for 5 (A), and 30 min (B, C and D). A) Many vibrios (*V.s.*) can be observed in the extracellular medium. The hemocyte does not show extensions of filopodia or pseudopodia and the plasma membrane is surrounded by granular electron dense material. Some bacteria can be also observed within lysosomes of different size (likely) resulting from fusion events (*, outlined with a black line). B) Detail of intact bacteria within a lysosome (lys), C) a phagosome (P) containing several intact bacteria. Bar 1 µm. D) Detail of vibrios within a large lysosome in close contact with the nucleus (N). Again, signs of fusion events between granules can be observed (*). Scale bars: 2 µm and 1 µm.

information on the interactions on *Mytilus* immune responses to *V. aestuarianus* 01/032.

The results obtained *in vitro* indicate that in *Mytilus* hemocytes *V.a.* does not affect LMS, evaluated as a marker of cellular stress. On the other hand, live *V.s.* severely affected LMS, with effects stronger than those previously recorded with the heat-killed strain [13]. Similar results were obtained in the absence of hemolymph serum (not shown). Both vibrios stimulated extracellular lysozyme release, with *V.s.* more than with *V.a.*, with a time course similar to that observed with other Gram-negative bacteria, but to a much lower extent [2].

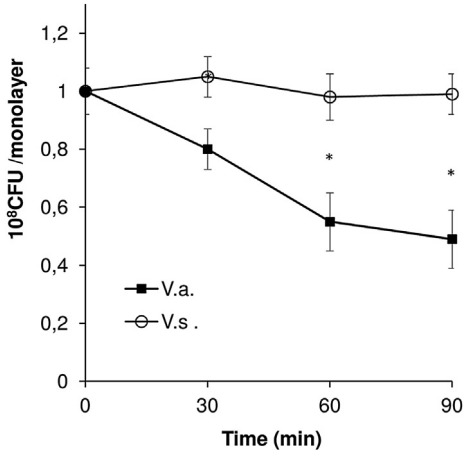

Fig. 5. *In vitro* bactericidal activity of hemocyte monolayers towards *V. aestuarianus* 01/032 (*V.a.*) and *V. splendidus* LGP32 (*V.s*). Results are expressed as the number of cultivable bacteria (CFU)/hemocyte monolayer. Data presented and statistic calculations as in Fig. 1. * = $p < 0.05$, all treatments vs control.

For comparison, the effects of both vibrios on LMS were evaluated in the hemocytes of *C. gigas* in the same experimental conditions. In oyster hemocytes, both *V.a.* and *V.s.* induced large destabilization of lysosomal membranes. In particular, *V.s.* showed stronger effects and also lead to hemocyte loss of adhesion (not shown). Interestingly, neither vibrio induced lysozyme release by *C. gigas* hemocytes, as previously observed in hemocytes of *Crassostrea virginica* incubated with *Vibrio vulnificus* [30]. The results clearly show that the hemocytes of *M. galloprovincialis* are less sensitive than those of *C. gigas* to these two oyster pathogens. These data indicate that lysozyme release does not appear as an efficient response of oyster hemocytes to vibrio challenge; on the other hand, the results underline that evaluation of LMS in live hemocytes represents a rapid and sensitive tool for comparing the effects of bacteria on the hemocytes of different bivalves.

The mechanisms involved in the effects of LGP32 on lysosomal function of mussel hemocytes were further investigated using inhibitors of different kinases [25,26]. In these cells, conserved

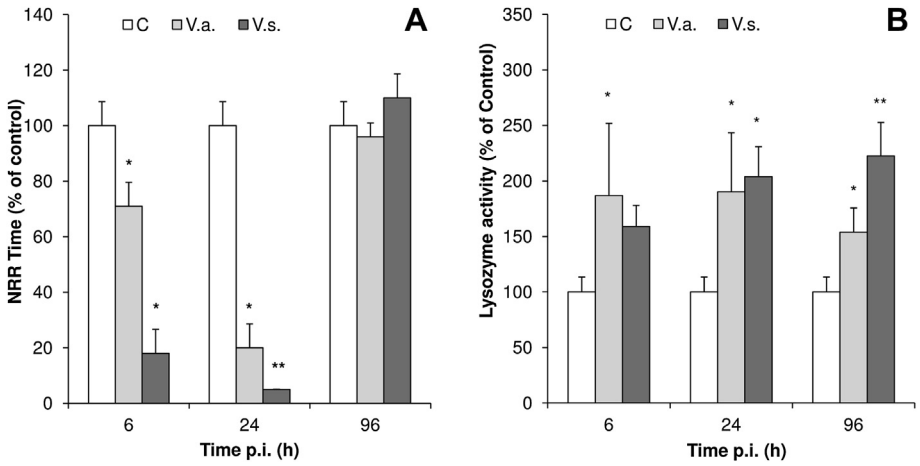

Fig. 6. Hemocyte lysosomal membrane stability-LMS (A) and serum lysozyme activity (B) in mussel hemolymph sampled at different times post-injection (6, 24 and 96 h) with *V. aestuarianus* 01/032 (*V.a.*) and *V. splendidus* LGP32 (*V.s*). Data (arithmetic mean ± SD), expressed as percentage of the value observed in mussels injected with PBS–NaCl considered as control (C), were obtained from 4 experiments each of them measured in triplicate. Statistical analysis was performed by using ANOVA followed by Tukey's post hoc test, * = $p < 0.05$, all treatments vs control; ** = $p < 0.05$ *V.s.* vs *V.a.*

components of kinase-mediated transduction pathways, including MAPKs-Mitogen Activated Protein Kinases, PKC-Protein Kinase C, PI-3Kinase, have been shown to play a key role in activation of the immune response [24,31,32]. However, certain bacterial species and strains, including heat killed *V.s*. LGP32, have been shown to induce disregulation of immune signaling, thus affecting the hemocyte response to bacterial challenge [20,24,31,32].

The results show that cell pre-treatment with the p38 MAPK inhibitor SB203580 lead to a significant, although small reduction of the effects of *V.s*. on LMS. On the other hand, inhibition of PKC was ineffective. Interestingly, the effect of this strain was fully abolished by the PI-3K inhibitor Wortmannin. PI-3K is involved in the immune responses of *M. galloprovincialis* hemocytes against pathogens, in particular in coordinating phagocytosis, in mediating ROS and NO production, and bactericidal activity [4,33]. The results here obtained underline a key role for PI-3K signaling in mediating the stressful conditions induced by live *V.s*. LGP32.

In professional phagocytes, pathogens are internalized into a membrane-bound vacuole, the phagosome, which undergoes a gradual maturation by fusion with endosomes and lysosomes to become an efficient microbicidal compartment, the phagolysosome, with PI-3Kinase(s) playing a crucial role in these processes [34–36]. Many pathogens have developed different strategies to manipulate phagocytosis and to gain entry into the host cells where they can establish a safe survival niche, by targeting the signaling pathways involved in activation of immune defenses, including PI-3K [34–36]. In particular, certain intracellular pathogens are able to affect phagosome/lysosome fusion by interfering with the PI3-K pathways [37,38].

When the interactions between *V.a*. and *V.s*. and mussel hemocytes were visualized by TEM, both vibrios were rapidly internalized by *M. galloprovincialis* hemocytes. With *V.a.*, no cellular or lysosomal damage were observed. Moreover, internalization was apparently efficient for this strain, with early signs of bacterial degradation within vacuoles at 30 min incubation: in parallel, progressive bactericidal activity was observed from 30 min, resulting in killing of about 50% of bacteria at 90 min.

On the other hand, *V.s*. induced rapid morphological alterations of the hemocytes, and several intact bacteria were observed within large phagosomes at all times of incubation. Moreover, *V.s*.

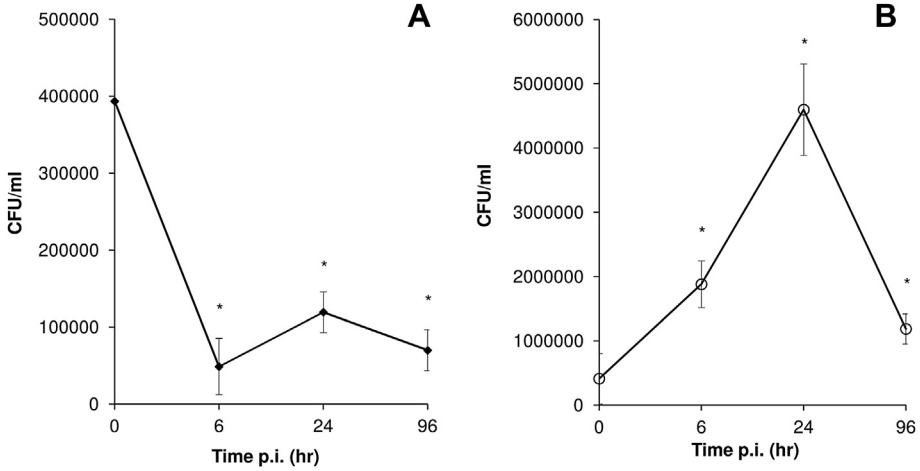

Fig. 7. Survival of *V. aestuarianus* 01/032 (A) and *V. splendidus* LGP32 (B) in whole hemolymph samples of *M. galloprovincialis* at different times p.i. with bacteria. Results are expressed as CFU/ml hemolymph. In this experiment, T_0 corresponded to 30 min p.i. (that is, the time interval required for hemolymph to reach equilibrium after bacterial injection), as described in Methods. Statistical analysis was performed by using ANOVA followed by Tukey's post hoc test, * = $p < 0.05$, all treatments vs control.

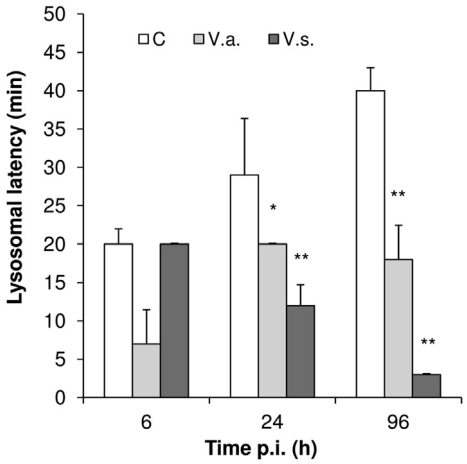

Fig. 8. Lysosomal membrane stability (LMS) in the digestive gland of mussels sampled at different times p. i. (6, 24 and 96 h) with *V. aestuarianus* 01/032 (*V.a.*) and *V. splendidus* LGP32 (*V.s*). For each set of experiments, data obtained from PBS–NaCl injected mussels considered as control (C) are also reported. Tissues were analyzed for latency of lysosomal *N*-acetyl-β-hexosaminidase activity as described in Methods. Data, expressed as lysosomal latency (min) are the mean ± SD ($n = 5$). Statistical analysis was performed by using ANOVA followed by Tukey's post hoc test. * = $p < 0.05$, all treatments vs control. ** = $p < 0.05$, *V.s.* vs control and vs *V.a.*

apparently induced early fusion events between lysosomes. These observations are in line with the LMS data, indicating that in mussel hemocytes *V.s.* severely affects the lysosomal system. Moreover, data on bactericidal activity show that hemocyte monolayers were unable to kill *V.s.* Overall, the results indicate that the LGP32 strain is rapidly phagocytized by *M. galloprovincialis* hemocytes; when internalized, it remains viable and culturable within intracellular vacuoles apparently escaping lysosomal degradation. Moreover, our data support the hypothesis that resistance of *V.s.* to the bactericidal activity of mussel hemocytes may involve alterations of PI-3K signaling, leading to impairment of the endo-lysosomal system.

In *C. gigas*, *V.s.* LGP32 uses the OmpU protein to attach and invade the hemocytes through Cg-EcSOD, the major plasma protein, that acts as an opsonin mediating recognition and promoting phagocytosis [18,19]. In this process, Cg-EcSOD is recognized through its RGD sequence by hemocyte β-integrins, leading to subversion of the cell actin cytoskeleton, inducing the expression of trafficking genes, and resulting in actin and clathrin polymerization. Capable of intracellular survival, LGP32 was shown to escape from host cellular defenses by avoiding acidic vacuole formation and by limiting ROS production [19].

In *Mytilus*, the major plasma protein repeatedly discovered and named 'serum protein band 1' (SBP1), Histidine-rich glycoprotein (HRG), 'heavy metal binding protein' (HIP) or 'extrapallial fluid major protein' (EP protein), shares no sequence homology with CgEcSOD [39], and references quoted therein]. However, both LMS data and TEM analysis showed no differences when hemocytes were incubated with LGP32 either in the presence of absence of hemolymph serum (not shown), indicating that interactions with hemolymph soluble factors are not crucial in determining the effects of this strain in mussel hemocytes. Moreover, in this work we made use for the first time in bivalve hemocytes of Dynasore, a blocker of the GTPase activity of dynamin, acting as a potent inhibitor of endocytic pathways by blocking coated vesicle formation [27,28]. The results show that the dynamin inhibitor did not afford protection from LGP32-induced lysosomal damage, suggesting the involvement of clathrin-independent processes. Therefore, the mechanisms involved in promoting *V.s.* adhesion and invasion in oyster and mussel hemocytes may be profoundly different, resulting in different effects.

The effects of *V.a* and *V.s.* were confirmed *in vivo*, in hemolymph samples from mussels injected with either live strain and sampled at different times p.i. In these conditions, both vibrios induced significant decreases in hemocyte LMS at 6 and 24 h p.i., with stronger effects of *V.s.*, followed by complete recovery at 96 h p.i. Moreover, both vibrios induced stimulation of lysozyme activity; with *V.s.* inducing a stronger response at longer times p.i.. When the bactericidal activity of whole hemolymph samples was evaluated, efficient killing of *V.a.*, but not of *V.s.*, was observed, this confirming the results obtained *in vitro*. Moreover, *V.s.* was actually able to grow within mussel hemolymph at all times p.i.; interestingly, bacterial growth peaked at 24 h p.i., when largest decreases in LMS were measured. Large but transient decreases in total cell count (THC) and in proportion of different hemocyte subpopulations (hyalinocytes, small and large granulocytes) have been described in mussels injected with *V.s.* (from 3 to 48 h) [12]. Although THC data were not collected in the present work, we observed that at longer times p.i. (96 h) full recovery of hemocyte LMS (a parameter that is measured in small and large granulocytes) was associated with highest lysozyme activity and reduction in bacterial growth within the hemolymph, indicating that, in these conditions, mobilization of granulocytes from the tissues, or hemocyte differentiation, or both, may occur, as previously suggested [12]. Such a capacity of recovery would account for the resistance of *Mytilus* spp. to infection not only with LGP32 and 01/032, but also to other *Vibrios*.

Finally, the results indicate that the mussel response to vibrio challenge is not limited to hemolymph components, since stressful conditions were observed also in the digestive gland or hepatopancreas. This tissue plays a central role in metabolism through intracellular digestion of food particles and nutrient distribution to the gonad, and can also participate with hemolymph components to eliminate entrapped bacteria by digestive function [2]. Challenge of *M. galloprovincialis* with different heat-killed bacteria, including *V.s.*, induced significant changes in lysosomal and oxidative stress biomarkers in the digestive gland, including a decrease in LMS [29]. The results here obtained indicate that the effects of live LGP32 on LMS were stronger than those previously observed with heat-killed bacteria. In particular, the extent of lysosomal membrane destabilization observed at longer times p.i. (below 80% of control values) corresponds to increased autophagic processes and stressful conditions in the tissue [40]. These results confirm that also in digestive cells the lysosomal system represents a sensitive target for vibrios.

Although the present work was aimed at investigating the functional responses of *Mytilus* to challenge with potential pathogenic *Vibrios*, the results obtained with *V.s.* are in line with those obtained by a transcriptomic approach. A new microarray platform, a mussel Immunochip, recently developed as a flexible tool for the experimental validation of immune-candidate sequences, was tested with hemolymph RNAs obtained from mussels challenged with *V.s.* at 3 and 48 h p.i. This approach revealed a total of 143 and 262 differentially expressed genes showing the early and late hemocyte response of the *V.s.*-challenged mussels [41]. A general down-regulation of antimicrobial peptides (AMP) and a delayed up-regulation expression of proteases and stress proteins were observed, indicating a functional decline, together with frequent up-regulation of proteins involved in cell shape and motility. The overall data indicate a mounting inflammatory response at shorter times p.i., followed by a later shift towards more general stress conditions. Moreover, in the same experimental conditions, *V.s.* was shown up-regulate components of the Toll pathway [42].

Overall, the results obtained with *V. splendidus* LGP32 indicate that, although this strain is not considered pathogenic to mussels, in *M. galloprovincialis* it is able to induce strong stressful conditions

not only in the hemocytes, but also at the tissue level, and to multiplicate within mussel hemolymph, thus significantly affecting the health status of the mussel. The involvement of PI-3K-mediated pathways in alteration of lysosomal functions may imply the participation of downstream effectors, such as the ser/thr kinase mTor, that regulates multiple cellular processes, including cell growth and survival, as well as autophagy and degradation of intracellular pathogens [43,44]. The presence of autophagy-related genes, including mTor in *Mytilus* has been recently described [45]. The possibility that certain bivalve pathogens may activate these pathways represents a promising field of investigation in host–pathogen interactions.

For what concerns *V. aestuarianus* 01/032, whose interactions with *Mytilus* spp. have been not investigated so far, the results indicate that this strain is efficiently cleared from the hemolymph, inducing less severe stressful conditions both in the hemocytes and digestive gland. The mechanisms involved in the interactions between *V. aestuarianus* 01/032 and mussel hemolymph components, leading to an efficient immune defense response, require further investigation.

Acknowledgments

This work was supported by the EU project BIVALIFE (FP7-KBBE-2010-4).

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