Aquaculture 470 (2017) 190–195

Contents lists available at ScienceDirect

Aquaculture

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

# The impact of catecholamine sensing on the virulence of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND)

Nguyen Thao Suong [a], Nguyen Van Hao [a], Nguyen Van Sang [a], Nguyen Dinh Hung [a], Nguyen Thi Ngoc Tinh [a], Le Hong Phuoc [a], Doan Van Cuong [a], Nguyen Thanh Luan [a], Do Viet Phuong [b], To Thi Thom [c], Phan Hong Thao [c], Peter Bossier [d], Patrick Sorgeloos [d], Tom Defoirdt [d,e,*]

[a] Research Institute for Aquaculture No.2, 160 Nguyen Dinh Chieu, District 1, Ho Chi Minh City, Viet Nam
[b] Virbac Regional RDL Center Asia, 17 Ba Huyen Thanh Quan, District 3, Ho Chi Minh City, Viet Nam
[c] Faculty of Bio-Food Technology and Environment, Ho Chi Minh University of Technology, 144/24, Dien Bien Phu, Binh Thanh District, Ho Chi Minh City, Viet Nam
[d] Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Coupure Links 653, 9000 Gent, Belgium
[e] Center for Microbial Ecology and Technology (CMET), Ghent University, Coupure Links 653, 9000 Gent, Belgium

ARTICLE INFO

*Article history:*
Received 17 September 2016
Received in revised form 20 December 2016
Accepted 22 December 2016
Available online 02 January 2017

*Keywords:*
Early mortality syndrome (EMS)
Host-microbe interaction
Microbial endocrinology
*Vibrio parahaemolyticus*

ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) is a severe shrimp disease that causes significant losses in the shrimp industry worldwide. In 2013, specific strains of *Vibrio parahaemolyticus* were found to be responsible for AHPND. Recently, inhibiting the detection of catecholamines has been reported to decrease the virulence of various pathogenic bacteria, including *Vibrio anguillarum*, *Vibrio campbellii* and human pathogenic *Vibrio parahaemolyticus*. Thus, in this study we investigated whether catecholamine sensing has any effect on the virulence of an AHPND-causing *V. parahaemolyticus* strain isolated from outbreaks in Vietnam. We found that the catecholamines norepinephrine and dopamine (50 μM) increased motility of *V. parahaemolyticus* ($P < 0.05$). Further, the catecholamine-induced motility could be neutralized by the prokaryotic catecholamine receptor antagonist LED209. Finally, pre-treatment of *V. parahaemolyticus* with catecholamines significantly increased its virulence to whiteleg shrimp (*Penaeus vannamei*), and pretreatment with the antagonist LED209 neutralized this effect ($P < 0.05$). LED209 increased the survival of shrimp challenged with catecholamine-pretreated *V. parahaemolyticus* to levels that were even higher than those observed in shrimp challenged with untreated *V. parahaemolyticus*, suggesting that this type of compounds might be useful to decrease losses due to AHPND in shrimp.



## 1. Introduction

Outbreaks of acute hepatopancreatic necrosis disease (AHPND), caused by *Vibrio parahaemolyticus*, have been particularly devastating in the cultivation of shrimp in a number of countries, with global losses of more than $ 1 billion per year (Tran et al., 2013; Han et al., 2015; Thitamadee et al., 2016). The disease has been reported to be responsible for an 80% decrease in shrimp production in Hainan, Guangdong, Fujian and Guangxi (China) during the first half of 2011 (Panakorn, 2012), a 33% decrease in Thai shrimp production during the first quarter of 2013 (Joshi et al., 2014), and about $ 118 million lost in Mexico in 2013 (Schryver et al., 2014). It has been estimated that 39,000 ha of shrimp ponds in the Vietnamese provinces Tra Vinh, Soc Trang, Bac Lieu and Ca Mau in the Mekong delta were affected by AHPND in 2011, with mortality rates of up to 100% (FAO, 2013).

Strategies to control AHPND mainly focus on prevention by pond renovation and disinfection. This approach is not capable of stopping the epidemiological situation once the disease has started and has been argued not to be the most effective strategy to prevent AHPND (Schryver et al., 2014). Furthermore, the use of antibiotics against *V. parahaemolyticus* to treat the disease has been documented to result in antibiotic resistance in the pathogen (Tran et al., 2015). Consequently, new approaches to tackle this problem (both preventive and curative) are urgently needed. One option is to disrupt the mechanisms the pathogen needs to infect its host, a strategy that has been termed antivirulence therapy (Defoirdt, 2014).

Catecholamines such as dopamine and norepinephrine are highly conserved in animals (both vertebrates and invertebrates). Elevated levels of these compounds have for a long time been associated with a decreased activity of the defense system of animals (including shrimp)

* Corresponding author at: Center for Microbial Ecology and Technology (CMET), Coupure Links 653, B-9000 Gent, Belgium.
*E-mail address:* Tom.Defoirdt@UGent.be (T. Defoirdt).

http://dx.doi.org/10.1016/j.aquaculture.2016.12.030
0044-8486/

(Cheng et al., 2006). More recent evidence showed that catecholamines are also used as host cues to enhance the virulence of pathogenic bacteria (Lyte, 2004). In various *Vibrio* species, including *V. anguillarum*, *V. harveyi* and human pathogenic *V. parahaemolyticus*, catecholamines have been reported to increase growth in media containing serum (which limits iron availability, thereby mimicking the in vivo situation), motility, biofilm formation and other virulence-related phenotypes (Nakano et al., 2007; Yang et al., 2014; Pande et al., 2015). Hence, catecholamine sensing might be an interesting target for the development of novel virulence inhibitors. A specific inhibitor of bacterial catecholamine receptors, N-phenyl-4-{[(phenylamino)thioxomethyl]amino}-benzenesulphonamide (LED209), has recently been reported (Rasko et al., 2008). Interestingly, LED209 also inhibited catecholamine-induced virulence in *V. harveyi*, both in vitro and in vivo in a challenge test with gnotobiotic brine shrimp larvae (Yang et al., 2014).

In this study, we investigated the impact of the catecholamines dopamine and norepinephrine and the bacterial catecholamine receptor antagonist LED209 on swimming motility (a virulence-related phenotype that is induced by catecholamines in many bacteria) in the Vietnamese AHPND-causing *V. parahaemolyticus* isolate CM1. We further determined the impact of these compounds on the virulence of strain CM1 towards white leg shrimp (*Penaeus vannamei)*. This work will lay the foundation for a better understanding of the factors that are involved in the AHPND epidemiology and provide a first step towards novel and innovative therapies to control the disease.

## 2. Materials and methods

### 2.1. Isolation and identification of V. parahaemolyticus

One hundred samples of AHPND-positive *P. vannamei* shrimp were collected from September 2014 to December 2014 from the provinces Ca Mau, Tien Giang and Ben Tre in the Mekong delta, Vietnam. At the collection time, aseptically excised tissue of the hepatopancreas (HP) was disaggregated and streaked on CHROMagar *Vibrio* agar (CHROMagar, Paris, France) plates. After incubation at 28 °C overnight, violet colonies on CHROMagar *Vibrio* agar plates were selected (Hara-Kudo et al., 2001). Individual colonies were re-streaked onto Luria Bertani agar plates (Himedia, India) containing 2% NaCl (LB +) to obtain pure isolates. These isolates were stored in glycerol at −80 °C. For bacterial identification, pure isolates on LB + plates were sent to Macrogen, Biochemistry Company, Vietnam to conduct 16S rDNA sequencing. Acquired 16S rDNA sequences were aligned and compared with a collection of 16S rDNA sequences in GenBank by using the NCBI Basic Local Alignment Search Tools, nucleotide (BLASTn) program in order to verify high identity with *Vibrio parahaemolyticus* sequences.

PCR detection of AHPND bacteria was performed according to Joshi et al. (2014), based on the amplification of unique DNA sequences of AHPND-causing *V. parahaemolyticus* isolates that are not present in non-AHPND *V. parahaemolyticus* isolates. The forward (F) and reverse (R) primer sets that were used are AP2F: 5′ - TCACCCGAATGCTCGCTTGTGG - 3′; and

AP2R: 5′ - CGTCGCTACTGTCTAGCTGAAG - 3′. The cycling conditions were 5 min at 94 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C and 60 s extension at 72 °C, plus a final 10 min extension at 72 °C. The amplified PCR products were analysed on 2% agarose gels, stained with ethidium bromide, and visualized under UV transillumination. Isolate CM1 (16S rDNA sequence submitted to GenBank under accession number KX619616) was retained for further experiments. This isolate gave positive PCR test results (bands at approximately 700 bp), while non-AHPND *V. parahaemolyticus* gave a negative result.

### 2.2. Growth conditions of AHPND-causing V. parahaemolyticus CM1

Isolate CM1 was stored in glycerol at −80 °C and re-streaked on an LB + plate. A single colony was inoculated into LB + broth (Himedia, India) and incubated at 28 °C under constant agitation (100 $min^{-1}$) overnight. Bacterial density was measured spectrophotometrically at 600 nm.

### 2.3. Catecholamines and the inhibitor of bacterial catecholamine receptors LED209

The catecholamines dopamine and norepinephrine were obtained from Sigma (Bornem, Belgium) and the receptor inhibitor LED209 were obtained from Cayman Chemicals (Michigan, USA). Stocks of dopamine and norepinephrine (10 mM) were prepared in distilled water and distilled water containing 0.1 N HCl, respectively. LED209 was dissolved in dimethyl sulfoxide at 1 mM. Dopamine and norepinephrine were tested at 50 μM and 100 μM each. LED 209 was tested at 0.05 μM and 0.1 μM. All stocks solutions were stored at −20 °C.

### 2.4. Swimming motility assays

Soft LB + plates containing 0.3% agar were used for performing swimming motility as described previously by Yang et al. (2014) and Pande et al. (2015). Overnight grown *V. parahaemolyticus* was diluted to $OD_{600} = 0.5$, and 10 μl aliquots were spotted in the center of the soft agar plates. Plates were incubated overnight, after which the diameters of the motility halos were measured.

### 2.5. Preparation of experimental shrimp

Specific-pathogen free (SPF) *P. vannamei* shrimp (2–5 g body weight) were purchased from commercial hatcheries (Vung Tau province, Vietnam). The animals were maintained in composite tanks containing aerated filtered seawater at 20 ppt salinity for 1 week before the start of the experiments. The shrimp health status was checked by monitoring swimming activity, luminescence, survival rate, muscle opaqueness, deformities, size variation, gut content, and colour of the hepatopancreas. Formulated feed for shrimp weighing 1–5 g (Monotech, Cargill) was used for feeding the shrimp and airlifts were used to supply oxygen.

### 2.6. Pathogenicity of V. parahaemolyticus CM1 to P. vannamei

Shrimp pathogenicity experiments were performed according to the method described by Joshi et al. (2014) with some modifications. Briefly, groups of ten randomly selected healthy shrimp were transferred to tanks containing 15 l of 20 ppt filtered seawater at 28 ± 0.5 °C. The shrimp were then challenged by inoculation of $5.10^7$, $1.10^7$, $6.10^6$, or $2.10^6$ CFU/ml *V. parahaemolyticus* cells in the rearing water, respectively. The control animals were maintained in fresh filtered seawater supplemented with a volume of LB broth equal to the *V. parahaemolyticus* inoculum. Gross signs of disease and cumulative mortality were recorded after 96 h. Histopathological examination of moribund shrimp was performed using the methods described by Tran (2013). The average lethal dose (LD50) values were calculated as described by Reed and Muench (1938). Each experiment was conducted in triplicate.

### 2.7. Impact of catecholamines and the inhibitor of the bacterial catecholamine receptor (LED209) on the virulence of V. parahaemolyticus CM1 towards P. vannamei

Groups of 16, 20 or 30 healthy white leg shrimp were distributed to glass tanks containing 30 l of filtered sea water (20 g $l^{-1}$ salinity) in experiment 1, 2 and 3, respectively. Shrimp were fed twice daily with a pelleted shrimp feed (Monotech, Cargill). In order to avoid a direct effect of catecholamines (50 μM) and LED209 (0.05 μM and 0.1 μM) on the animals, *V. parahaemolyticus* was grown overnight in LB + with the compounds, after which the cultures were washed with autoclaved sea water (20 ppt) supplemented with 10% (v/v)