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Infection dynamics of a *V. splendidus* strain pathogenic to *Mytilus edulis*: *In vivo* and *in vitro* interactions with hemocytes

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ABSTRACT

The pathogenic strain *V. splendidus* 10/068 1T1 has previously been reported for its virulence to the blue mussel and for its capacity to alter immune responses. In this study, we expanded the knowledge on hemocyte-pathogen interactions by using *in vitro* and *in vivo* assays. *V. splendidus* 10/068 1T1 severely inhibited cell adhesion and acidic vacuole formation unlike the innocuous phylogenetically related *V. splendidus* 12/056 M24T1 which had no effect on these cell functions. Furthermore, the virulent bacteria decreased hemocyte viability (59% of viability after 24 h). Infection dynamics were explored by using a model based on water tank cohabitation with septic mussels infected by GFP-tagged *V. splendidus* 10/068 1T1. Experimental infections were successfully produced (16.6% and 45% mortalities in 3 days and 6 days). The amount of GFP *Vibrio* in seawater decreased during the experiment suggesting its horizontal transfer from diseased animals to healthy ones. At the same time periods, bacteria were detected in hemocytes and in various organs and caused necrosis especially in gills. Total hemocyte count and viability were affected. Taken together, our results indicate that the pathogen *V. splendidus* 10/068 1T1 colonizes its host both by bypassing external defense barriers and impairing hemocyte defense activities.

1. Introduction

Heterotrophic bacteria belonging to the genus *Vibrio* are highly abundant in the aquatic environment, mostly in seawater [1]. These ubiquitous microorganisms persist in a variety of geographic areas in interaction with eukaryotic marine hosts including zooplankton, sponges, corals and molluscs [1]. *Vibrio* sp. Show a remarkable biodiversity. Until now, more than 110 species of *Vibrios* have been identified, displaying a variety of host association modalities that extend from symbiosis to virulent pathogenicity [2,3].

Many species of pathogenic *Vibrios* are known to be responsible for diseases in terrestrial or marine vertebrates and invertebrates. *Vibrio cholerae, Vibrio vulnificus* and *Vibrio parahaemolyticus* in particular cause severe disorders in humans [4,5]. In numerous aquatic organisms including fish [6], corals [7], shrimp [8] and shellfish [9], some *Vibrios* have been associated with serious infections. Because of the high economic loss generated in the aquaculture sector, many studies are dedicated to bacterial diseases, particularly in farmed bivalves [3,9,10]. Among the etiological agents, bacteria from the clade *Splendidus* have been repeatedly described in relation to mortality events. This polyphyletic group include 16 species with contrasted pathotypes [11,12]. Different strains have been implicated in mortalities of various bivalves, e.g. the Pacific oyster *Crassostrea gigas* [13–17], the Atlantic scallop *Pecten maximus* [18,19], the carpet shell clam *Ruditapes philippinarum* [20], the greenshell mussel *Perna canaliculus* [21] and recently the blue mussel *Mytilus edulis* [11].

While infection mechanisms are well studied in human invaders, little is known in the specific case of invertebrate pathogens. Some results have been gathered in different species, especially concerning host-pathogen interactions [11,22–24]. However, invasion processes remain poorly documented. Understanding infection dynamics is an essential step for developing diseases management strategies [25]. In particular, there is a need for robust and standardized experimental models of *Vibrio*-bivalve interactions.

In a previous work, from mortality events reported by mussel farmers, we have isolated a *Vibrio* strain virulent to the blue mussel. *V. splendidus* 10/068 1T1 has been shown to alter hemocyte phagocytosis capacities, a key parameter of the immune defense system in mussels. Furthermore, a stable GFP-tagged *Vibrio* strain was constructed to facilitate the study of interactions between the

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microorganism and immune cells [11]. Fluorescent proteins (FP)-tagged microorganisms constitute a useful tool to monitor colonization processes. They have been used to elucidate early invasion events in squids [26,27] and bacterial dynamics in filter feeding oysters [28].

In the present study, we have investigated infection dynamics of *V. splendidus* 10/068 1T1 in *Mytilus edulis*. We describe (i) the development of an experimental infection model by water tank cohabitation with septic mussels, (ii) the localization of GFP bacteria in infected animals with the corresponding tissue lesions and (iii) *in vitro*/*in vivo* interactions between the pathogenic *Vibrio* strain and *Mytilus edulis* hemocytes.

2. Material and methods

2.1. Mussel collection

Adult mussels, *M. edulis* with shell length ranging from 4 to 5 cm, were collected on the intertidal rocky shore of Yport (0°18′52″E:49°44′30″N, France) between December 2015 and March 2016, immediately transported to the laboratory and placed in a temperature-controlled (10 °C) aerated Biotop Nano Cube 60 seawater tank (Sera, Heinsberg, Germany), equipped with mechanical and activated biological filtering. The animals were fed with algae (*Isochrysis galbana*) and maintained in these conditions for at least one week before use.

2.2. Bacterial strains and culture conditions

Two parental and GFP-tagged *V. splendidus*-related strains were used in this study: a virulent *V. splendidus* 10/068 1T1 isolated from mussel mortality events reported by professional (French national surveillance network REPAMO) in 2010 and an innocuous *V. splendidus* 12/056 M24T1 isolated from mussel microflora in absence of mortality in the context of Bivalife European project in 2012 [11]. Bacteria were routinely cultivated overnight in LBS [Luria Bertani complemented with salt, NaCl 20 g L^{-1} (f.c.)] at 22 °C. Stock cultures were stored at −80 °C in LBS with glycerol 15% (v/v) supplemented with kanamycin 100 μg L^{-1} for GFP-tagged strains.

2.3. In vitro hemocyte challenge

2.3.1. Hemolymph collection

Hemolymph was withdrawn from the posterior adductor muscle sinus, by gentle aspiration with a 1 mL syringe equipped with a 22G needle. Quality of samples was systematically checked by microscopic observation before using in bioassays. Samples containing protozoa, tissue fragments, low number of hemocytes were discarded.

2.3.2. Hemocyte adhesion

Cells were incubated with bacteria at a ratio of 10 bacteria/hemocyte, or with sterile physiological water (NaCl 9 g L^{-1}), in a 24-well tissue-culture plates (Greiner). After 2, 4 and 6 h at 15 °C, the number of non adherent cells in the supernatant was counted by flow cytometer.

2.3.3. Acidic vacuole formation

Crude hemolymph was placed into individual wells of 24-well tissue-culture plates (Greiner) for cytometry or in 35 mm μ-Dish (Ibidi) for microscopy. Cells were exposed to *Vibrio* strains at 10:1 ratio (bacteria:hemocytes) for 2 h at 15 °C.

Lysotracker (LysoTracker® Green DND-26, life technologies) at 2 μM was added and cells were incubated for 30 min at 15 °C in the dark. Hemocyte fluorescence was quantified by flow cytometry. For microscopy imaging, cells were washed with the marine physiological saline solution [MPSS (470 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 10 mM HEPES, 48,7 mM $MgSO_4$), pH 7.8, 0.2 μm filtered]. Hemocyte nuclei were counterstained with hoechst 33342 (5 μM, 15 min) and imaged by epifluorescence microscopy.

2.3.4. Hemocyte viability

Hemocytes were exposed to bacteria (10/068 1T1 and 12/056 M24T1) at 10^8 CFU mL^{-1} for different time periods (2, 4, 6, 18 and 24 h) at 15 °C. At each time point, propidium iodide was added (20 μM) and cell viability was measured by flow cytometry.

2.4. In vivo challenge

2.4.1. Mussel infection by water tank cohabitation model

Bacteria were prepared at OD_{600nm} of 1 as described in Ben Cheikh et al. [11]. Animals were anesthetized for 2–3 h at 16 °C in a magnesium chloride solution (50 g L^{-1}, 1/4: v/v seawater/freshwater) under aeration. Subsequently, a volume of 100 μL of bacterial suspension (2.10^8 CFU mL^{-1}) or filtered sterile seawater (FSSW) for the negative control was injected into the posterior adductor muscle. After injection, the animals were transferred to tanks (3 replicate tanks, 10 mussels per tank) filled with 2 L of UV-treated and filtered seawater supplemented with 50 mL of phytoplankton (*Isochrysis galbana*). After 24 h, moribund animals were sacrificed by severing their adductor muscle and placed in cohabitation with a group of 10 apparently healthy mussels. For the negative control, mussels injected with FSSW (alive) were sacrificed and used in the same conditions. After 72 h of cohabitation, injected mussels were removed. During the experiment, animals were maintained under static conditions at 16 °C with aeration. Mortality was monitored each day over a six days period. Animals were considered to be dead when the valves did not close following stimulation. Newly dead mussels were removed from the tanks.

2.4.2. Bacteria counting in seawater

Seawater was sampled from cohabitation tanks each day during the experiment period. 100 μL of samples serially diluted in sterile physiological water (NaCl 9 g L^{-1}) was plated on LBS agar supplemented with kanamycin 100 μg L^{-1}. After 48 h at 22 °C, colonies were counted. The presence of GFP colonies was verified under a fluorescence stereo microscope (Leica microsystems).

2.4.3. Hemocyte cellular parameters analysis

Hemolymph was sampled from exposed mussels and mixed with cold Alsever's solution (300 mM NaCl, 100 mM Glucose, 30 mM sodium Citrate, 26 mM citric acid, 10 mM EDTA, pH 5.4) for cytometry analysis.

Variation in hemocyte count and the percentage of cells containing GFP-tagged bacteria was determined. Cell viability was investigated by adding propidium iodide (20 μM).

2.4.4. Histological and immunohistochemical analyses

During cohabitation infection, mussels were sampled at different time periods and removed from their shell. Tissues were fixed in Davidson's solution (20% formaldehyde 36%, 30% FSSW, 10% glycerol, 30% ethanol 95% and 10% acetic acid) for 48 h, dehydrated in a graded series of ethanol, and embedded in paraffin. Consecutive 3–5 μm thick sections were adhered to Superfrost [hematoxylin-eosin (HE) staining] or Superfrost Plus [immunohistochemistry (IHC)] microscope slides.

For tissue examination, sections were stained by classical hematoxylin-eosin protocol. Presence of listed pathogens (*Bonamia* sp., *Marteilia* sp., *Perkinsus* sp. and *Mikrocytos* sp.) was examined as well as trematods, copepods, *Mytilicola*, ciliates and gregarines. For lesions, we noticed the presence of necrosis, bacterial “foyer”,