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Short communication

Quorum sensing-disrupting compounds protect larvae of the giant freshwater prawn *Macrobrachium rosenbergii* from *Vibrio harveyi* infection

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ABSTRACT

Vibriosis outbreaks caused by *Vibrio harveyi* and related species are amongst the major obstacles for the further expansion of giant freshwater prawn (*Macrobrachium rosenbergii*) larviculture. *Vibrio harveyi* regulates virulence gene expression through quorum sensing, bacterial cell-to-cell communication, and consequently, quorum sensing disruption has been suggested as an alternative strategy to control infections caused by these bacteria. Previous studies have shown that quorum sensing-disrupting compounds are able to disrupt quorum sensing in *Vibrio harveyi*. In this study, we demonstrated that the quorum sensing-disrupting compounds cinnamaldehyde, (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone and (*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)metoxy)-4-oxobutanoic acid increased the survival of giant freshwater prawn larvae when challenged to pathogenic *Vibrio harveyi*. Our *in vivo* challenge test showed that cinnamaldehyde and the thiophenone can protect the larvae from *Vibrio harveyi* infection when dosed to the culture water at 1 μM and 10 μM, whereas the brominated furanone offered protection at 1 μM but resulted in complete mortality at 10 μM. Although there were significant differences in survival between challenged larvae with and without addition of quorum sensing-disrupting compounds, there were no differences in growth (as determined by the larval stage index).

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii*, is one of the most important freshwater crustaceans from a commercial point of view. Freshwater prawn farming has expanded enormously since 1980, and in 2009 the global production reached a level of up to 80 times the production reported in 1980 (New and Nair, 2012). However, disease outbreaks are amongst the major obstacles to produce healthy and high quality seed for the further expansion of giant freshwater prawn culture (Nhan et al., 2010). Some studies have shown that *Vibrio* spp. are a major cause of disease (Kennedy et al., 2006; New et al., 2010; Tonguthai, 1997). In general, vibriosis is prevalent in the early life stages (eggs, larvae, and postlarvae) of giant freshwater prawn (Bhat and Singh, 1999), all of which need brackish water to survive (New et al., 2010). One of the species that has been isolated from affected giant freshwater prawn larvae is the luminescent bacterium *Vibrio harveyi* (Bhat and Singh, 1999; Tonguthai, 1997). *Harveyi* clade vibrios (including *V. harveyi* and closely related species such as *V. campbellii* and *V. parahaemolyticus*) are amongst the most important bacterial pathogens of aquatic animals, and cause significant losses in the aquaculture industry worldwide (Austin and Zhang, 2006; Ruwandeepika et al., 2012).

The frequent use of antibiotics to control vibriosis in hatcheries has led to the development and spread of antibiotic-resistant bacteria (Karunasagar et al., 1994; Moriarty, 1998), and alternative methods are needed to control these bacterial infections. Recently, it has become clear that the virulence of *V. harveyi* is under control of quorum sensing, a regulatory mechanism based on secreting and sensing small signal molecules called autoinducers (Cao and Meighen, 1989; Chen et al., 2002; Defoirdt et al., 2008; Henke and Bassler, 2004; Natrah et al., 2011). We previously reported that *V. harveyi* quorum sensing regulates its virulence towards giant freshwater prawn larvae (Pande et al., submitted for publication). Consequently, the application of quorum sensing-disrupting agents might be a valid strategy to control vibriosis in this species. Quorum sensing-disrupting compounds such as cinnamaldehyde, brominated furanones and brominated thiophenones

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(Fig. 1) have been reported before to protect brine shrimp larvae from *V. harveyi* (Brackman et al., 2008; Defoirdt et al., 2007, 2012). In this study, we aimed at investigating the impact of these compounds on the survival and growth of giant freshwater prawn larvae when challenged to pathogenic *V. harveyi*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

In this study we used *Vibrio harveyi* BB120 (=ATCC BAA-1116). The bacterium was stored at −80 °C in 40% glycerol and the stocks were streaked onto Luria-Bertani agar containing 12 g l^{-1} synthetic sea salt (LB_{12}). After 24 h of incubation at 28 °C, a single colony was inoculated into 5 ml LB_{12} broth and incubated overnight at 28 °C under constant agitation (100 min^{-1}). Bacterial density was measured spectrophotometrically at 600 nm.

2.2. Quorum sensing-disrupting compounds

Cinnamaldehyde (Sigma) was dissolved in distilled water at 1 mM and the brominated furanone (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone (Sigma) was dissolved in methanol at 100 μM. The brominated thiophenone (*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiopen-3-yl)methoxy)-4-oxobutanoic acid was synthesized as described before (Defoirdt et al., 2012) and dissolved in ethanol at 10 mM. All compounds were stored at −20 °C.

2.3. Preparation of axenic Artemia nauplii as feed for the prawn larvae.

Decapsulation and hatching of axenic *Artemia* nauplii for larval feeding were performed as described by Marques et al. (2004). 200 mg *Artemia* cysts (Ocean Nutrition Europe, Essen, Belgium) were hydrated in 18 ml distilled water for 1 h. Sterile cysts and nauplii were obtained via decapsulation using 660 μl NaOH (32%) and 10 ml NaOCl (50%). During the reaction, filtered aeration was provided. The decapsulation process was carried out under a laminar flow hood. The reaction was stopped after 2 min by adding 14 ml $Na_2S_2O_3$ (10 g l^{-1}). The decapsulated cysts were washed with fresh autoclaved brackish water (12 g l^{-1} synthetic sea salts) over a 100 μm sieve and transferred to two sterile falcone tubes, each containing 30 ml brackish water. The falcons with decapsulated cysts were incubated at 28 °C for 24 h on a rotor under constant light.

2.4. Giant freshwater prawn experiments

Giant freshwater prawn experiments were performed as described in Pande et al. (submitted for publication). Briefly, prawn broodstock maintenance was performed according to Cavalli et al. (2001) and water quality parameters were adjusted according to New (2003). The larvae were obtained from a single oviparous female breeder. A matured female which had just completed its pre-mating moult was mated with a hard-shelled male as described before (Baruah et al., 2009). The female with fertilized eggs was then maintained for 20 to 25 days to undergo embryonic development. When fully ripe (indicated by dark grey color of the eggs), the female was transferred to a hatching tank (30 l) containing slightly brackish water (containing 6 g l^{-1} Instant Ocean synthetic sea salt, Aquarium System Inc., Sarrebourg, France). The water temperature was maintained at 28 °C by a thermostat heater. After hatching, the newly hatched larvae with yolk were left for 24 h in the hatching tank. The next day, prawn larvae with absorbed yolk were distributed in groups of 25 larvae in 200 ml glass cones containing 100 ml fresh autoclaved brackish water (12 g l^{-1} synthetic sea salts). The glass cones were placed in a rectangular tank containing water maintained at 28 °C and were provided with aeration. The larvae were fed daily with 5 *Artemia* nauplii/larvae and acclimatized to the experimental conditions for 24 h.

During the experiments, water quality parameters were kept at minimum 5 mg l^{-1} dissolved oxygen, maximum 0.5 mg l^{-1} ammonium-N and maximum 0.05 mg l^{-1} nitrite-N. Prawn larvae were challenged with *Vibrio harveyi* by adding the strains at 10^6 CFU ml^{-1} to the culture water on the day after first feeding. The quorum sensing-disrupting compounds were also added to the culture water on the day after first feeding. Survival was counted daily in the treatment challenged to wild type *Vibrio harveyi* without addition of the quorum sensing-disrupting compounds and the challenge test was stopped when more than 50% mortality was achieved. At this time point, larval survival was determined in all treatments by considering that only those larvae presenting movement of appendages were alive. The larval stage index (LSI) was estimated according to Maddox and Manzi (1976) by randomly sampling 5 larvae from each and calculated as:

$$LSI = \Sigma S_i / N$$

S_i : stage of the larva $(i = 1 \text{ to } 12)$

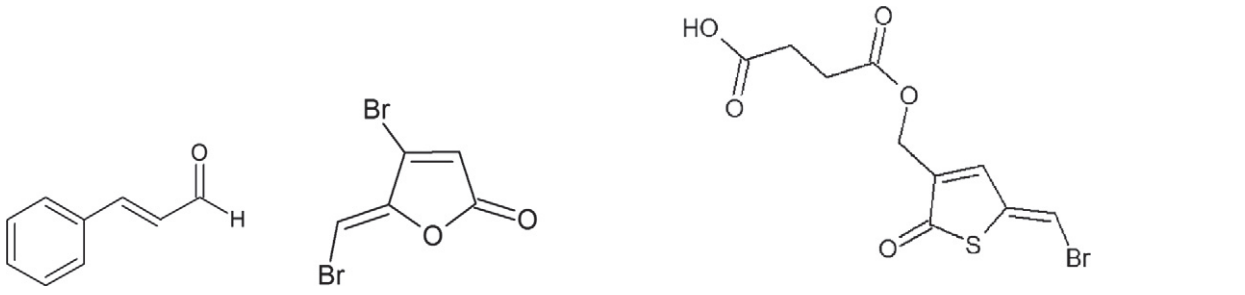
N : the number of larvae examined.

2.5. Statistical data analysis

Statistical analyses were performed using the SPSS software, version 20. The larval survival data were arcsin transformed in order to satisfy normal distribution and homoscedasticity requirements. Data were analysed by one-way ANOVA, followed by Tukey multiple range tests with a significance level set at 0.05.

3. Results and discussion

In this study we investigated the impact of the addition of three different quorum sensing-disrupting compounds, including cinnamaldehyde, the brominated furanone (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-



Cinnamaldehyde | (*Z*-)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone | (*Z*)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)methoxy)-4-oxobutanoic acid

Fig. 1. Structure of the quorum sensing-disrupting agents used in this study.