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The major outer membrane protein OmpU of *Vibrio splendidus* contributes to host antimicrobial peptide resistance and is required for virulence in the oyster *Crassostrea gigas*

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Abstract:

Vibrio splendidus, strain LGP32, is an oyster pathogen associated with the summer mortalities affecting the production of *Crassostrea gigas* oysters worldwide. *Vibrio splendidus* LGP32 was shown to resist to up to 10 µM *Cg*-Def defensin and *Cg*-BPI bactericidal permeability increasing protein, two antimicrobial peptides/proteins (AMPs) involved in *C. gigas* immunity. The resistance to both oyster *Cg*-Def and *Cg*-BPI and standard AMPs (polymyxin B, protegrin, human BPI) was dependent on the *ompU* gene. Indeed, upon *ompU* inactivation, minimal bactericidal concentrations decreased by up to fourfold. AMP resistance was restored upon ectopic expression of *ompU*. The susceptibility of bacterial membranes to AMP-induced damages was independent of the *ompU*-mediated AMP resistance. Besides its role in AMP resistance, *ompU* proved to be essential for the adherence of *V. splendidus* LGP32 to fibronectin. Interestingly, in vivo, *ompU* was identified as a major determinant of *V. splendidus* pathogenicity in oyster experimental infections. Indeed, the *V. splendidus*-induced oyster mortalities dropped from 56% to 11% upon *ompU* mutation (Kaplan–Meier survival curves, $P < 0.01$). Moreover, in co-infection assays, the *ompU* mutant was out competed by the wild-type strain with competitive indexes in the range of 0.1–0.2. From this study, *ompU* is required for virulence of *V. splendidus*. Contributing to AMP resistance, conferring adhesive properties to *V. splendidus*, and being essential for in vivo fitness, the *OmpU* porin appears as an essential effector of the *C. gigas/V. splendidus* interaction.

Keywords: mollusk, invertebrate, antimicrobial peptide, innate immunity, virulence factor, colonization.

Introduction

Antimicrobial peptides/proteins (AMPs) are an ancient class of host defense molecules, the presence of which has been reported in almost all living organisms, including bacteria, fungi, plants and animals. AMPs play a central role in the innate immunity of both vertebrates and invertebrates (Sorensen et al., 2008). Expressed in phagocytes and epithelial barriers, they are believed to form a first line of defense against invading microbes. AMP structures include α-helical peptides (*e.g* cecropins), disulfide bond-containing peptides (*e.g.* defensins), some of which form β-hairpins (*e.g.* protegrins, tachyplesins), and peptides with an over-representation of some amino acids (*e.g.* proline-rich peptides) (Bulet et al., 2004). The amphipathicity and cationic charge of AMPs are considered essential for their binding and insertion into microbial membranes, which are subsequently damaged by pore formation or detergent effect (Brogden, 2005).

Contrary to vertebrates, invertebrates lack an acquired immunity based on antibody production. Their defense against microbes is mediated by the so-called innate immunity. With the development of genetic and reverse genetic tools (transgenesis, mutagenesis, RNA interference), AMPs have been shown to play a major role in invertebrate defense. For instance, AMP gene expression could be unambiguously correlated to the resistance to infectious diseases in insects (Lemaitre et al., 1996), nematodes (Alegado and Tan, 2008; Pujol et al., 2008), and crustaceans (de la Vega et al., 2008; de Lorgeril et al., 2008).

Resistance to AMPs is now recognized as an important virulence phenotype in many human pathogenic bacteria. However, with rare exceptions (Derzelle et al., 2004), the resistance to host AMPs has been poorly studied in invertebrate-pathogen interactions. The mechanisms by which pathogenic bacteria escape the host antimicrobial response include the reduction of the net negative charge of the bacterial cell envelope (so that the electrostatic interactions with the cationic AMPs are diminished), the production of bacterial proteases degrading AMPs, and the active efflux of AMPs by drug exporters (for review see (Peschel and Sahl, 2006)). In *Vibrio* species, AMP-resistance has been mainly studied on the human pathogen *V. cholerae*. Thus, the *ompU* gene, which encodes a major outer membrane protein, and the *vexAB* genes, which encode a RND-family efflux system were shown to be involved in the resistance

of *V. cholerae* to polymyxin B or to P2, a synthetic peptide derived from human BPI (Mathur and Waldor, 2004; Mathur et al., 2007; Bina et al., 2008).

Several *Vibrio splendidus* strains have been isolated from oysters during the major summer mortality outbreaks that have affected the *Crassostrea gigas* production over the past fifteen years (Gay et al., 2004a). Among them, *V. splendidus* LGP32 was shown to cause mortalities when injected to oysters (Gay et al., 2004b; Le Roux et al., 2007). The genome of *V. splendidus* LGP32 was sequenced (Le Roux et al., 2009) and genetic tools have been developed that offer the opportunity to decipher the basis of the *V. splendidus* virulence by the disruption of candidate genes (Le Roux et al., 2007). This led to the identification of the Vsm metalloprotease as a major determinant of toxicity of *V. splendidus* LGP32 extracellular products (ECP) (Binesse et al., 2008). However, the metalloprotease deletion mutant did not display altered virulence in oyster experimental infections (Le Roux et al., 2007).

To face pathogens from their environment, *C. gigas* oysters possess an arsenal of AMPs, which include defensins (Gueguen et al., 2006; Gonzalez et al., 2007a), proline-rich peptides (Gueguen et al., 2009), and a bactericidal/permeability-increasing protein (Gonzalez et al., 2007b), which are concentrated in cells and tissues rather than in a plasma-soluble form. How pathogenic *Vibrio* species circumvent their host immune response and cause disease is an important question to address. While previous studies have examined the role of *ompU* in the adaptation of *Vibrio* species to environmental parameters such as bile, pH (Wang et al., 2003; Duret et al., 2007; Kao et al., 2009), and bacterial AMPs (Provenzano et al., 2001; Mathur and Waldor, 2004), this is the first time, to our knowledge, that the OmpU-mediated AMP-resistance is examined in a natural host-Vibrio interaction. Thus, we have examined the role of the outer membrane protein OmpU of the oyster pathogen *V. splendidus* LGP32 in AMP-resistance and virulence in oysters. To address this question, we have constructed an *ompU* deletion mutant of *V. splendidus* LGP32, and compared it to the wild-type strain (i) *in vitro,* in terms of AMP-resistance, through antimicrobial and membrane permeability assays, and (ii) *in vivo,* in terms of virulence, through standardized experimental infections and colonization assays. Our data show that OmpU contributes to AMP-resistance and is required for virulence of *V. splendidus* in oyster experimental infections.

Results

Identification of the ompU gene

An *ompU* sequence (VS_2494) similar to that of other *Vibrio* species was found by homology searches on the chromosome I of *Vibrio splendidus* LGP32 (Le Roux et al., 2009). The *ompU* gene encodes a 348 amino acid protein (UniProtKB**,** B7VJI7**)** presenting 77, 71 and 64% identities with the OmpU sequences of *V. vulnificus* (strain CMCP6), *V. parahaemolyticus* (strain RIMD2210633) and *V. cholerae* (strain N16961) respectively. The identities with the OmpU sequences of two other *V. splendidus* strains were 87 and 79% for strain 12B01 and Med222, respectively. In addition, it displays the conserved site for signal peptidase and the typical Tyr-Asp-Phe (YDF) C-terminal motif (data not shown). After removal of the putative 21-amino acid signal peptide, the OmpU of *V. splendidus* is predicted to be a 327 amino acid-protein with a calculated mass of 35.2 kDa and a theorical pI of 4.19. Synteny analysis with the Magnifying Genome (MaGe) interface (http://www.genoscope.cns.fr/agc/mage) revealed that the genome sequence context surrounding *ompU* is conserved in many *Vibrio* species, in particular the marine invertebrate pathogens *V. harveyi* ATCC BAA1116, and *V. nigripulchritudo* SFn1, whose genome annotation is in progress. Interestingly, neighbor genes encode the penicillin-binding protein 4, DacB (VS_2495), involved in β-lactam resistance in *Pseudomonas aeruginosa* (Moya et al., 2009), as well as AcrA (VS_2500) and AcrB (VS_2499), two proteins involved in multidrug efflux in Gram-negative bacteria including *Haemophilus influenzae* or *Enterobacter clocae* (Dean et al., 2005 ; Perez et al., 2007) (Figure S1). Those four neighbor genes are conserved in all sequenced *Vibrio* genomes from the MaGe interface (data not shown).

Fig.S1

Construction of an ompU deletion mutant and ectopic complementation.

In order to evaluate the contribution of outer-membrane protein OmpU to the AMP-resistance and virulence of *V. splendidus* LGP32, a Δ*ompU* isogenic mutant was constructed by allelic exchange. A 256-bp sequence ranging from position 517 to 772 was deleted from the *ompU* sequence. The truncated OmpU protein deduced from the Δ*ompU* nucleotide sequence is composed of the first 172 amino acids of OmpU fused to an extra 33 amino acid out-of-frame C-terminal fusion. By subjecting the Δ*ompU* mutant to ectopic complementation, we generated Δ*ompU* P_{BAD}*::ompU*, in which the *ompU* gene is under the control

of the arabinose-inducible P_{BAD} promotor. The mutant strains were controlled by comparing the wild-type *V. splendidus* LGP32, the Δ*ompU* isogenic mutant and Δ*ompU* P_{BAD}*::ompU*, for their *ompU* DNA and RNA content as well as for the presence of OmpU in an outer membrane protein extract. By PCR amplification, the wild-type and Δ*ompU Vibrio* genomes were shown to carry the full-length and the deleted *ompU* gene, respectively. As expected, both the full-length and the deleted *ompU* were evidenced in the complemented strain (Figure 1A). RT-PCR was then performed using primers specific for the amplification of the full-length *ompU* mRNA, which showed that only the wild-type and complemented strains cultured in the presence of 0.2 % arabinose expressed the full-length *ompU* (Figure 1B). Finally, the expression of the OmpU protein was observed by SDS-PAGE as a band migrating at ~35 kDa only observed in the outer membrane protein fraction of the wild-type and complemented strains (Figure 1C). Altogether, these data validated the Δ*ompU* mutant and the Δ*ompU* P_{BAD}*::ompU* complemented strain at the genomic and expression levels.

Fig.1

Growth rate of V. splendidus LGP32 in oyster plasma is altered by the ompU mutation

When observed under a microscope, the Δ*ompU* mutant appeared smaller than the wild-type *V. splendidus* (data not shown). Besides, no detectable growth defect was observed in rich medium. Indeed, the wild-type *Vibrio* and the Δ*ompU* mutant displayed similar kinetics of growth and final absorbance at 600 nm in Zobell medium (Figure 1D), indicating that the *ompU* mutation had no deleterious effect in terms of growth. Both strains were then cultured in oyster plasma (cell-free hemolymph), a compartment likely to contain endogenous antimicrobials. While both the wild-type *V. splendidus* LGP32 and the Δ*ompU* mutant grew in oyster plasma, the cultures reached a final absorbance at 600 nm (at 16 h) ~ 5 times weaker than in Zobell medium (Figure 1D). In addition, the Δ*ompU* mutation significantly altered the *Vibrio* growth rate (Figure 1D), as indicated by a final absorbance at 600 nm of 0.285 ± 0.004 and 0.211± 0.021 ($p<0.05$), for the wild-type and Δ*ompU* mutant, respectively. Because growth is not prevented but significantly altered, this suggests that *ompU* is not required for but contributes to *V. splendidus* resistance to oyster plasma.

OmpU contributes to the resistance of V. splendidus LGP32 to antimicrobial peptides and proteins including the oyster Cg-BPI and Cg-Def

The wild-type *V. splendidus* LGP32, the Δ*ompU* isogenic mutant, and the Δ*ompU* P_{BAD}*::ompU* obtained by ectopic complementation were first exposed to standard cationic antimicrobial peptides (AMPs) and proteins including synthetic protegrin (a β-hairpin AMP from porcine), synthetic polymixin B (PmB, a cyclic AMP from bacteria), and recombinant human BPI (hBPI, a large bactericidal protein) in the range of 0.02 - 10 µM. For all three antimicrobials, minimal bactericidal concentrations (MBCs) measured in PB-NaCl medium were reduced by 2 to more than 4-fold upon *ompU* mutation (Table 1). The complementation was complete for both protegrin and PmB, and partial for the bactericidal protein hBPI. Thus, the MBC value of hBPI, which was above 10 µM against the wild-type *V. splendidus*, decreased to 2.5 µM upon *ompU* mutation, and was restored to 5 µM upon complementation.

We then examined the sensitivity of *V. splendidus* LGP32 to oyster immune effectors, *Cg*-Def and *Cg*-BPI. In standard antimicrobial assays, the MBCs of both AMPs were above 10 µM (Table 1). We therefore developed a more sensitive assay, the CFU assay, in which bacteria were exposed for 1 h to increasing AMP concentrations before CFU counting. In the CFU assay, AMP activity is measured in a mineral medium (artificial sea water), preventing the absorption of the AMPs to organic compounds, such as those found in a liquid broth. The actual concentration of AMPs in contact with the bacterial membranes is consequently increased, and the assay is therefore more sensitive. Under such conditions, both *Cg*-Def and *Cg*-BPI induced a dose-dependent loss of culturability of *V. splendidus* LGP32 (Figure 2). Moreover, as with standard AMPs, the CFU counts were systematically lower for the Δ*ompU* mutant than for the wild-type and complemented strain (Figure 2). Therefore, the *ompU* mutation increased the susceptibility of *V. splendidus* to the standard AMPs tested and to the oyster antimicrobials, *Cg*-Def and *Cg*-BPI.

Table 1

Fig. 2

AMP-induced membrane damages are not dependent on ompU

Because cationic AMPs are frequently membrane active, we compared the permeability of the membranes of AMP-treated *V. splendidus* LGP32 in the wild-type strain and Δ*ompU* isogenic mutant. Before AMP-treatment, the membrane integrity and functionality of both strains was assessed in three

183 independent experiments. Stationary phase cultures of the wild-type *V. splendidus* LGP32 and Δ*ompU*
184 mutant displayed a similar percentage of cells with intact membranes, *i.e.* 95.9 ± 1.8 % and 92.3 ± 5.0 %
185 of propidium iodide (PI)-negative cells, respectively. The respiratory function of the membrane was also
186 similar for both strains with a percentage of cells containing 5 cyano-2,3-ditolyl tetrazolium chloride (CTC)
187 crystals of 89.4 ± 3.9 % and 90.9 ± 1.7 %, for the wild-type and Δ*ompU* mutant, respectively.
188 Both strains were then exposed for 1 h to AMPs before staining with PI and Syto9. AMPs were the
189 oyster defensin *Cg*-Def and tachyplesin, an invertebrate AMP known to induce membrane permeability
190 (Ohta et al., 1992). Both 2 µM tachyplesin and 10 µM *Cg*-Def induced major membrane-damages in *V.*
191 *splendidus* LGP32, as indicated by a high proportion of PI-stained cells (91.4 % and 99.5% for *Cg*-Def and
192 tachyplesin-treated cells, respectively), which were almost absent (0.2 %) from the untreated control (table
193 2). This showed that, like tachyplesin, the oyster *Cg*-Def induces inner membrane permeability in *V.*
194 *splendidus* LGP32. However, the susceptibility of the inner membrane to both AMPs appeared
195 independent of the Δ*ompU* mutation, a similar percentage of PI-stained cells being observed in the AMP-
196 treated samples of the wild-type and Δ*ompU* mutant (table 2).
197
198 *OmpU is required for virulence of V. splendidus LGP32 in oyster experimental infections*
199 To measure a virulence phenotype of the Δ*ompU* mutation in oysters, we standardized an experimental
200 infection assay (see experimental procedures). Three doses of *V. splendidus* LGP32 wild-type strain (2 x
201 10^8 CFU ; 5 x 10^8 CFU ; 7.5 x 10^8 CFU) were injected per oysters (n=30 oysters). A significant dose-
202 dependent effect was observed on oyster mortality (Kaplan-Meier $p < 0.01$), the LD50 at day 4 (96 h)
203 being obtained for an injection of 5 x 10^8 CFU per animal (Figure S2A). The LD50 was then validated in
204 four independent infections (30 oysters each) with 5 x 10^8 CFU per animal. Kaplan-Meier survival curves
205 were generated proving the lack of statistical difference between the four experiments ($p > 0.4$) and
206 validating our experimental infection assay (Figure S2B).
207 The virulence of the wild-type *V. splendidus* LGP32 and the Δ*ompU* isogenic mutant were then compared
208 by injecting a dose of 5 x 10^8 CFU per animal, corresponding to the LD50 for the wild-type strain. Groups
209 of 60 oysters were monitored for 5 days after infection. A major loss of virulence was observed for the
210 Δ*ompU* mutant as indicated by the comparison of Kaplan-Meier survival curves ($p < 0.01$) generated for

oysters injected either with the wild-type *V. splendidus* LGP32 or the Δ*ompU* mutant (Figure 3). Indeed, the mortalities at day 4 were reduced from 56% for the wild-type strain to 11% for the Δ*ompU* mutant. We then compared the mortalities observed when injecting an identical dose of the Δ*ompU* mutant or a *V. splendidus*-related avirulent control, *V. tasmaniensis* LMG 20012T. Both strains induced identical mortalities (data not shown). Fig. S2

Fig. 3

ompU is not required for oyster colonization but confers major competitive colonization advantage to V. splendidus LGP32 in oyster experimental infections

The ability *V. splendidus* LGP32 wild-type and Δ*ompU* mutant to colonize oyster tissues was monitored over 2 days. When injected separately, both strains colonized oysters to similar extents. Indeed, CFU counts on TCBS plates varied from 4.1×10^6 (day 0) to 3.9×10^4 per g of oyster (day 2) for the wild-type strain, and from 3.4×10^6 (day 0) to 4.4×10^4 per g of oyster (day 2) for the Δ*ompU* mutant (Figure 4A). Interestingly, when oysters were co-injected with a 1:1 mixture of the wild-type and Δ*ompU* mutant, the mutant was rapidly out competed by the wild-type *Vibrio*, with competitive indexes of 0.122 ± 0.034 at day 0 and 0.206 ± 0.138 at day 2 (Figure 4B). Interestingly, *in vitro* competition assays in Zobell medium showed an identical growth of both the wild-type and Δ*ompU* mutant, as indicated by a competitive index of 1 all over the 24 h-time course (data not shown). This indicates that the competitive colonization advantage of the wild-type strain in oyster experimental infections does not result from a toxic effect of the wild-type against the mutant, but rather from a clearance by the oyster immune system.

Fig. 4

OmpU confers adhesive properties to V. splendidus LGP32

Puzzled by the competitive colonization advantage of the wild-type strain, we compared the adhesion properties of the wild-type and Δ*ompU* mutant in a fibronectin-binding assay. Results showed that *ompU* is required for the binding of *V. splendidus* LGP32 to fibronectin. Indeed, while the wild-type strain bound to fibronectin in a dose-dependent and specific way (no binding to BSA), the Δ*ompU* mutant did not display any specific binding (Figure 5).

Fig. 5

Discussion

Results showed that the major outer-membrane protein OmpU of *Vibrio splendidus* LGP32 contributes to its resistance to antimicrobial peptides (AMPs) including oyster AMPs, and plays a major role in virulence in *Crassostrea gigas* oysters. This is the first time, to our knowledge, that the OmpU-mediated AMP-resistance is examined in a natural host-*Vibrio* interaction.

V. splendidus LGP32 was shown here to be resistant to rather high doses of oyster AMPs, with MBCs above 10 µM for Cg-BPI and Cg-Def in standard antimicrobial assays. Besides, *V. splendidus* was able to grow in oyster plasma, either due to its resistance to oyster antimicrobials or to insufficient AMP concentrations in oyster plasma. To investigated the role of *ompU* in AMP-resistance and in virulence in oysters, we constructed a *ΔompU* mutant of *V. splendidus* LGP32, and found the *ΔompU* mutation not to modify its growth rate in rich medium (Zobell). Similarly, a large deletion of the *ompU* gene did not modify the growth rate of *V. cholerae* (Provenzano et al., 2001) and *V. anguillarum* (Wang et al., 2003). Moreover, the growth of *V. splendidus* in oyster plasma was altered but not prevented upon *ompU* mutation. Therefore, like *ompU* is not required for the resistance of *V. cholerae* to human serum (Provenzano et al., 2001), it is not required for but contributes to the resistance of *V. splendidus* to oyster plasma. One possible reason for that is the higher resistance to antimicrobials conferred by *ompU* to *V. splendidus* LGP32. Indeed, we showed here that *ompU* confers resistance to recombinant human BPI, which displayed MBC more than 4-fold lower against the *ΔompU* mutant than against the wild-type *V. splendidus*. This supports previous observation by Mathur and Waldor, who reported a higher sensitivity of V. cholerae to P2, a synthetic peptide derived from human BPI, upon *ompU* mutation (Mathur and Waldor, 2004). While contradictory results were reported on the sensitivity of *V. cholerae ompU* mutants to polymyxin B (PmB) (Provenzano et al., 2001; Mathur and Waldor, 2004), a synthetic cyclic peptide naturally produced by prokaryotes, we found here that *ompU* contributes to PmB-resistance in *V. splendidus,* with MBCs twice lower against the mutant than against the wild-type and complemented strains. Therefore, together with the *V. cholerae* studies, our results support the role of *ompU* in the resistance of *Vibrio* species to cationic antimicrobial peptides and proteins.

Interestingly, *ompU* was also shown to contribute to the resistance to oyster AMPs including *Cg*-BPI and *Cg*-Def. While both antimicrobials induced a loss of cultivability of the oyster pathogen, the effect was enhanced upon *ompU* mutation. To our knowledge, this is the first time the *ompU*-mediated AMP-

resistance is studied with the effectors of a natural host-pathogen interaction. However, because *ompU* conferred only moderate resistance to oyster AMPs as compared to standard cationic antimicrobials, we think that other bacterial genes are required for the resistance of *V. splendidus* to the oyster AMPs. AMP-resistance may be conferred by the physical nature of the *V. splendidus* cell envelope. Besides, resistance mechanisms may be specific of the *C. gigas* - *V. splendidus* interaction. Indeed, most infectious microbes are highly adapted to specific hosts, and host cationic AMPs are believed to have co-evolved with microbial resistance (Peschel and Sahl, 2006). However several effectors of AMP-resistance are widespread in the bacterial genomes, some of which are present in *V. splendidus* LGP32.

Thus, one interesting finding from this study is that *ompU* belongs to a genomic region that contains several putative effectors of antimicrobial/antibiotic resistance, namely AcrAB (Dean et al., 2005; Perez et al., 2007) (VS_2500 and VS_2499) and the penicillin-binding protein 4, DacB (VS_2495) (Moya et al., 2009). Therefore, rather than *ompU* alone, this synthenic group of genes, which ranges from *ompU* to *acrA* and which is conserved among *Vibrio* species, is likely involved in the resistance of *V. splendidus* to AMPs. Besides, homologues of AMP-resistance genes were found elsewhere in the genome of *V. splendidus* LGP32 and may participate to AMP-resistance in this species. These include (1) *vexAB* (VS_0064 and VS_0063), which encode resistance/nodulation/division efflux pumps conferring AMP-resistance in *Neisseria gonorrhoeae* (Shafer et al., 1998) and *Vibrio cholerae* (Bina et al., 2008), and (2) *phoPQ* (VS_0031 and VS_0030)**,** a conserved two component regulatory system mediating AMP-resistance in many species including *Salmonella enterica* sevovar Typhimurium (Miller et al., 1990), *Pseudomonas aeruginosa* (Macfarlane et al., 1999), or *Photorhabdus luminescens* (Derzelle et al., 2004).

How OmpU participates to AMP-resistance in *V. splendidus* LGP32 remains to be established. We showed here that like tachyplesin, the oyster defensin *Cg*-Def induces severe damages of the *V. splendidus* membranes. However, both peptides induced similar membrane permeability in a wild-type or Δ*ompU* background. Similarly, the *ompU* mutation did not alter the susceptibility of *V. cholerae* outer membrane to the BPI-derived P2 peptide (Mathur and Waldor, 2004). Both studies support the idea that membrane damages are not sufficient to explain the antimicrobial activity of AMPs, and as a consequence, to explain the OmpU-mediated AMP-resistance. We found here that the OmpU proteins from *V. splendidus* and *V. cholerae* are 64% identical and display the conserved C-terminal YXF motif

295 proposed to signal the envelope stress response through DegS activation (Walsh et al., 2003). Therefore,
296 as in *V. cholerae* (Mathur et al., 2007; Davis and Waldor, 2009), OmpU may signal the envelope stress
297 response in *V. splendidus* exposed to cationic AMPs. Indeed, the genes encoding proteins that signal the
298 response to cationic AMPs in *V. cholerae* (Mathur et al., 2007) are present in the genome of *V. splendidus*
299 LGP32. These include *rpoE* (VS_2625), which encodes the extracytoplasmic stress factor σ^E, *rseA*
300 (VS_2624), which encodes the anti-sigma factor RseA, and *degS* (VS_0425), which encodes the protease
301 cleaving RseA, thereby releasing σ^E (for review see (Ades, 2008)). The very anionic calculated isoelectric
302 point (pI = 4.19) of *V. splendidus* OmpU protein makes it a good candidate for sensing cationic AMPs
303 attracted by the negative net charge of *Vibrio* outer membranes. Upon interaction with AMPs, OmpU may
304 expose its YDF motif, thereby activating DegS, and cell surface repair mechanisms.

305 Finally, one major finding from this study is that *ompU* is essential for the virulence of *V. splendidus*
306 LGP32 in oysters. To our knowledge, this is the first mutation described that induces a loss of virulence in
307 an oyster pathogenic *Vibrio*. Indeed, deletion of metalloprotease genes drastically lowered the toxicity of
308 *Vibrio* ECPs but did not alter the virulence in oyster experimental infections (Le Roux et al., 2007 ;
309 Hasegawa et al., 2008). Like in *V. splendidus* LGP32, OmpU was previously reported to be required for
310 virulence in *V. vulnificus,* with a 10 fold-increased LD50 in mice upon *ompU* mutation (Goo et al., 2006).
311 Conversely, OmpU was not essential for *V. anguillarum* to cause disease in fish (Wang et al., 2003). Our
312 data showed that the loss of virulence of the Δ*ompU* mutant did not result from an inability to colonize the
313 host but corresponded to a marked disadvantage in competitive colonization assays, with competitive
314 indexes (CI) in the range of 0.11-0.20. Such a loss of competitivity was also observed for *V. cholerae* in
315 infant mouse intestine colonization, when deleted from the *ompU* paralogue *vca1008* (CI = 0.025) (Osorio
316 et al., 2004), whereas in similar competition assays, a large deletion of *ompU* itself did not severely alter
317 the *in vivo* competitivity of *V. cholerae* (CI = 0.56) (Provenzano et al., 2001). Noteworthy, since the *ompU*
318 mutation had no effect on the *in vitro* fitness of *V. splendidus* LGP32 *(*CI = 1), the rapid clearance of the
319 Δ*ompU* mutant in competitive colonization assays is not due to a toxic effect of the wild-type over the
320 mutant but rather to host defense effectors induced in co-infection assays. Together with its role in AMP-
321 resistance, this shows that OmpU is an essential determinant of the *C. gigas* / *V. splendidus* interaction.

While the molecular basis of the severe loss of virulence and *in vivo* competitity of *V. splendidus* upon *ompU* mutation remain to be established, several hypotheses can already be made. On the one hand, the *ompU* mutation may alter the expression of virulence factors, although data on *V. cholerae* do not support this hypothesis (Provenzano et al., 2001). On the other hand, this study showed that OmpU has a dual role in (1) resistance to host defenses (oyster plasma and antimicrobials), and (2) host recognition (fibronectin-adhesive properties). On that basis, we think that the loss of competitivity of the Δ*ompU* mutant in co-infection assays could be attributed to host defenses induced by the wild-type strain and primarily active against the Δ*ompU* mutant. Indeed, as in *V. vulnificus* (Goo et al., 2006) and *V. cholerae* (Sperandio et al., 1995), the OmpU of *V. splendidus* LGP32 was found here to a ligand of fibronectin, one major extracellular matrix component of mammalian cells. Like mammalian fibronectin, the extracellular *Cg*-EcSOD of oysters possesses an RGD motif and co-localizes with a beta-integrin-like receptor of hemocytes (Gonzalez et al., 2005). The *ompU*-mediated adhesive properties of *V. splendidus* LGP32 could therefore be of prime importance in non-self recognition. Such a role of *ompU* is host recognition is also supported by its major role in avoiding the adherence of the squid symbiont *V. fisheri* to its host hemocytes (Nyholm et al., 2009). Altogether, this identifies OmpU as a key determinant of the *C. gigas* / *V. splendidus* interaction. Future studies on this host-pathogen interaction will help deciphering the molecular basis of the *ompU*-mediated virulence of *V. splendidus* in *C. gigas* oysters.

Experimental procedures

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in Table 3. *Escherichia coli* strains were grown in Luria-Bertani (LB) or, for strain □3813, Mueller-Hinton (MH) broth, at 37℃. *Vibrio* strains were grown either in LB medium (Difco) supplemented with NaCl 0.5M (LB NaCl), artificial sea water (ASW) (Saulnier et al., 2000) supplemented with 4 g/l bactopeptone and 1 g/l yeast extract (referred to as Zobell medium), or TCBS (Difco) at 20℃. Chloramphe nicol (12.5 mg/l), Thymidine (0.3 mM) and diaminopimelic acid (DAP) (0.3 mM) were added as supplements when necessary. Induction of *ccdB*

Table 3

expression under the control of P_{BAD} promoter was achieved by the addition of 0.2 % L-arabinose to the growth media, and conversely, this activity was repressed by the addition of 1 % D-glucose.

Vector construction for ompU deletion and ectopic complementation

The *ΔompU* mutant was constructed by allelic exchange using the suicide vector pSW4426T described previously (Le Roux et al., 2007). Briefly, this vector contains the *pir*-dependent R6K replicative origin, and can be transferred by RP4-based conjugation to *V. splendidus.* It also carries the plasmid F toxin gene (*ccdB*) gene under the control of the P_{BAD} promoter. This genetic system allows the efficient counter-selection of integrated plasmids in the presence of arabinose in *V. splendidus.* The *V. splendidus ompU* gene was PCR-amplified from *V. splendidus* LGP32 genomic DNA using primers 2494-1 and 2494-2, which contains an *Eco*R1 site at 5' end (table 3). The resulting amplicon (848 bp) was digested by *EcoR1* and the digestion product (816 bp) was cloned into the *EcoR1* site of pUC18 (Pharmacia). An *ompU* allele carrying an internal deletion (*ΔompU*) was obtained by inverse PCR using the primers 2494-3 and 2494-4 (table 3), which contain *Xho*I sites at 5' ends, and the pUC18 derivative as a template. The PCR product was finally digested with *XhoI* and self-ligated. The resulting *ΔompU* allele was recovered from pUC18 by *EcoRI* digestion (566 bp) and gel extraction, and introduced by ligation into pSW4426T previously linearized with *EcoRI.* This led to pSW□2494T.

Ectopic complementation of the *ompU* mutation was performed by introducing the *ompU* gene into the genome of *V. splendidus* LGP32 *ΔompU* under the control of the P_{BAD} promoter. Insertion was targeted to a non-essential multi-copy gene encoding the ISVisp1 transposase, using the strategy previously described (Le Roux et al., 2007). The plasmid was generated by a two-step PCR construction (Matsumoto-Mashimo et al., 2004). The *araC-pBAD* and *ompU* genes were PCR-amplified independently using the primer pairs 2494-9 / 2494-10 (1267 bp), and 2494-11 / 2494-12 (1044 bp), respectively (table 3). After gel purification, 100 ng of the two PCR products were mixed and a final PCR amplification was carried out using the most external primer pairs 2494-9 and 2494-12, which contain *Xho*1 sites at 5' ends. After gel purification, the PCR product, referred to as the *araC-pBAD::ompU,* was *Xho1* digested and ligated into the *XhoI* site of IS*Visp1 orfBΔ*allele carried by pSWδ6720T. This led to pSWδ6720T-*ompU.*

375 The pSWδ2494T and pSWδ6720T-*ompU* constructs were then cloned into *E. coli* π3813. After
376 sequencing, both plasmids were transferred to *E. coli* β3914 donor cells. The conjugation experiments
377 with *V. splendidus* strains were performed according to the filter-mating procedure using a donor/recipient
378 ratio of 1/10 as previously described (Le Roux et al., 2007). Selection against the Δ*dapA* donor *E. coli*
379 β3914 was achieved by plating onto LB NaCl medium devoid of DAP, and supplemented with 1 % glucose
380 and 12.5 mg/l chloramphenicol. Antibiotic-resistant colonies were grown in LB NaCl medium and spread
381 on plates containing 0.2 % arabinose, and screened by PCR-amplification using the primer pair: 2494-5 et
382 2494-6, which amplify a 616 bp- and a 365 bp-fragment on the wild-type and Δ*ompU* strain, respectively.
383 To validate the mutant and complemented strains at the expression level, an RT-PCR was also performed
384 on total RNAs isolated with Trizol reagent (Invitrogen) from stationary phase grown cultures containing
385 0.2 % arabinose. The M-MLV reverse transcriptase was purchased from Invitrogen. Primers 2494-7 and
386 2494-8 specifically amplified the wild type *ompU* allele (177 bp PCR product), primer 2498-8 being
387 designed in the deleted region of *ompU*.
388
389 *Animals and hemolymph collection*
390 Adult diploid C*rassostrea gigas* were purchased from a local oyster farm in Mèze (Gulf of Lion, France).
391 When needed, hemolymph was collected by withdrawing 1 to 2 ml from the posterior adductor muscle
392 sinus using a 2 ml syringe equipped with a 23G-needle. Cell-free hemolymph (plasma) was obtained by
393 centrifugation (1,000 x *g*, 15 min, 4°C) and passage of the supernatant through a 0.22 µm-pore size filter.
394
395 *Standardized experimental infections*
396 Before an infection, oysters were maintained for 4 days in tanks of UV-treated and biologically filtered sea
397 water. The temperature was maintained at 16°C and an air-bubbler was added. After 4 days, the
398 culturable endogenous *Vibrio* species were stabilized at ~100 colony forming units (CFU) / ml of oyster
399 hemolymph and ~200 CFU / g of oyster tissue, as estimated on TCBS plates. To allow the intramuscular
400 injection of bacteria, a cut was made in the oyster shell next to the adductor muscle. Bacteria were
401 prepared as follow. *V. splendidus* LGP32 wild-type and Δ*ompU* as well as the *V. splendidus*-related strain

V. tasmaniensis LMG20012^T (Thompson et al., 2003), used as an avirulent control (Binesse et al., 2008), were grown overnight at 20°C in Zobell medium and washed 3 times by centrifugation (10 min, 1,000 x *g*, 20°C) in sterile sea water (SSW) obtained by autoclaving. For every experimental infection, 30 oysters were injected at day 0 with 5 x 10^8 CFU (colony forming unit) /animal under 100 µl. SSW (100 µl) was used as a control. Oysters were placed in 50 liter glass tanks (30 animals / tank). Those tanks were equipped with biological filters and sea water was thermo-regulated at 20°C. Mortalities were monitored daily over five days. The non-parametric Kaplan–Meier test was used to estimate Log-Rank and Wilcoxon values for comparing the survival curves (Kaplan and Meier, 1958). A confidence limit of 95% was used to test the significance of differences between groups. All experimental infections were performed according to the Ifremer animal care guideline and policy.

Colonization and competition assays

Experimental infections were performed as above. At day 0 (*i.e.* 1 h post-injection), and day 2 (48 h p.i.), 6 oysters were separately grinded in sterile sea water using an Ultra Turax T25 homogenizer, and serial dilutions were plated onto TCBS plates. CFU were counted after 48 h at 20°C. To distinguish wild-type and Δ*ompU* mutants of *V. splendidus* LGP32 from the natural *Vibrio* species of oysters, colonies isolated from every grinded oyster were analyzed by PCR using primers 2494-9 and 2494-10 designed in LGP32-specific regions of the *ompU* sequence, which encompass the Δ*ompU* deletion. The amplicon size on the *V. splendidus* LGP32 *ompU* and Δ*ompU* genomic DNA was 732 bp and 382 bp, respectively. Primer specificity for the *ompU* sequence of *V. splendidus* LGP32 was assessed by the lack of amplification on genomic DNAs extracted from 8 different *Vibrio* strains, including other *V. splendidus* species. We also verified the absence of *V. splendidus* LGP32 in control oysters injected with sterile sea water.

In vivo competition assays were performed by simultaneous injection of wild-type *V. splendidus* and Δ*ompU mutant* to oysters. Again, 100 µl of a mixture containing 2.5 x 10^8 CFU of each strain (1:1 ratio) was injected per animal. At day 0 and day 2, 4 oysters were grinded as above and serial dilutions were plated onto TCBS. Wild-type *V. splendidus* LGP32 were distinguished from the Δ*ompU* mutants in every grinded oyster by subjecting 30 randomly selected colonies to the PCR procedure described above.

Competitive index (C.I.) was calculated as the ratio of wild-type to mutant in the input divided by the ratio of wild-type to mutant in the output.

In vitro competition assays were performed as follows. Co-cultures were performed in Zobell medium inoculated with 5 x 10^4 CFU/mL of the wild-type and Δ*ompU* mutant, and grown over 24 h at 20°C. CFU were counted by plating onto Zobell plates at 0, 15 min, 1 h, 5 h, 8 h, and 24 h. Wild-type *V. splendidus* LGP32 were distinguished from the Δ*ompU* mutants oyster by subjecting 30 randomly selected colonies to the PCR procedure described above. C.I. were calculated as above.

Preparation of outer membrane proteins

Cultures in stationary phase of growth were prepared in Zobell medium containing 0.2 % arabinose. Cells were harvested by centrifugation (15 min at 3,000 g) and washed in 200 mM Tris-HCl pH 8. After centrifugation (15 min at 3,000 g), cells were resuspended in in 200 mM Tris-HCl pH 8 containing 1M sucrose, 10 mM EDTA, and 1.5 mg/ml lysozyme. After 30 min at 4°C, cells (spheroplasts) were eliminated by centrifugation (15 min at 16,000 g). A fraction containing outer membrane proteins was obtained by ultracentrifugation of the supernatant (1 h at 40,000 g). The protein concentration in the outer membrane protein fraction was estimated using the Bradford method with Micro-BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). Outer membrane proteins were separated on a 15 % sodium dodecyl sulfate-polyacrylamide gel and stained with silver nitrate.

Antimicrobial peptides and proteins

Recombinant expression and purification of *C. gigas* antimicrobials, namely the *Cg*-Def defensin, and the *Cg*-BPI, bactericidal/permeability increasing protein was performed as described previously (Gueguen et al., 2006; Gonzalez et al., 2007b). Recombinant human BPI ($hBPI_{23}$) was a generous gift from Ofer Levy. Chemical synthesis of standard antimicrobial peptides including polymyxin B, tachyplesin and protegrin-1 was performed on an Abimed AMS 422 synthesizer by Fmoc chemistry as previously described (Gueguen et al., 2009).

Antimicrobial assays

457 *Minimal Bactericidal Concentrations (MBCs)* – Liquid growth inhibition assays (Hetru and Bulet, 1997)
458 were performed in Poor Broth medium supplemented with 0.5 M NaCl (PB-NaCl, 1% bactotryptone, 0.5 M
459 NaCl w/v, pH 7.5). Growth was monitored spectrophotometrically at 620 nm for 24 h at 20°C on a
460 Multiscan microplate reader colorimeter (Dynatech) and the 100 µl-content of wells displaying no apparent
461 growth was plated onto Zobell agar plates to monitor the loss of cell culturability. MBC values were
462 determined after 48 h at 20°C as the lowest concent ration of peptides at which no CFU could be detected.
463 *CFU assay* - Peptide activity on cell culturability was assayed in sterile ASW. Bacteria were grown to
464 stationary phase in Zobell medium, washed three times in ASW and adjusted to an input concentration of
465 10^6 CFU/ml. The bacterial suspensions (100 µl) were then incubated for 1 h at 20°C with 0.1 to 10 µM
466 peptide (10 µl) in ASW. Controls were performed for every culture in the absence of peptide (10 µl of
467 water). After incubation, serial dilutions of the bacterial suspensions were plated onto Zobell agar plates
468 and incubated for 24 h at 20°C until colonies were counted.
469
470 *Fibronectin-binding assay*
471 The wild-type *V. splendidus* and Δ*ompU* isogenic mutant were tested in the fibronectin cell adhesion
472 assay (3H biomedical, Uppsala, Sweden). Briefly, cultures of wild-type (*black bars*) and Δ*ompU* (*white*
473 *bars*) *V. splendidus* in mid-log phase of growth were serially diluted in Zobell medium. Two hundred µl of
474 each cell suspension (ranging from 3.13 x 10^7 to 2.5 x 10^8 CFU/ml) were applied onto the microplate of
475 the fibronectin cell adhesion assay coated either with fibronectin or bovine serum albumine (BSA), a large
476 protein used as a negative control. In the negative controls (BSA), the cell suspensions were adjusted to
477 the highest value (2.5 x10^8 CFU/ml). After a 90 min-incubation at room temperature, unbound bacteria
478 were washed away with PBS, and fixed for 10 min in PBS containing 0.1% glutaraldehyde. Bacteria were
479 then stained following the manufacturer recommendation. The adhesive properties of the two strains were
480 measured as an absorbance at 590 nm indicative of their binding to fibronectin.
481
482 *CTC reduction assay*
483 The CTC (5 cyano-2,3-ditolyl tetrazolium chloride) reduction assay was used as a direct method to assess
484 bacterial respiration (Rodriguez et al., 1992). A 50 mM stock solution of CTC (Polysciences Europe,

485 Eppelheim) was freshly prepared just before experiment by dissolving the fluorogenic ester in sterile distilled
486 water. The CTC solution was added to the bacterial suspension (10^6 cells/ml in ASW) to a final concentration
487 of 4 mM and incubated in the dark for 1 h at room temperature. CTC reduction was stopped by the addition
488 of formaldehyde (4% final concentration) and stored at 4°C until microscopic observation. The fixed *Vibrio*
489 suspensions previously incubated with CTC were counter-stained with 4'-6-Diamidino-2-phenylindole (DAPI)
490 in Tris-HCl pH 7.1 for 15 min in the dark at a final concentration of 2.5 mg/ml. Microscopic observations
491 were performed under an Olympus Provis epifluorescence microscope. Stained cells were captured by
492 microfiltration through a 0.2 µm-pore size Nucleopore black polycarbonate filter (47 mm diameter). Filters
493 were air-dried and mounted with immersion oil on glass microscope slides. Preparations were examined
494 under immersion with 100 X objective lens. Approximately 500 total bacterial cells were counted. Respiring
495 bacteria were counted with excitation at 420 nm and total bacteria were counted at 357 nm. The fractions of
496 CTC-positive cells were calculated as the ratio of CTC-positive to DAPI cell counts.
497
498 *Flow cytometry assessment of membrane permeability*
499 The membrane permeability of the *Vibrio* strains exposed to antimicrobial peptides was tested by flow
500 cytometry after treatment with the LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes). Briefly,
501 stationary phase cultures of wild-type and mutant *V. splendidus* were prepared as in CFU assay, and 100
502 µl of the bacterial suspension (10^7 cells / ml) were exposed to 2 µM tachyplesin, or 10 µM *Cg*-Def. An
503 equivalent volume of water (10 µl) was used in controls. After 1 h at 20°C, 890 µl ASW were added to the
504 bacterial suspension to dilute the peptides. The membrane permeability of the bacteria was monitored as
505 previously published (Passerat et al., 2009) by adding 3 µl of a 1:1 (v/v) mixture of SYTO9 and propidium
506 iodide (PI) from the LIVE/DEAD kit to the bacterial suspension (1 ml at 10^6 cells/ml). Incubation was
507 performed for 15 min in the dark, at room temperature. Flow cytometry analysis were performed on a
508 FACSCalibur (Becton Dickinson, San Jose,Calif., USA), with 488 nm excitation from a blue laser at 15
509 mW argon ion. Analyses were run at low speed (15 µl/min) for a 2 min-acquisition time. The green
510 fluorescence of SYTO9 was measured at 530 nm (FL1 channel), and the red fluorescence of PI was
511 measured above 670 nm (FL3 channel). Cells with damaged membrane (PI-positive cells) were

differentiated from those with intact membrane by their signature in a plot of green *versus* red fluorescence.

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651 **FIGURES**

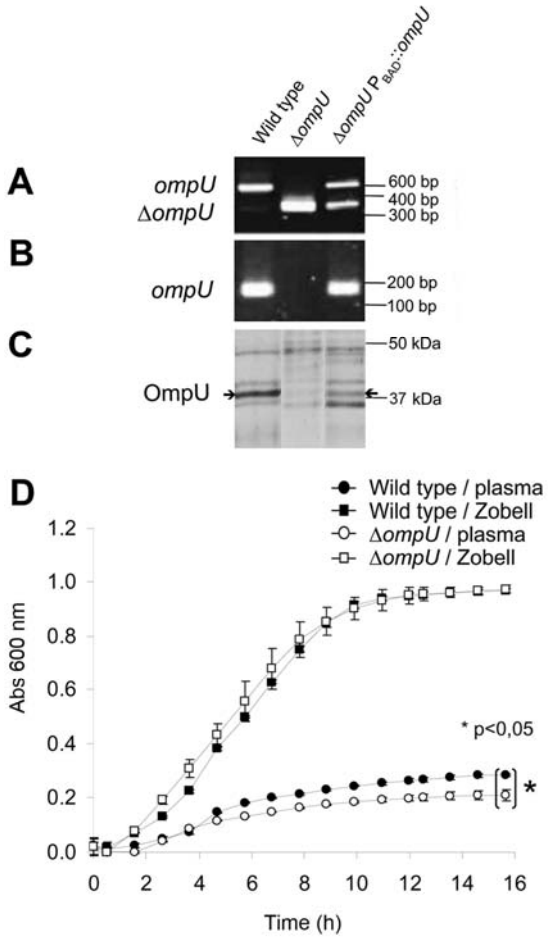

Figure 1

652

653 **Figure 1. Inactivation of the OmpU protein does not impair the growth of *V. splendidus* LGP32 in**
654 **Zobell medium and oyster plasma.**

655 **(A)** PCR-amplification of genomic DNA. The 616 bp- and 365 bp-amplicons corresponding to the wild-type
656 (full-length) and the deleted *ompU* alleles, are observed in the wild-type and Δ*ompU* mutant, respectively.
657 Both alleles are evidenced in the complemented strain. **(B)** Specific RT-PCR amplification (177 bp) of the
658 full-length *ompU* mRNA. Amplicons are observed for the wild-type and complemented strains only. **(C)**
659 Silver-stained SDS-PAGE of outer membrane proteins. The OmpU protein (*arrow*) is observed for the
660 wild-type and complemented strains, only. **(D)** Growth of the wild-type *(closed symbols)* and Δ*ompU*
661 mutant *(open symbols)* in Zobell medium *(boxes)* and oyster plasma *(circles)*. No significant difference is
662 observed in Zobell medium, while a significant difference ($p<0.05$) is observed in plasma.

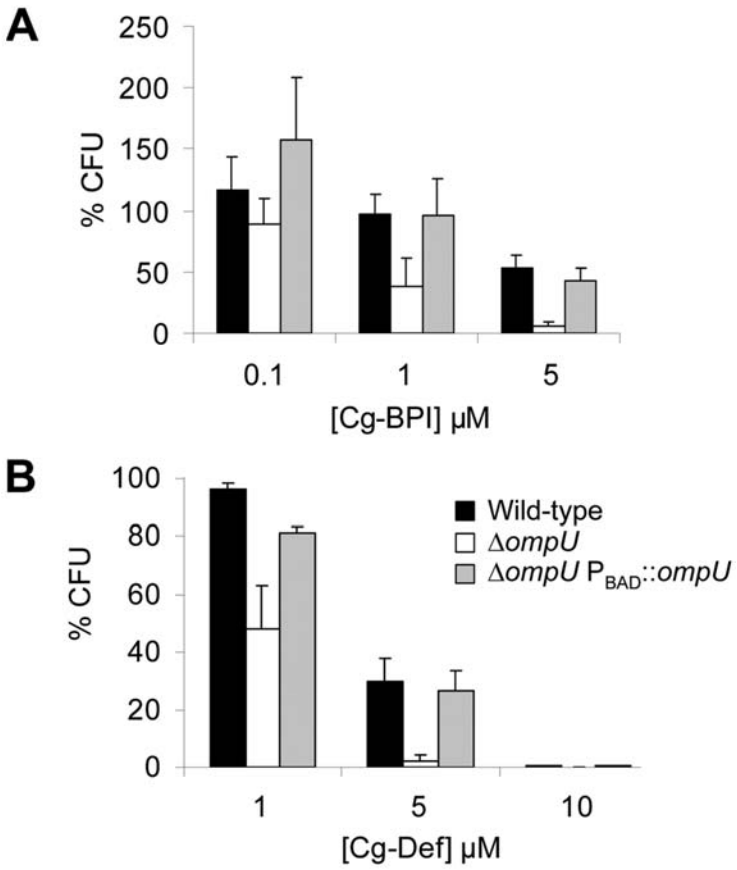

Figure 2

Figure 2. OmpU contributes to the resistance of *V. splendidus* LGP32 to *Cg*-BPI and Cg-Def

The wild-type *V. splendidus* LGP32, the Δ*ompU* isogenic mutant, and the Δ*ompU* P_{BAD}*::ompU* obtained by ectopic complementation were exposed to increasing concentrations of *Cg*-BPI (A), and *Cg*-Def (B), or an equivalent volume of water (controls). CFU were counted after a 1 h-incubation for all three strains. The graph shows the culturability of the wild-type (*black rectangles*), Δ*ompU* mutant (*white rectangles*) and the complemented mutant (*gray rectangles*) as a percentage of the CFU counted in their respective controls without peptide ($1.03 \times 10^5 \pm 4.62 \times 10^4$ CFU/ml, $1.03 \times 10^5 \pm 2.4 \times 10^3$ CFU/ml, and $9.39 \times 10^4 \pm 3.3 \times 10^3$ CFU/ml, for the wild-type, Δ*ompU*, and Δ*ompU* P_{BAD}*::ompU* controls, respectively). Data are the mean of three independent experiments ± SEM.

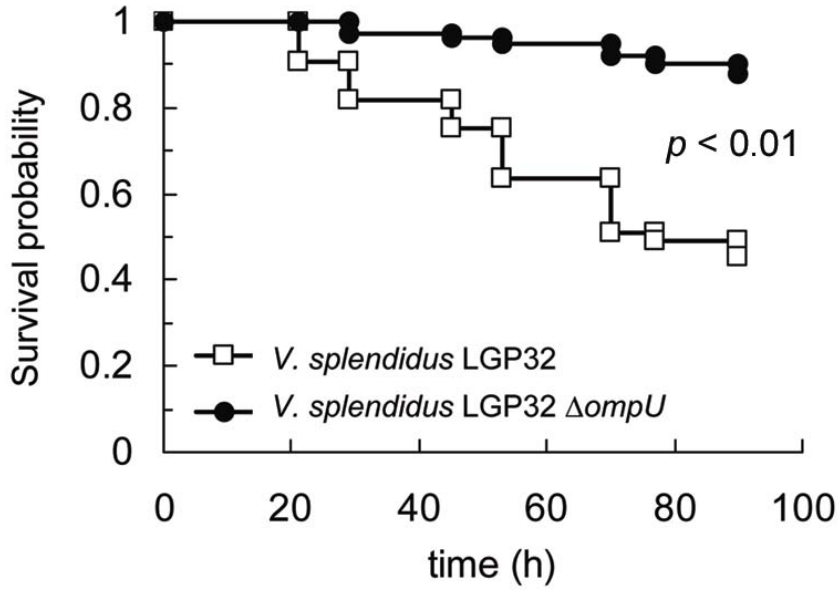

Figure 3

Figure 3. Inactivation of *ompU* results in a loss of virulence of *V. splendidus* LGP32 in oyster experimental infections

The wild-type *V. splendidus* LGP32 and the Δ*ompU* isogenic mutant were injected to oysters at a dose of 5 x 10^8 CFU per animal. Groups of 60 oysters were monitored for four days after infection. Kaplan-Meier survival curves were generated for oysters injected with the wild-type *V. splendidus* LGP32 (*open boxes*) and the Δ*ompU* mutant (*closed circles*). Data are representative of three independent survival experiments.

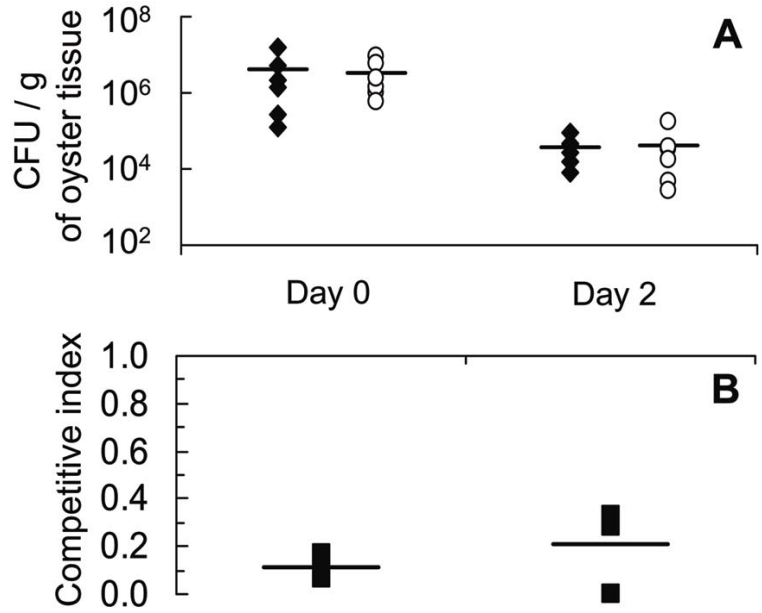

Figure 4

Figure 4. The *ompU* deletion mutant is competed out by wild-type *V. splendidus* LGP32 in oyster experimental infections

(A) Six oysters were injected with 5 x 10^8 CFU of either *V. splendidus* LGP32 (*closed diamonds*) or the Δ*ompU* isogenic mutant (*open circles*). CFU were counted in oyster tissues at day 0 and day 2 by plating on TCBS agar plates. Data points represent CFU counted in each oyster, and bars represent the CFU average for the six oysters. **(B)** Groups of four oysters were co-injected with a 1:1 ratio of a mixture of the *ompU* mutant and the wild-type *V. splendidus* LGP32 (5 x 10^8 CFU per oyster). The competitive index (CI) of the Δ*ompU* mutant was calculated for every oyster at day 0 and day 2. Plots represent CI values in individual oysters and bars represent the average calculated for four oysters. A CI < 1 indicates domination by *V. splendidus* wild-type strain.

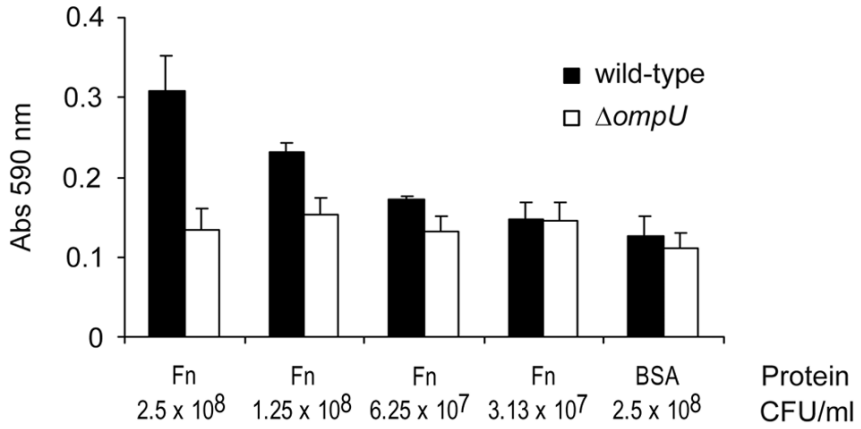

Figure 5

699

700 **Figure 5. *ompU* confers fibronectin-adhesive properties to *V. splendidus* LGP32**

701 Mid-log phase cultures of wild-type (*black bars*) and Δ*ompU* (*white bars*) *V. splendidus* serially diluted in
702 Zobell medium were subjected to the fibronectin-binding assay. In this assay, the adhesive properties of
703 the two strains are measured by a colorimetry (absorbance at 590 nm), which is indicative of their binding
704 to fibronectin (Fn) or bovine serum albumine (BSA), a large protein used as a negative control.

705

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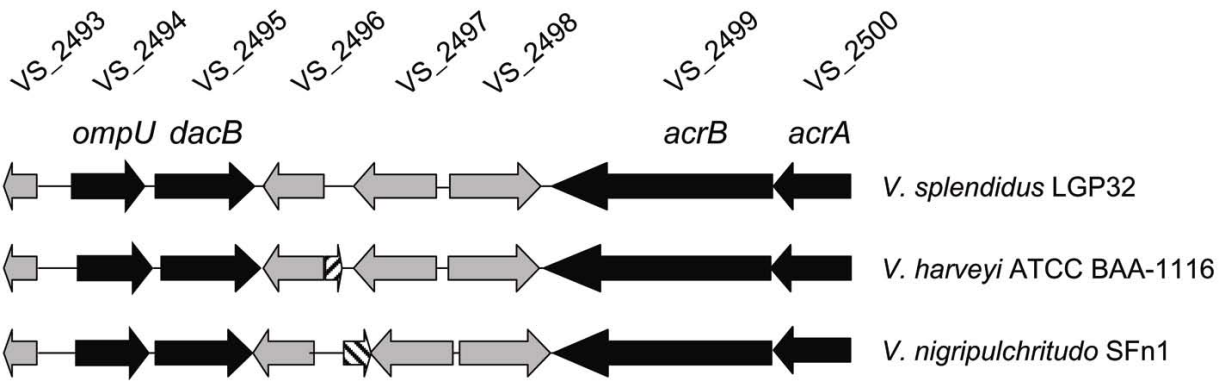

Figure S1

Figure S1. Genetic organization of the *ompU* gene region. The region flanking *ompU* localized on chromosome 1 of *V. splendidus* LGP32 is compared to the corresponding region on the chromosome 1 of *V. harveyi* ATCC BAA-1116 and *V. nigripulchritudo* SFn1. Gray and black arrows are conserved between the three genetic regions. Dashed arrows refer to genes absent from the genome sequence context surrounding *ompU* in *V. splendidus* LGP32. Black arrows refer to genes with a potential role in resistance to antimicrobials/antibiotics. The gene names and labels are displayed above the arrows.

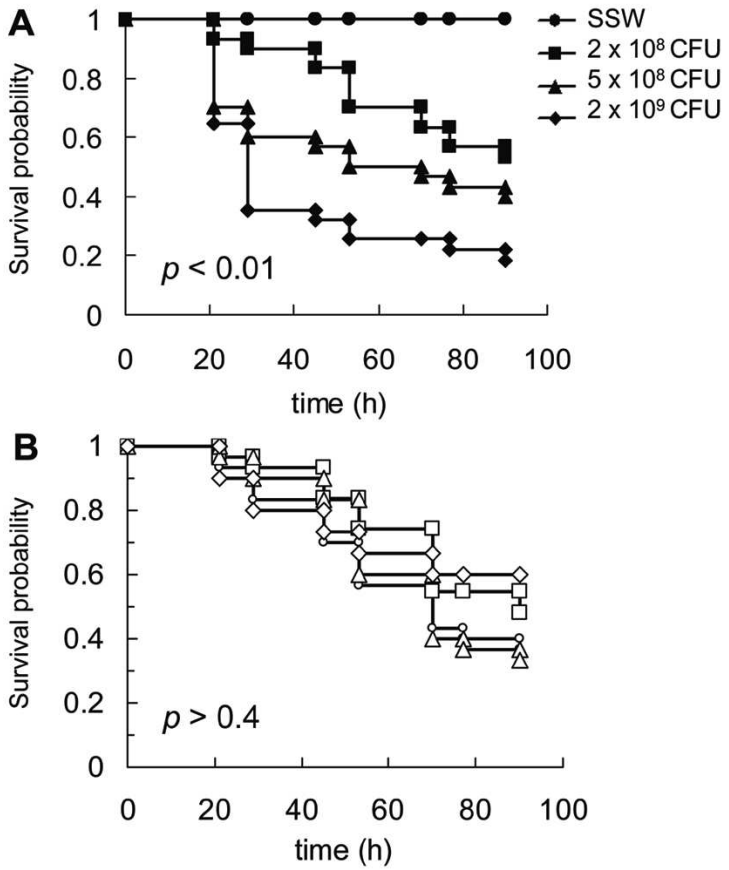

Figure S2

Figure S2. Standardization of oyster experimental infections by *V. splendidus* LGP32

(A) Three groups of 30 oysters were injected with wild-type *V. splendidus* LGP32 at the following doses: 2 x 10^8 (*closed boxes*), 5 x 10^8 (*closed triangles*), or 2 x 10^9 (*closed diamonds*) CFU per animal. A control was performed by injection of sterile sea water (SSW, *closed circles*). Oyster mortalities were monitored over four days and Kaplan-Meier survival curves were generated. **(B)** The wild-type *V. splendidus* LGP32 was injected to oysters at a dose of 5 x 10^8 CFU per animal. Groups of 30 oysters were monitored for 4 days after infection. Kaplan-Meier survival curves were generated for four independent survival experiments. A different *open symbol* was attributed to every of the four replicates.