Major role of the outer-membrane protein OmpU in the *Vibrio splendidus/ Crassostrea gigas* interaction

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Introduction

Vibrio splendidus LGP32 is an oyster pathogen associated with the summer mortality syndrome that dramatically affects the production of *Crassostrea gigas* oysters worldwide (Gay et al., 2004; Saulnier et al., 2010). We have recently shown that the outer membrane protein OmpU is required for the virulence of *V. splendidus* in oysters (Duperthuy et al., 2010). In order to better understand the role of OmpU in the host-pathogen interaction, we have constructed green fluorescent protein (GFP)-labeled variants of the wild-type and Δ*ompU V. splendidus* and studied their fate *in vivo* and *in vitro.*

Materials and methods

Bacterial strains
GFP-expressing *Vibrio* strains were derived from wild-type and Δ*ompU V. splendidus* (Duperthuy et al., 2010) by introduction of the *gpf3* allele under the Ptrc promoter according to the previously published procedure (Le Roux et al., 2007).

Binding of V. splendidus to oyster plasma
Plasma (12.5 µg) was coated onto a 96 well-microplate. GFP-labeled *V. splendidus* cultures washed in sterile sea water were added to each well. After 1 hour, unbound cells were washed off with sterile sea water (SSW) and the fluorescence measured (λex 395 nm / λem 509 nm).

Phagocytosis assay
Hemocytes (2.5 x 10^5 cells) were allowed to form monolayers in a 24 well-culture plate. Wild-type or *ΔompU V. splendidus* LGP32 (4 x 10^8 CFU) were added to wells containing either unwashed monolayers (total hemolymph), monolayers washed with SSW (washed hemocytes), or washed hemocytes to which plasma was added back (hemocytes + plasma). After a 30 min-contact, extracellular bacteria were eliminated with trypsin-EDTA 0.02 %. Hemocyte monolayers were then lysed with 1 mL of cold 0.05 % Triton X-100 to release intracellular bacteria. After centrifugation, the fluorescence was measured in bacterial pellets (λex 395 nm / λem 509 nm).

Oyster bacterial challenge
Oysters were immersed at 20 °C in sea water containing 5 x 10^5 CFU/ml of GFP-labeled wild-type or *ΔompU V. splendidus* LGP32. Oyster hemolymph was collected after 24 h and analyzed by flow cytometry to count the number of free GFP-labeled bacteria in plasma.

Results and discussion

The role of the OmpU protein in the *V. splendidus* LGP32 – *C. gigas* interaction was first studied *in vitro* by a contact between the oyster hemolymph and the GFP-labeled wild-type

and Δ*ompU V. splendidus* LGP32. After elimination of the extracellular bacteria, the bacteria-associated fluorescence was counted within hemocyte lysates. The wild-type cells were found to be avidly engulfed by hemocytes, *i.e.* the oyster immunocompetent cells, as indicated by intense fluorescence signals (Figure 1). Conversely, Δ*ompU V. splendidus* were poorly engulfed, giving 4-fold less intense fluorescence signals (Figure 1), indicating that OmpU is required for phagocytosis.

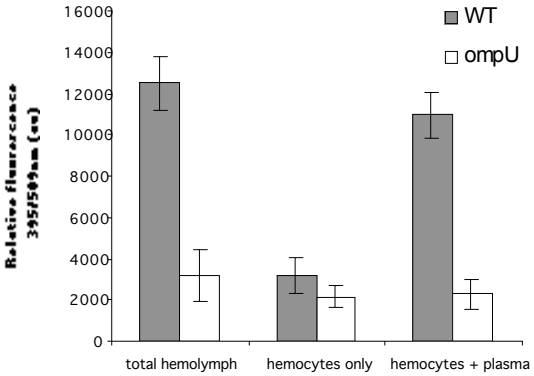

Figure 1. *In vitro* uptake of GFP-labeled wild-type (WT) and Δ*ompU V. splendidus* LGP32 by oyster hemocytes. *Vibrio* strains were incubated with oyster hemocytes in the presence/absence of plasma (total hemolymph, washed hemocytes, or washed hemocytes to which plasma was added back). The fluorescence associated to intracellular bacteria is expressed as relative fluorescence units (λex 395 nm / λem 509 nm). Data are representative of three independent experiments.

Interestingly, when washed hemocytes were used instead of total hemolymph in phagocytosis assays, the wild-type strain lost its ability to be engulfed by oyster hemocytes. Engulfment was restored upon addition of plasma (cell-free hemolymph) to the assay (Figure 1). This strongly suggested that a plasmatic component operates as opsonin for *V. splendidus*. Interestingly, since the presence/absence of plasma had no effect on the uptake of the Δ*ompU V. splendidus,* it was proposed that this plasmatic component specifically recognizes OmpU at the bacterial outer membrane.

In order to demonstrate the role of a plasma component in OmpU recognition, we designed binding assays in which oyster plasma was immobilized onto a microtiter plate. We then compared the binding of the GFP-labeled wild-type and Δ*ompU V. splendidus* LGP32 to the immobilized plasma. In this assay, we found that upon expression of OmpU the fluorescence signals indicative of bacteria binding increased by two-fold (Figure 2). Altogether, our data show that OmpU is essential for recognition by oyster hemocytes and that the OmpU-mediated phagocytosis requires a plasmatic opsonin, which remains to be identified.

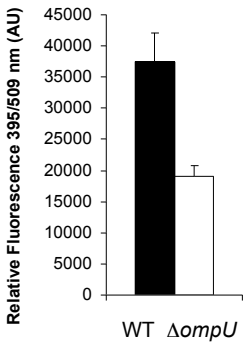

Figure 2. Binding of GFP-labeled wild-type and Δ*ompU V. splendidus* LGP32 to immobilized plasma. Binding is expressed as relative fluorescence units (λex 395 nm / λem 509 nm). Data are representative of two independent experiments, each performed with 5 oysters per condition.

The role of OmpU in the *V. splendidus / C. gigas* interaction was also analyzed in experimental infections. Oysters were bathed for 24 hours in sea water containing Δ*ompU V. splendidus* or the wild-type *V. splendidus*. After contact, green fluorescent bacteria were counted in cell-free hemolymph, showing that oysters exposed to Δ*ompU V. splendidus* displayed a significantly higher bacterial load (7.42 x 10^4 cells/ml of plasma) than oysters exposed to wild-type *V. splendidus* (1.31 x 10^4 cells/ml of plasma), $p<0.05$. This is in agreement with the *in vitro* phagocytosis data, which showed a significantly higher phagocytosis of the wild-type *Vibrio*.

Altogether, our data show that OmpU is essential for recognition and phagocytosis of the oyster pathogen *V. splendidu*s by the oyster immune cells. This is consistent with the proposed role of OmpU in mediating recognition by and avoidance of host hemocytes in pathogenic and symbiotic squid-*Vibrio* interactions, respectively (Nyholm et al., 2009). Both studies show that OmpU is a key determinant of the interaction between hemocytes of marine invertebrates and their associated symbiotic or pathogenic *Vibrio* species.

Conclusions

We showed here that OmpU is required for the recognition and phagocytosis of *V. splendidus* by oyster hemocytes, and that this phenomenon is mediated by a plasmatic protein, which specifically recognizes OmpU, and serves as an opsonin in the phagocytic process. Together with our previous study showing the major role of OmpU in virulence (Duperthuy et al., 2010), our results strongly suggest that the OmpU-mediated phagocytosis is an essential step in the pathogenesis of *V. splendidus* in oysters. Such a role of outer membrane proteins in adherence to immune cells is reminiscent of pathogenesis of other bacterial species such as *Klebsilla pneumoniae* (Soulas et al., 2000).

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