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Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*

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Abstract :

OmpU porins are increasingly recognized as key determinants of pathogenic host *Vibrio* interactions. Although mechanisms remain incompletely understood, various species, including the human pathogen *Vibrio cholera*, require OmpU for host colonization and virulence. We have shown previously that OmpU is essential for virulence in the oyster pathogen *Vibrio splendidus* LGP32. Here, we showed that *V. splendidus* LGP32 invades the oyster immune cells, the hemocytes, through subversion of host-cell actin cytoskeleton. In this process, OmpU serves as an adhesin/invasin required for β-integrin recognition and host cell invasion. Furthermore, the major protein of oyster plasma, the extracellular superoxide dismutase *Cg*-EcSOD, is used as an opsonin mediating the OmpU-promoted phagocytosis through its RGD sequence. Finally, the endocytosed bacteria were found to survive intracellularly, evading the host defense by preventing acidic vacuole formation and limiting reactive oxygen species production. We conclude that (*i*) *V. splendidus* is a facultative intracellular pathogen that manipulates host defense mechanisms to enter and survive in host immune cells, and (*ii*) that OmpU is a major determinant of host cell invasion in *Vibrio* species, used by *V. splendidus* LGP32 to attach and invade oyster hemocytes through opsonisation by the oyster plasma *Cg*-EcSOD.

Keywords : host–pathogen interaction ; innate immunity ; invertebrate ; mollusk ; oxidative burst

Vibrio splendidus LGP32 is an oyster pathogenic strain isolated from massive mortality events in the production of *Crassostrea gigas* oysters (1). However, up to now, little is known on the route of infection and pathogenic processes of LGP32 (2, 3). A metalloprotease has been associated to toxicity (4, 5) and the outer membrane protein (OMP) OmpU was shown to be a major determinant of LGP32 virulence (6).

As bacterial surface components, OMPs are both used by hosts for pathogen recognition, and by pathogens for interaction with and invasion of host cells, serving as adhesion proteins (adhesins) (7-9) or invasion proteins (invasins) (10, 11). OmpU is a major porin from *Vibrio* species. Recent studies have shown that it mediates host-*Vibrio* interactions, being involved in the resistance to antimicrobial peptides (AMPs) (12, 13), the adherence to host cells (14), or pathogen/symbiont recognition (15). Similarly, in LGP32, OmpU participates to the resistance to oyster AMPs and displays adhesive properties (6). To date, little is known on the molecular basis of the OmpU-mediated host-*Vibrio* interactions. However, in *V. vulnificus*, OmpU was reported to bind to fibronectin as a process for invasion of HEp-2 cells (14).

Hemocytes are the immune cells of mollusks. As such, they are equipped with immune receptors and effectors involved in pathogen recognition and control. Recent advances in mollusk-pathogen interactions have shown the existence of highly diverse somatically generated receptors that give high specificity to pathogen recognition (16, 17). So far, in oysters, recognition is mediated by germ-line encoded receptors such as a β-integrin involved in hemocyte phagocytosis (18). It was proposed to serve as a receptor for the major plasma protein, the extracellular superoxide dismutase *Cg*-EcSOD, which contains a RGD cell-binding domain (19). Besides, a broad diversity of immune effectors has been characterized in oyster hemocytes ranging from reactive oxygen species (ROS) (20, 21) to AMPs (22-24).

Some oyster pathogens target hemocytes to invade their host. For instance, the parasite *Perkinsus marinus* enters *Crassostrea virginica* hemocytes through recognition by a specific receptor, the *Cv*-Gal galectin, which promotes its phagocytosis (25). Inside hemocytes, it avoids the induction of the oxidative burst as a mechanism of immune evasion, proliferates, and spreads throughout the host (26, 27).

Here, we have studied the role of OmpU in the interaction of LGP32 with oyster hemocytes. Green fluorescent protein (GFP)-expressing derivatives of LGP32 and its Δ*ompU* mutant were constructed. By *in vitro* hemocyte invasion assays and experimental infections, we showed that LGP32 is a facultative intracellular pathogen that invades the oyster immune cells by using OmpU as an adhesin and invasin. In this process, the plasma *Cg*-EcSOD is used as an opsonin recognized through its RGD sequence by hemocyte β-integrins. OmpU-recognition was shown to subvert the host cell actin cytoskeleton, inducing the expression of host cell trafficking genes and resulting in actin and clathrin polymerisation. Capable of intracellular survival, LGP32 was shown to escape from host cellular defenses by avoiding acidic vacuole formation, and by limiting ROS production.

Results

V. splendidus LGP32 invades and survives in oyster hemocytes, impairing immune defense fonctions. A GFP-expressing LGP32 was constructed, which carries the *gfp*3 gene under the P_{TRC} constitutive promoter in a genomic locus. After a 2 h-*in vitro* contact between the GFP-expressing LGP32 and oyster hemolymph, 31.7 ± 5.6 % hemocytes were GFP-positive, as determined by flow cytometry (Fig. 1A). A massive engulfment of LGP32 was observed on confocal microscopy optical sections (Fig. 1B, C). Although less intense, the engulfment of GFP-expressing LGP32 by hemocytes was also observed *in vivo,* 1 h after injection to oysters (Fig. 1D). Interestingly, counts of intracellular vibrios monitored by plating hemocyte lysates from *in vitro* contacts were stable at 10^6 colony forming units (CFU)/ml over

16 h (Fig. 2A). This showed that LGP32 remains viable and culturable within oyster hemocytes. In contrast, the avirulent control LMG20012^T (6, 28), which was also avidly engulfed, did not survive within hemocytes, CFU counts dropping by 4 Logs over time (Fig. 2A). The bacterial load due to the oyster-associated environmental microflora remained below 10 CFU/ml over the time-course.

Hemocyte responses were measured 1 h after engulfment of LGP32 or LMG20012^T, when similar intracellular bacterial loads (10^5 to 10^6 CFU/ml) are present in both samples (Fig. 2A). Unchallenged hemocytes were used as a control. Interestingly, the lysotracker fluorescence indicative of acidic vacuole formation was similar in control and LGP32-containing hemocytes but significantly higher in LMG20012^T-containing hemocytes ($p<0.05$) (Fig. 2B). Similarly, the DCFH-DA fluorescence indicative of ROS production was significantly higher in LMG20012^T- than in LGP32-containing hemocytes ($p<0.05$), although both were higher than in control hemocytes (Fig. 2B). Thus, defense reactions involved in the efficient control of phagocytosed bacteria such as LMG20012^T are significantly attenuated in hemocytes invaded by LPG32.

LGP32 uses β-integrin recognition and subverts the host cell actin cytoskeleton for hemocyte invasion. The role of β-integrin in recognition and binding of LGP32 was studied by incubating oyster hemolymph with anti-β-integrin or an irrelevant antibody before contact with GFP-expressing LGP32. Strikingly, flow cytometry counts of GFP-positive hemocytes dropped by 72% upon anti-β-integrin treatment ($p<0.001$) while they remained stable with the irrelevant antibody (Fig. 3A). Similarly, incubation of hemolymph with an Arg-Gly-Asp (RGD) tripeptide inhibited invasion by 72% ($p<0.01$), while an irrelevant Glu-Gly-Phe (EGF) tripeptide had no significant effect (Fig. 3B). This showed that the RGD-recognition domain of β-integrin is essential for hemocyte invasion.

Contacts between oyster hemolymph and GFP-expressing LGP32 were then performed in the presence of hypertonic concentrations of sucrose, which inhibit receptor-mediated phagocytosis by inducing abnormal clathrin polymerization (29). Sucrose inhibited hemocyte invasion in a dose-dependent manner, from 24% ($p<0.01$) at 0.1 M up to 68 % ($p<0.001$) at 0.5 M (Fig. 3C). Similarly, cytochalasin D used as an inhibitor of actin polymerization inhibited hemocyte invasion from 13 % at 1 µg/mL ($p<0.05$) to 71% at 10 µg/mL ($p<0.001$) (Fig. 3D). This showed that hemocyte invasion by LGP32 uses host cell clathrin and actin polymerization.

Hemocyte invasion requires the OmpU porin and a plasma opsonin that specifically binds OmpU. The ability of an avirulent Δ*ompU* mutant (6) to invade hemocytes was studied by constructing a GFP-expressing derivative. Hemocyte / *Vibrio* interactions were monitored by flow cytometry. Strikingly, the number of GFP-positive hemocytes shifted from 31.7 ± 5.6 % when exposed to LGP32 down to 2.5 ± 1 % when exposed to the Δ*ompU* mutant ($p<0.01$) (Fig. 4A). In agreement, confocal microscopy showed little to no intracellular GFP-expressing bacteria (Fig. 4B), indicating that OmpU is required for hemocyte invasion. Besides, invasion of hemocytes by wild-type LGP32 dropped dramatically from 31.7 ± 5.6 % to 7.5 ± 2.3 % ($p<0.05$) upon plasma withdrawal, and returned to 32.0 ± 6.0 % when plasma was added back (Fig. 4A & S1). This strongly suggests that a plasma component operates as an opsonin. To determine how OmpU and plasma are involved in the host-pathogen interaction, we tested the ability of LGP32 and Δ*ompU* to bind to immobilized plasma proteins. Results showed that OmpU is required for the dose-dependent binding of LGP32 to plasma (Fig. S2). Altogether, this strongly suggests that a plasma opsonin mediates recognition and promotes phagocytosis of LGP32 by binding to OmpU.

Oyster *Cg*-EcSOD binds to OmpU in an RGD-dependent manner and promotes hemocyte invasion by LGP32. To identify OmpU-binding plasma proteins, plasma was fractionated by HPLC and fractions were tested for binding to GFP-expressing LGP32 and Δ*ompU*. Only three HPLC fractions referred to as f6, f7 and f8 showed differential binding ($p<0.05$) (Fig 5A & C). On SDS-PAGE, they displayed a 30 kDa band

predominant in f6 and f7, which was absent from the fractions displaying no differential binding (Fig 5B). An LC-MS/MS analysis of f6 and f7 unambiguously identified the proteins as variants of *Cg*-EcSOD (GenBank AAY60161.1) (Fig. S4). The aberrant migration of *Cg*-EcSOD variants at ~30 kDa instead of 20 kDa is in agreement with previous studies (19, 30). f6 and f7, which could contain different variants or foldamers of *Cg*-EcSOD as presumed from their different chromatographic behavior (31), displayed similar binding to LGP32 (Fig. 5C). The binding of LGP32 to immobilized *Cg*-EcSOD was OmpU-dependent, dose-dependent, and linear in the range of concentrations tested (Fig. 6). Preincubation of LGP32 with RGD tripeptide resulted in a significant loss of binding by more than two fold ($p<0.01$) while an EGF tripeptide (control) had no significant effect (Fig. 6). Altogether, this shows that the RGD sequence of *Cg*-EcSOD is essential for the OmpU-mediated binding of *Cg*-EcSOD to LGP32.

This prompted us to investigate the role of *Cg*-EcSOD in the plasma opsonisation previously observed. GFP-expressing wild-type or Δ*ompU* LGP32 were treated with purified *Cg*-EcSOD before being exposed to washed hemocytes, unable to be invaded by LGP32. This fully restored the ability of LGP32 to invade oyster hemocytes, as evidenced by both flow cytometry (Fig. 4A) and confocal microscopy (Fig. S1). Taken together, those results identify *Cg*-EcSOD as an oyster opsonin recognizing OmpU of LGP32.

Expression of cell remodeling genes is OmpU-dependent in LGP32-experimentally infected oysters. To determine the role of OmpU on hemocyte responses *in vivo,* oysters were infected with 10^8 CFU of wild-type or Δ*ompU* LGP32, or SSW (control). Expression of oyster hemocyte genes involved in actin dynamics and cell motility was monitored 24 h post-injection. Among them, β-actin is involved in cell motility, structure, and integrity through microfilament building. Intersectin-1 is a cytoplasmic membrane-associated protein that coordinates endocytic membrane traffic with the actin assembly machinery, and regulates the formation of clathrin-coated vesicles (32). Fascin is an actin cross-linking protein involved in the assembly of actin filaments into bundles (33). Finally, calreticulin is a multifunctional protein that serves as a cell surface receptor for the C1q component of complement and mediates phagocytosis upon binding to major plasma proteins (34, 35). While the number of actin transcripts was similar in *Vibrio*- and SSW-injected oysters, all other three genes had a significantly higher number of transcripts in LGP32-injected oysters ($p<0.1$) (Fig. 7). Interestingly, Δ*ompU*-injected oysters did not differ significantly from the SSW-injected controls (Fig. 7). That this lack of response is due to OmpU-expression is indicated by the statistical difference observed for intersectin-1 and fascin between LGP32- and Δ*ompU*-injected oysters ($p<0.1$). Although calreticulin responded similarly, the response to an injection by the wild-type or the Δ*ompU* mutant was not statistically different ($p=0.16$) (Fig. 7). Altogether, these data show that ompU activates the expression of genes involved in cell remodeling. Conversely, the expression of *Cg*-EcSOD and β-integrin, which mediate recognition, was not statistically different between LGP32- and Δ*ompU*-injected oysters (Fig S3).

Discussion

Results showed that *V. splendidus* LGP32 is a facultative intracellular pathogen that uses OmpU to attach and invade oyster hemocytes through opsonisation by the plasma extracellular superoxide dismutase, Cg-*Ec*SOD. Although usually considered as extracellular pathogens, several *Vibrio* species support intracellular stages. This was shown for the coral pathogen *V. shiloi,* which invades epithelial cells (36), and for the human pathogen *V. cholerae,* which is endocytosed by amoebas (37) and phagocytes in which it retains viability (38). That Vibrio species can have intracellular stages is also supported by the emerging evidence that most pathogenic microbes are capable of intracellular survival, at least during some stages of the infection and disease cycle. Hence, Casedevall proposed that the capacity for intracellular life may be the rule rather than the exception (39).

The major role of hemocyte invasion in the pathology of LGP32 is shown here by the impaired ability of an avirulent mutant (6) to invade oyster hemocytes (Fig. 4). Hemocyte invasion, which was observed both *in vivo* and *in vitro*, had deleterious consequences on oyster cellular defenses. Indeed, the presence of viable and culturable intracellular LGP32 was associated with the lack of acidic vacuole formation and an attenuated ROS production, which were clearly observed upon phagocytosis and elimination of the avirulent LMG20012^T (Fig. 2). Whether those mechanisms rely on the intracellular secretion of bacterial effectors remains to be established. Such an intracellular secretion was recently shown for type VI secretion (T6SS) effectors of *V. cholerae* (38), which are also present in the genome of LGP32 (40). The lack of acidic vacuole formation (Fig. 2), indicative of defective phagolysosome maturation, is a mechanism of immune evasion for intracellular pathogenic bacteria like *Mycobacterium tuberculosis* and *Salmonella typhimurium* (41), which greatly differs from that of *Vibrio aestuarianus,* another pathogen associated to the major episodes of mortality in oysters. Indeed, *V. aestuarianus* avoids phagocytosis by the secretion of inhibitory extracellular products (42) while LGP32 uses phagocytosis as part of its pathogenic process (this study). This process is actually more similar to that of a third oyster pathogen, the parasite *Perkinsus marinus,* which invades oyster hemocytes but fails to elicit an oxidative response (27).

OmpU was found here to serve as an adhesin for hemocyte recognition and cell entry. Such a role in host cell recognition was recently reported in other Vibrio species (14, 15). Like OmpU, OMPs of intracellular pathogens can mediate adherence to host cells, stimulating host transduction pathways required for bacterial entry. One remarkable example is that of OpC from *Neisseria meningitis*, which binds to human serum factors like activated vitronectin and to a lesser extent fibronectin to attach to and invade human brain endothelial cells (11). There, fibronectin/vitronectin serve as molecular bridges between the pathogen and the host RGD-recognizing $\alpha v\beta 3$-integrin receptors. As previously shown for fibronectin (6, 14), the major protein of oyster plasma known as *Cg*-EcSOD was shown here to specifically recognize OmpU. From this study, it mediates attachment to and invasion of hemocytes, serving as an opsonin which promotes phagocytosis of LGP32 (Fig. 4). This provides the multifunctional *Cg*-EcSOD with another immune function, the protein having also LPS-binding, iron-binding, and antioxidant properties (19, 43). Binding of *Cg*-EcSOD to OmpU-expressing LGP32 was dependent on the RGD sequence (Fig 5B), which is found in both fibronectin and *Cg*-EcSOD (19) and was reported to mediate the interaction of OmpU from *V. vulnificus* with fibronectin (14). Thus, like fibronectin binds to OpC in *N. meningitis, Cg*-EcSOD binds to OmpU in LGP32 and mediates its interaction with *C. gigas* host cells.

We showed here that the OmpU-dependent hemocyte invasion by LGP32 is β-integrin-mediated (Fig. 3). This is consistent with the identification of β-integrins as major targets for adhesin-dependent phagocytosis, being particularly prone to hijacking by invasive bacteria (44). Integrin-mediated invasion by binding of host major plasma proteins to OMPs has actually been documented in various intracellular pathogens (*e.g. N. meningitis* (11), *Orientia tsutsugamushi* (45) and *Moraxella catarrhalis* (46)). Experimental infections of oysters showed that LGP32 signals cytoskeletal remodeling in an OmpU-dependent manner. Indeed, genes like calreticulin, intersectin-1, and fascin, which mediate recognition, clathrin-dependent endocytosis and actin polymerisation are overexpressed in hemocytes from animals injected with wild-type but not Δ*ompU* LGP32 (Fig. 7). Those *in vivo* data support the OmpU-promoted subversion of oyster hemocyte actin cytoskeleton as a major step in the infectious process of LGP32.

Altogether, the data from the present study unravel the cellular and molecular mechanisms associated to the OmpU-dependent virulence of LGP32 evidenced in our previous study (6). We propose a model of hemocyte invasion by LGP32 in which LGP32 is recognized by β-integrin at the surface of oyster hemocytes through an OmpU-specific opsonisation by *Cg*-EcSOD (Fig. S5). Intracellular bacteria then evade hemocyte defenses (antimicrobial peptides and ROS) by avoiding acidic vacuole formation and limiting the production of ROS. Whether oyster hemocytes are the final target of LGP32 or whether they

serve as vehicles for infection of tissues remains to be established. Aside from the present model, the increasing number of reports identifying OmpU as a key mediator of pathogenic interactions indicates that OmpU cell adhesive and invasive properties could be of prime importance in many biological models of infections, including human diseases, where fibronectin could serve as a bridging molecule instead of *Cg*-EcSOD between the pathogen and the host cell β-integrins.

Materials and Methods

Bacterial strains. *Vibrio* strains (Table 1) were grown at 20°C in Zobell medium. When needed, 12.5 µg/ml chloramphenicol (Cm) was added. Green fluorescent protein (GFP)-expressing derivatives of LGP32 and its Δ*ompU* isogenic mutant were obtained by allelic exchange as described previously (6). The *gfp* gene under the PTRC promoter was integrated in a non essential transposase gene of LGP32 using the pSW3654T suicide vector (Table 1). Primers for pSW3654T construction were Tr32s-Pst1 and Tr32as-BamH1 (Table 1).

Animals and hemolymph collection. Adult diploid Crassostrea gigas were purchased from local oyster farms in Mèze and Palavas-les-Flots (Gulf of Lion, France). Hemolymph was collected from the posterior adductor muscle sinus using a 2 ml syringe equipped with a 23G-needle. Cell-free hemolymph (plasma) was obtained by centrifugation (3,000 x g, 15 min, 4°C) and filtration through a 0.22 µm-pore size filter.

Oyster bacterial challenge. Experimental infections were performed at 20°C as previously described (6). Oysters were injected with 1 x 10^8 CFU (colony forming unit) /animal of wild-type or ΔompU LGP32. Control animals were injected with Sterile Sea Water (SSW). For every condition, oysters were placed for 24 h in three separate in 50 l-tanks of sea water. For gene expression analysis, hemolymph was collected from 30 oysters per experimental conditions (10 oysters per tank). Hemocytes were collected from pools of 10 hemolymphs (one pool per tank) by centrifugation (15 min, 1000 x g, 4°C) and directly processed for RNA extraction. For confocal microscopy, hemolymph was collected 1 h after injection of GFP-expressing LGP32 (1 x 10^8 CFU).

Hemocyte invasion assays. GFP-labeled LGP32 in stationary phase of growth and oyster hemolymph or hemocytes were incubated in a 50:1 ratio. When needed, a 1 h-preincubation was performed with either plasma, purified *Cg*-SOD, or various inhibitors, including sucrose (Fluka), cytochalasin D (Sigma), 0.4 mg/ml of RGD or EGF tripeptides (Sigma), or 8 µg/ml of anti-β-integrin antibody (generous gift from Dr. Marie-Christine Lebart). After fixation with 3.7 % formaldehyde, hemocytes were washed twice with SSW and counter-stained with Evan's blue (Sigma), Alternatively, hemocytes were stained with phalloidin-tetramethylrhodamine B isothiocyanate and DAPI (Sigma). Hemocyte invasion was monitored after a 30 min-contact by confocal microscopy, and after 2 h by flow cytometry.

Confocal microscopy. Images were acquired on a Leica SPE confocal laser scanning system connected to a Leica DM 2500 upright microscope. Lasers were used at λex 488nm for GFP (λem 505-530nm), λex 532nm for rhodamine-phalloidin (λem 560-630nm), λex 405nm for DAPI (λem 420-480nm), and λex 405nm for Evan's blue (λem 670-750nm). A 40X ACS APO 1.15oil Leica objective was used. Images were collected sequentially to avoid cross-contamination between fluorochromes and scanned at a 1024X1024 pixel resolution. Series of optical sections were collected.

Flow cytometry. The green fluorescence of LGP32 in hemocytes was monitored in a FACscan apparatus using a 488 nm argon-ion laser. Hemocyte invasion was monitored using at least a total of 30,000 events. Data were analyzed with the Cell Quest software .

Viability of intracellular bacteria. Monolayers of 2.5 x 10^5 hemocytes were exposed to LGP32 or LMG20012^T in a 50:1 ratio. After 1, 4 and 16 h, extracellular bacteria were eliminated with trypsin-EDTA 0.02 % and hemocytes were lysed in 0.05 % Triton X-100 before plating on Zobell medium for CFU counting.

Quantification of hemocytes responses. Hemocyte suspensions exposed for 1h to LGP32 or LMG20012^T in a 50:1 ratio were treated with 0.1 mM green-lysotracker (Invitrogen) or 1 mM 2'7'-dichlorofluorescein diacetate (DCFH-DA, Sigma). Fluorescence indicative of acidic vacuole formation and reactive oxygen species (ROS) production was monitored on a Tecan microplate reader at λex 504 / λem 511 and λex 492 / λem 530, respectively. Unchallenged hemocytes were used as controls.

Plasma fractionation. Pooled plasma from 10 oysters was acidified to pH 3.8 with 2 M HCl. Proteins (2 mg) were separated on a UP5NEC-25QS HPLC column (Interchim) equilibrated in 0.05% trifluoroacetic acid (TFA). Fractionation was performed with a linear gradient of 0-70% acetonitrile (ACN) in TFA 0.05 % over 35 min at a flow rate of 0.7 ml/min. Absorbance was monitored at 225 nm. Fractions were lyophilized and reconstituted in PBS before SDS-PAGE analysis or binding assays.

LC-MS/MS analysis. Protein bands were excised from SDS-PAGE, dehydrated, and incubated with 0.15 µg trypsin (Promega) in 25 mM NH_4HCO_3 for 16 h at 37°C. Peptides were extracted from gel pieces on a robot (EVO150, Tecan) with three sequential 30 µL-extractions in 50% ACN, 5% formic acid, and 100% ACN. Peptide extracts were pooled and dried under vacuum before LC-MS/MS analysis on a Ultimate 3000 HPLC (Dionex) coupled with a LTQ-ORBITRAP discovery (Thermo Fisher Scientific) mass spectrometer. The MS-MS sequenced peptides were analyzed by the Mascot search engine for protein identification, against the 29,745 unique ESTs available for *C. gigas* (http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html).

Plasma- and Cg-EcSOD-binding assay. Plasma, HPLC fractions or purified *Cg*-EcSOD were coated on a microplate for 16 h at 4°C. Bound proteins were then overlaid for 1 h at room temperature with GFP-expressing bacteria in stationary phase of growth (10^8 per well). In specific assays, bacteria were preincubated for 30 min with 400 µg/ml RGD or EGF tripeptides (Sigma) before overlay. Unbound bacteria were washed off with SSW before GFP reading (λex 395 nm / λem 509 nm) on a Tecan Infinite200 microplate reader.

RNA isolation and qPCR analysis of oyster gene expression. RNAs were extracted in Trizol (Invitrogen) from oyster hemocytes (3 pools of 10 oysters per condition). cDNA was synthesized with M-MLV reverse transcriptase (Invitrogen) and diluted for qPCR reactions with LightCycler 480 master mix (Roche). Primers used for intersectin-1 [Genbank CU995003], fascin [Genbank FP007130], calreticulin [Genbank CU993006], and β-actin [GenBank EW779066] are listed in Table 1. qPCR were carried out in triplicates on the LightCycler 480 System (Roche). Relative expression was calculated using the 2-ΔΔCq method (47), with normalization to the C. gigas RPL40 [GenBank FP004478]. Statistics used the non-parametric ANOVA-Kruskal-Wallis test (STATISTICA software).

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Figures

Figure 1 : LGP32 invades oyster hemocytes. (A) Flow cytometry analysis of hemocyte invasion by GFP-expressing LGP32. Cytograms show hemocyte cell counts *vs.* GFP-fluorescence (FL-1) after contact with LGP32 (black curve) or not (grey curve). The percentage of GFP-positive hemocytes ± s.e.m. is indicated. Data are representative of three independent experiments. (B, C and D) Confocal microscopy optical sections of invaded hemocytes. (B) Evan's blue-stained hemocytes (red) invaded by GFP-expressing LGP32 (green). (C and D) GFP-expressing LGP32 (green) in hemocytes upon phalloidin staining of actin (red) and DAPI staining of nucleic acids (blue). A, B and C show *in vitro* data, D shows *in vivo* data.

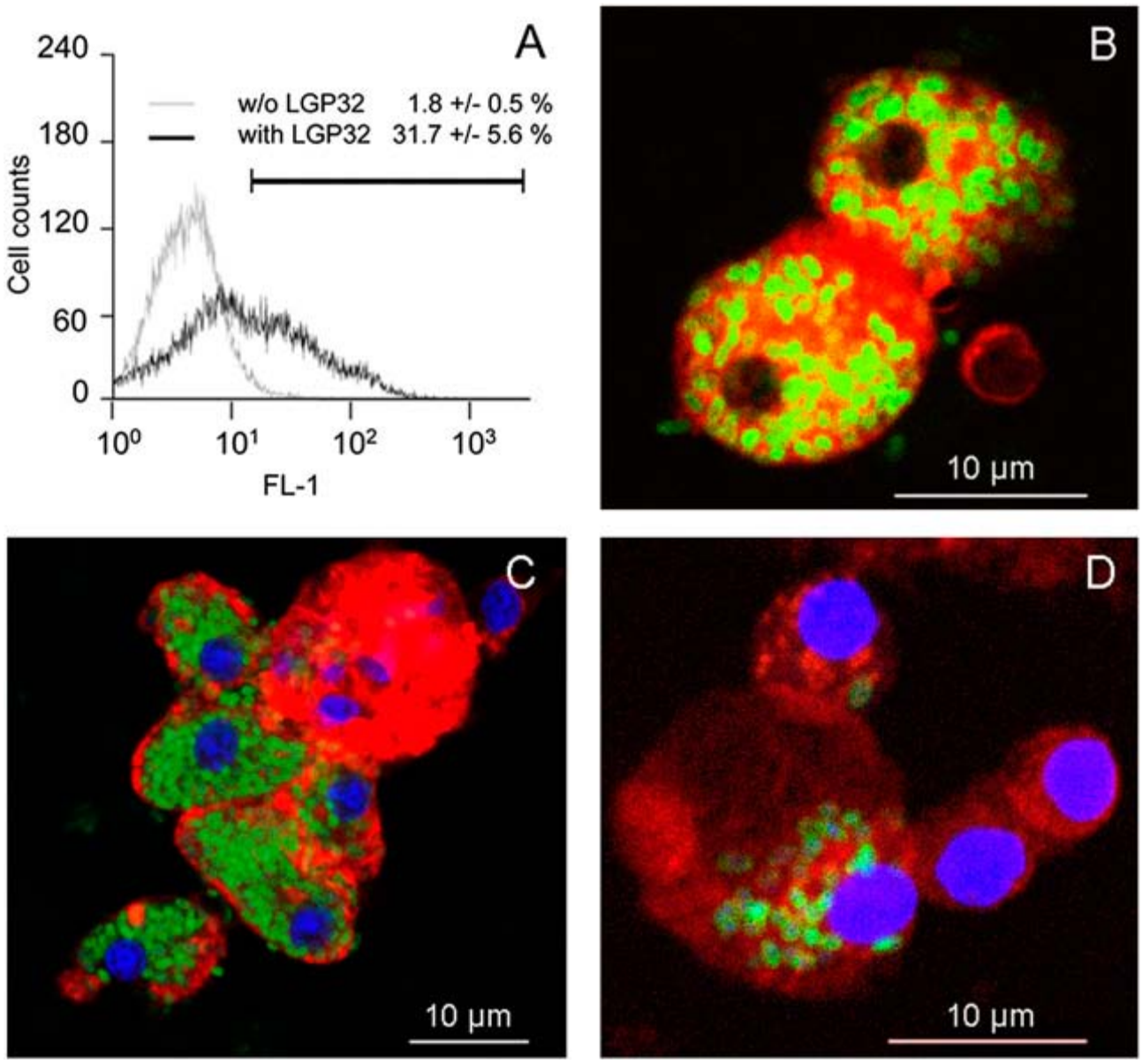


Figure 2 : LGP32 survives in oyster hemocytes. (A) Culturable intracellular bacteria (CFU/ml) in hemocytes. LGP32 (black), LMG20012^T (grey), and control without bacteria (white). (B) Acidic vacuole formation (Lysotracker) and ROS production (DCFH-DA) in hemocytes after a 1h-contact with LMG20012^T (grey) or LGP32 (black). Results are expressed in a relative fluorescent arbitrary unit (AU) after subtracting the fluorescence from the control without bacteria. All data are representative of three independent experiments (* p<0.05).

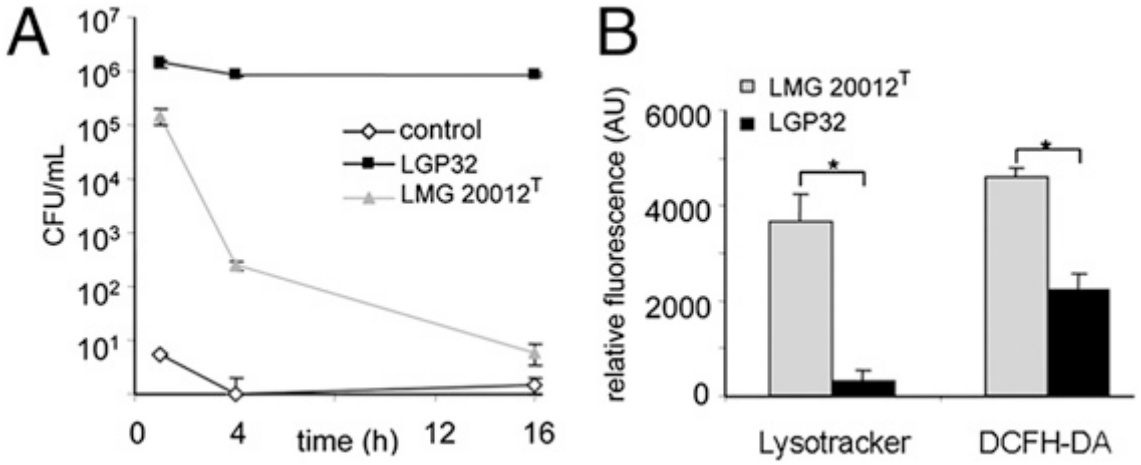


Figure 3 : Integrin- and RGD-dependent hemocyte invasion by LGP32 uses the polymerization of clathrin and actin microfilaments. Histograms display the inhibition of hemocyte invasion (%) as measured by flow cytometry after treatment of hemolymph with (A) anti-β-integrin or an irrelevant antibody (8 µg/ml), (B) an RGD or an EGF tripeptide (0.4 mg/ml), (C) sucrose (0.1, 0.2 and 0.5 M), and (D) cytochalasin D (1, 5 and 10 µg/ml). The 100% reference was attributed to untreated controls. Data are representative of three independent experiments. Statistical differences with untreated controls are displayed (* p< 0.05, **p<0.01 and *** p<0.001)

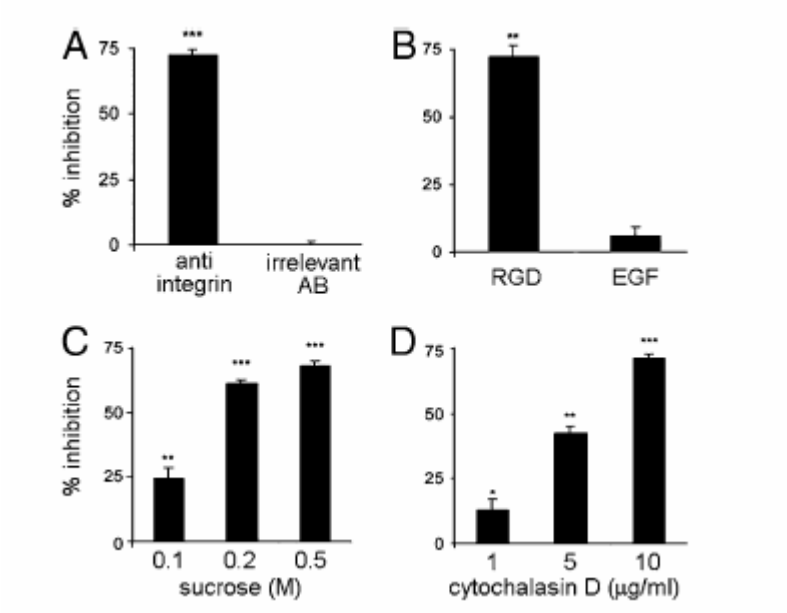


Figure 4 : Hemocyte invasion by LGP32 is OmpU- and plasma-dependent. (A) Percentage of GFP-positive hemocytes (flow cytometry data) obtained by incubating hemolymph, washed hemocytes, or washed hemocytes supplemented with plasma or purified *Cg*-EcSOD, with wild-type (*black bars)* or Δ*ompU* (*white bars)* GFP-expressing LGP32. Data are representative of three independent experiments (* $p<0.05$, ** $p<0.01$). (B) Confocal microscopy optical sections of Evan's blue counter-stained hemocytes after exposure to wild-type or Δ*ompU* GFP-expressing LGP32.

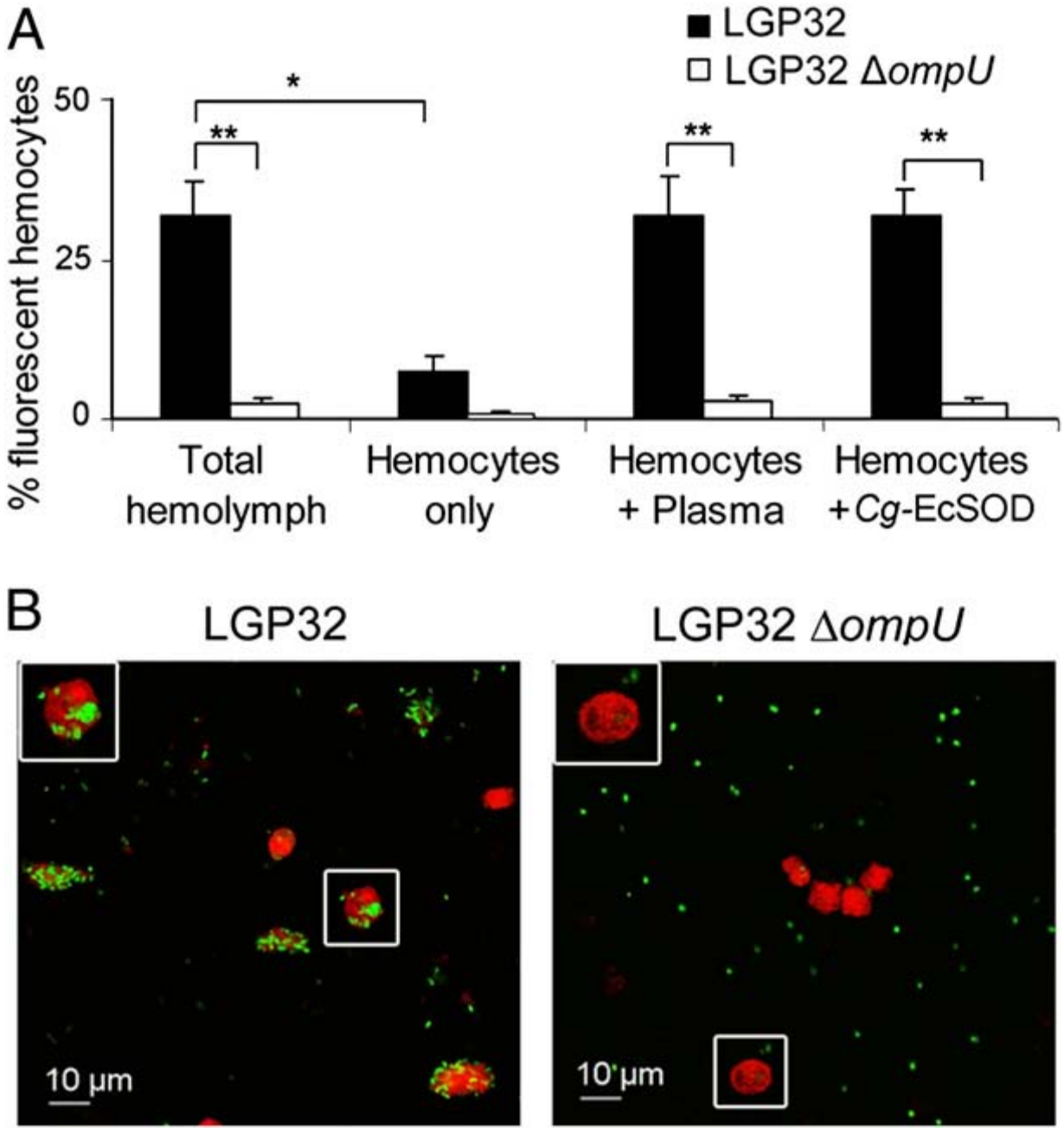


Figure 5 : Identification of the OmpU-binding proteins. (A) Plasma proteins separated by RP-HPLC on a linear gradient of 0-70% ACN in 35 min. Fractions are referred to as f1 to f9. Sequences of trypsic fragments from OmpU-binding fractions f6 and f7 are shown. (B) Coomassie blue-stained SDS-PAGE (12%) of HPLC fractions. (C) Binding of GFP-expressing wild-type and Δ*ompU* LGP32 to the immobilized HPLC fractions. *Black bars* refer to wild-type LGP32 and *white bars* to Δ*ompU*. Results are expressed in a relative fluorescent arbitrary unit (AU) after subtracting the fluorescence from the control without bacteria. Significant difference in binding to wild-type and Δ*ompU* is indicated (**p*<0.05, ***p*<0.01).

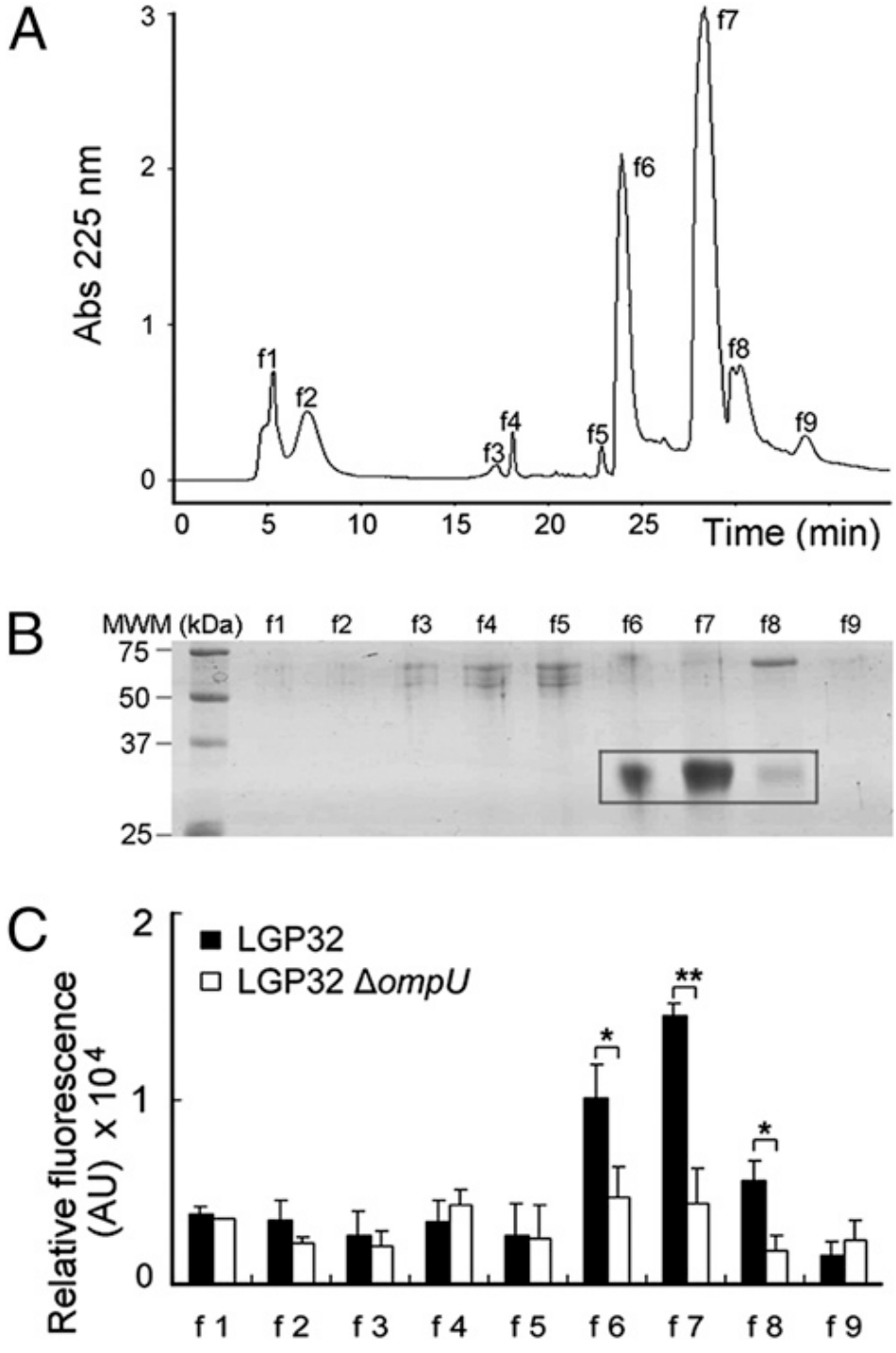


Figure 6 : OmpU-dependent binding of LGP32 to immobilized *Cg*-EcSOD requires the RGD sequence. The relative fluorescence due to binding is compared for GFP-expressing LGP32 (*black*), RGD- (*grey*) and EGF-treated (*hatches*) LGP32, and untreated *⊿ompU* (*white*). Data are representative of three independent experiments (**p*<0.05, ***p*<0.01).

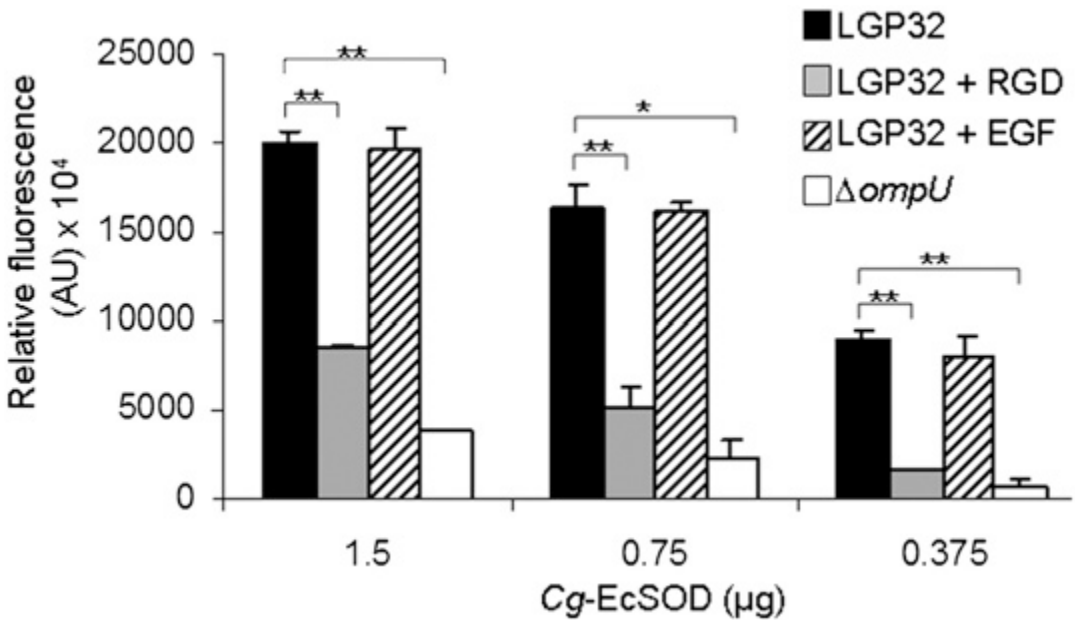
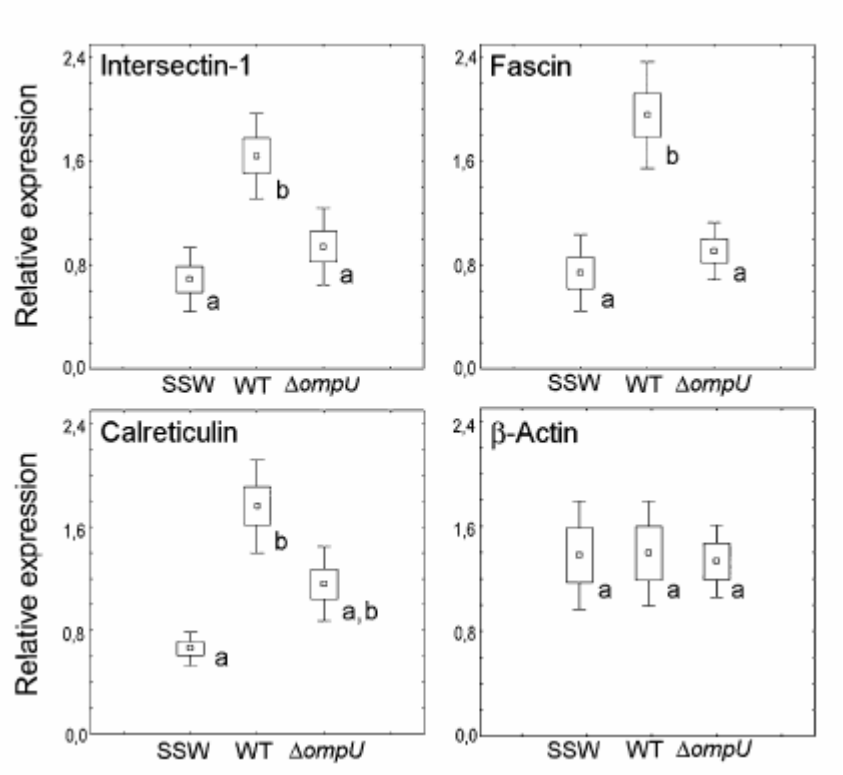


Figure 7 : OmpU-dependent induction of hemocyte recognition and cell-trafficking genes in experimentally infected oysters. Hemocyte gene expression in oysters injected with LGP32, *ΔompU*, or Sterile Sea Water (SSW) was monitored by qPCR. Relative expression was calculated by normalization to *C. gigas* RPL40. Results are expressed as mean values ± standard deviation (whiskers) and ± standard error (boxes). Significant differences between conditions ($p < 0.1$) were determined by the ANOVA-Kruskal-Wallis test. They are indicated by different lowercase letters (*a* or *b*). The absence of significant difference is indicated by the use of identical lowercase letters. *a,b* is used when a condition is neither different from *a* nor from *b*.



Tables

Table 1. Strains, plasmids, and oligonucleotides

Strains, plasmids, or oligonucleotides	Description or sequence	Reference
Bacterial strains		
LGP32	*V. splendidus* LGP32	(1)
Δ*ompU*	*V. splendidus* LGP32 Δ*ompU*	(6)
LMG20012^T	*V. tasmaniensis* (*V. splendidus*-related)	(28)
Plasmids		
pSW3654T	pSW23T::P_{TRC}-gfp-Tr32; *ori*$V_{R6K\gamma}$-*ori*T_{RP4} (Cm^r)	Present study
Oligonucleotides		
Tr32s-Pst1	5′-GCCCCTGCAGCCCTAACAAACGCTTC AAGAGGG-3′	
Tr32as-BamH1	5′-GCCCGGATCCGTATGAAAGAACGAC TCCACCTCCGC-3′	
Cg-fascin F	5′-CATGTAAAACTGTTGTAGCC-3′	
Cg-fascin R	5′-ACTCCACATCACTATAACTG-3′	
Cg-intersectin-1F	5′-AAGTGATCCGTACTGTGAGG-3′	
Cg-intersectin-1R	5′-GGTCCTTGATTGTGAACTGC-3′	
Cg-calreticulin F	5′-ACTGGGATGACGAGATGGAC-3′	
Cg-calreticulin R	5′-GCCAAAGATCAAATCCAACG-3′	
Cg-β-actin F	5′-CCATGTACGTCGCCATCCAG-3′	
Cg-β-actin R	5′-GATCACGTCCAGCGAGATCC-3′	
Cg-RPL40 F	5′-AATCTTGCACCGTCATGCAG-3′	
Cg-RPL40 R	5′-AATCAATCTCTGCTGATCTGG-3′	