Journal of Invertebrate Pathology 133 (2016) 27–33

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

Following the infection process of vibriosis in Manila clam (*Ruditapes philippinarum*) larvae through GFP-tagged pathogenic *Vibrio* species

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ARTICLE INFO

Article history:
Received 21 September 2015
Revised 17 November 2015
Accepted 18 November 2015
Available online 25 November 2015

Keywords:
Vibriosis
Manila clam
Larvae
Vibrio spp.
Green fluorescent protein (GFP)

ABSTRACT

Vibriosis represents the main bottleneck for the larval production process in shellfish aquaculture. While the signs of this disease in bivalve larvae are well known, the infection process by pathogenic *Vibrio* spp. during episodes of vibriosis has not been elucidated. To investigate the infection process in bivalves, the pathogens of larvae as *V. tubiashii* subsp. *europaensis*, *V. neptunius* and *V. bivalvicida* were tagged with green fluorescent protein (GFP). Larvae of Manila clam (*Ruditapes philippinarum*) were inoculated with the GFP-labeled pathogens in different infection assays and monitored by microscopy. Manila clam larvae infected by distinct GFP-tagged *Vibrio* spp. in different challenges showed the same progression in the infection process, defining three infection stages. GFP-tagged *Vibrio* spp. were filtered by the larvae through the vellum and entered in the digestive system through the esophagus and stomach and colonized the digestive gland and particularly the intestine, where they proliferated during the first 2 h of contact (Stage I), suggesting a chemotactic response. Then, GFP-tagged *Vibrio* spp. expanded rapidly to the surrounding organs in the body cavity from the dorsal to ventral region (Stage II; 6–8 h), colonizing the larvae completely at the peak of infection (Stage III) (14–24 h). Results demonstrated for the first time that the vibriosis is asymptomatic in Manila clam larvae during the early infection stages. Thus, the early colonization and the rapid proliferation of *Vibrio* pathogens within the body cavity supported the sudden and fatal effect of the vibriosis, since the larvae exhibited the first signs of disease when the infection process is advanced. As a first step in the elucidation of the potential mechanisms of bacterial pathogenesis in bivalve larvae the enzymatic activities of the extracellular products released from the wild type *V. neptunius*, *V. tubiashii* subsp. *europaensis* and *V. bivalvicida* were determined and their cytotoxicity was demonstrated in fish and homeothermic cell lines for the first time. That activity was lost after heat treatment.

1. Introduction

Vibriosis is the most important bacterial disease affecting the larval stages of different species of marine mollusks in shellfish aquaculture. Outbreaks of disease due to pathogenic *Vibrio* spp. have been described worldwide in larvae of several bivalve species cultured in hatcheries (Prado et al., 2005, 2015; Beaz-Hidalgo et al., 2010; Travers et al., 2014; Rojas et al., 2015).

Bivalve larvae are more susceptible to vibriosis than adults since the resistance to bacterial infection significantly increases with age of the bivalves (Gómez-León et al., 2008). The pathogens *V. tubiashii* subsp. *europaensis*, *V. neptunius* and *V. bivalvicida* have been identified as the etiological agents responsible for larval and spat mortality episodes in clam and oyster cultures leading to important economic losses for the shellfish hatcheries (Prado et al., 2005, 2015; Dubert et al., in press). Extracellular products (ECPs) should be studied since virulence is usually associated with these products released by some pathogenic *Vibrio* species (Shinoda and Miyoshi, 2011). Signs of disease include an important reduction of larval motility, erratic swimming, closing of the valves, velum detachment, and bacterial swarming inside and around the larvae (Prado et al., 2005; Beaz-Hidalgo et al., 2010; Rojas et al., 2015). While the clinical signs of vibriosis are well

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http://dx.doi.org/10.1016/j.jip.2015.11.008
0022-2011/

known, the processes of colonization and infection by pathogenic *Vibrio* spp. in bivalve larvae have not been elucidated. Vibriosis has only been monitored by means of molecular tagging of the pathogens in adult mollusks (Cabello et al., 2005; Travers et al., 2008; Cardinaud et al., 2014).

In order to investigate the pathogenesis of vibriosis in bivalve larvae, we investigated the colonization and infection process by the pathogens *V. neptunius*, *V. tubiashii* subsp. *europaensis* and *V. bivalvicida*, tagged with green fluorescent protein (GFP), in bivalve larvae. Considering the economic importance of clam production for shellfish aquaculture and the high susceptibility of larval batches to vibriosis, Manila clam (*Ruditapes philippinarum*) larvae were chosen as infection model. Additionally, enzymatic activities of wild type pathogenic *Vibrio* spp. and their ECPs were determined. Finally, the cytotoxicity of the ECPs was evaluated in fish and homoeothermic cell lines.

2. Materials and methods

2.1. Bacterial strains, plasmid and culture conditions

Larval pathogens *V. neptunius* PP-145.98, *V. tubiashii* subsp. *europaensis* CECT 8136^T and *V. bivalvicida* CECT 8855^T were grown at 25 °C and shaken 200 rpm in YP broth, constituted by yeast extract (0.5%, w/v) (Thermo Fisher Scientific, USA) and peptone (0.1%, w/v) (BD, France) at pH 7.6, supplemented with 3% (w/v) of sea salts (YP30) (Instant Ocean, USA). Streptomycin (Sm^{200}; 200 μg ml^{-1}, Sigma–Aldrich, USA) was only added to YP30 for mating assays since wild type strains were resistant to Sm.

In a previous study, Zhao et al. (unpublished results) designed the plasmid pRhokHi-2-GFP, which has chloramphenicol (Cm^R) and kanamycin (Km^R) resistance markers, and derivative from pRhokHi-2-FbFP (Piekarski et al., 2009) with *gfp* gene under the control of the constitutive promoter of the aminoglycoside phosphotransferase II (P*aphII*). Plasmid pRhokHi-2-FbFP is 7.38 kb, carrying Cm^R and Km^R resistances, P_{T7} *FbFP* constructed under the control of P*aphII* from the conjugally transferable and stably maintained plasmid pBBR1MCS. The resulting plasmid pRhokHi-2-GFP was introduced in *Escherichia coli* Sm10 (*thi thr leu tonA lacY supE recA* RP4-2 Tc::Mu::KmR, λ pir) by electroporation. In the present study, *E. coli* Sm10 pRhokHi-2-GFP was cultured in Luria-Bertani plus 10% NaCl (LB10) supplemented with Cm^{20} (20 μg ml^{-1}; Sigma, USA) at 37 °C while shaking at 200 rpm.

In all cases, 1.5% agar (w/v) (BD, France) was added when the solid media were required.

2.2. Mating assays and selection of GFP-tagged strains

Plasmid pRhokHi-2-GFP carried was introduced into larval pathogens from *E. coli* Sm10 by conjugation following the procedure described by Milton et al. (1992) with slight modifications. Briefly, overnight cultures of each *Vibrio* spp. and *E. coli* Sm10 pRhokHi-2-GFP were grown for the different mating assays as described above. Then, *Vibrio* culture was mixed at 1:1 ratio in Nine Salt Solution (NSS) (Varina et al., 2008) and *E. coli* in 10 mM $MgSO_4$ and 100 μl of each one were diluted again in 2.5 ml of NSS and $MgSO_4$ respectively. Bacterial suspensions were combined and vacuum filtered onto a 0.22 μm nitrocellulose membrane (Millipore, USA), which was placed onto YP plates plus 1.5% (w/v) of sea salts (YP15) and incubated overnight at 25 °C. Subsequently, the cells were removed from the filter by vigorous mixing in YP30 broth and 100 μl were spread onto three YP30 plates supplemented with Sm^{200} and Cm^5 and incubated at 25 °C until *Vibrio* transconjugants were observed (usually 24 to 72 h).

2.3. Confirmation of the transconjugants

Transconjugants were isolated from the mating assays and their morphology, motility and green fluorescence was checked by epifluorescence microscopy (40×) (Zeiss Axioskop 2, Germany) using the filter for green signal (FITC, 488 nm). Growth on TCBS plates (Oxoid, USA) was also evaluated. DNA of the GFP-tagged *Vibrio* spp. was extracted with Instagene kit (Bio-Rad, UK) and their 16S rRNA gene was amplified and sequenced to confirm their identities using specific bacterial primers (27F and 1510R) (Lane, 1991). Sequences were analyzed with the Lasergene Seqman (DNAstar, USA) and identified using the BLAST database (Altschul et al., 1997). Moreover, growth curves of wild type *Vibrio* spp. and GFP-tagged strains were determined by optical density measurements at 600 nm (OD_{600}) to detect differences in the growth speed. Hence, stationary phase cultures were adjusted to 1.0 absorbance, diluted 1:100 in YP30 broth and incubated at 25 °C and 200 rpm. Bacterial concentrations (OD_{600}) were estimated per duplicate at 2, 4, 6, 8, 10, 24 and 48 h. At the same time, the green fluorescence of the GFP-tagged strains were evaluated at 96 h by epifluorescence microscopy (40×) to guarantee that the expression of the green fluorescence protein gene lasted for this length of time in absence of selective pressure due to the antibiotic.

2.4. Colonization and infection assays

A previously developed challenge model (Prado et al., 2015) was used to determine the colonization and infection process for each of the GFP-tagged *Vibrio* species. Healthy ten-day-old *R. philippinarum* veliger larvae (120 μm, umbonate veliger) were placed in wells in 12 well microplates (NUNC; ThermoFisher Scientific, USA) filled with 3 ml of sterile seawater (SSW) at a density of 15–20 larvae ml^{-1}. Wild type pathogenic *Vibrio* spp., used as positive controls, were grown overnight in YP30 plates as described above, whereas GFP-tagged strains were cultured in YP30 plates supplemented with Sm^{200} and Cm^5 under the same incubation conditions. Strains, including wild type and GFP-tagged, were re-suspended in SSW and inoculated in different duplicate wells at a final concentration of 10^6 CFU ml^{-1}. Bacterial concentrations were confirmed by serial dilution and spread plating onto YP30 agar plates. In addition, wells containing only larvae in SSW were used as negative controls. Larval cultures were kept in dark at 20 °C in a rotary shaker at 50 rpm. Positive and negative controls were checked at 0, 24 and 48 h. Wells inoculated with each GFP-tagged *Vibrio* spp. were sampled at 0, 2, 6, 8, 14, 24 and 36, 48 h for monitoring the infection. Advance of the clinical signs of vibriosis and the integrity of the larvae were checked directly from the microplates by phase contrast (10/20×) in an inverted microscopy (Olympus CK40, Japan). Larvae from each corresponding well were collected from the wells, centrifuged at 3000 rpm 10 s and observed by epifluorescence microscopy (40×) (Zeiss Axioskop 2, Germany) in a concave slide. Samples were evaluated with different filter sets FITC (488 nm), Cy3 (546 nm) and Cy5 (633 nm) to distinguish between the fluorescence due to the GFP and the autofluorescence of the larvae.

2.5. Cytotoxicity of the extracellular products (ECPs)

The ECPs were obtained from liquid media as described Romalde (1992). Briefly, wild type *Vibrio* spp. were grown in flasks (50 ml) containing 10 ml of YP30 broth and incubated at 25 °C (24 h, 200 rpm). Cultures were centrifuged at 8000 rpm (5510*g*) for 5 min (Eppendorf Centrifuge 5418), filtered through a 0.20 μm cellulose acetate filter (Sartorius, Germany) and frozen at −20 °C. The protein concentration was determined using the Protein Assay (Bio-Rad, USA), based on the Bradford dye-binding