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QUORUM SENSING INHIBITION AS AN ALTERNATIVE METHOD TO PROTECT PRAWN LARVAE FROM BACTERIAL INFECTION

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Abstract

Aquaculture is the fastest-growing food-producing sector worldwide. One of the major aquaculture species is the giant freshwater prawn, Macrobrachium rosenbergii. However, diseases caused by various opportunistic pathogenic bacteria such as Vibrio spp. constitute a significant obstacle for the further expansion of M. rosenbergii cultivation. The wide and frequent use of antibiotics to control infections in aquaculture has resulted in the development and spread of antibiotic resistance. As this is gradually rendering antibiotic treatments ineffective, new strategies to control bacterial infections are needed for a sustainable further development of the aquaculture industry. Many bacterial aquaculture pathogens regulate their virulence gene expression through quorum sensing (QS) mechanism, the bacterial cell-to-cell communication, and consequently, quorum sensing inhibition has been suggested as a novel strategy to control infections. In this study, we performed enzymatic inactivation of quorum sensing signal molecules by application of QS signal molecule-degrading bacteria. The bacteria strain was isolated from the microalgae Chaetoceros muelleri culture. This isolate are able to degrade signal molecules i.e. acylhomoserine lactones (AHL) and protect M. rosenbergii larvae from disease caused by pathogenic Vibrio harveyi. Since the degradation of quorum sensing signal molecules has been shown to protect prawn larvae from bacterial infection, this method might be interesting novel biocontrol strategy for use in aquaculture.

Keyword: AHL-degrading bacteria, larviculture, vibriosis.

1 INTRODUCTION

Aquaculture comprises the farming of aquatic organisms, including fish, mollusks, crustaceans and aquatic plants. As the fastest growing food producing sector (Bostock et al., 2010), aquaculture plays an important role in the economic development worldwide, especially in many developing countries. One of the commercially important aquaculture species is the giant freshwater prawn Macrobrachium rosenbergii (de Man, 1879). Disease outbreaks are considered to be amongst the major obstacles to produce healthy and high quality seed of giant river prawn culture. Previous studies have shown that Vibrio spp., including Vibrio harveyi, are a major cause of diseases in the early life stages (larvae and postlarvae) of this species (Tonguthai, 1997).

The frequent use of antibiotics to control vibriosis in shrimp hatcheries has led to the development and spread of antibiotic-resistant bacteria (Karunasagar et al., 1994), 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 (Henke & Bassler, 2004; Defoirdt et al., 2008; Natrah et al., 2011). *V. harveyi* contains a three-channel quorum sensing system, with three different types of signal molecules (HAI-1, AI-2 and CAI-1, respectively) feeding a common signal transduction cascade (Ruwandeepika et al., 2012). We previously reported that *V. harveyi* quorum sensing regulates its virulence towards giant freshwater prawn larvae, in which the HAI-1 and the AI-2 mediated channels of the *V. harveyi* quorum sensing system are essential for full virulence to giant river prawn larvae (Pande et al., 2013).

The use of signal molecule-degrading bacteria is one of the most intensively studied strategies to interfere with quorum sensing (Pande et al., 2015). The ability to inactivate acylhomoserine lactones (AHLs), one of the types of quorum sensing molecules, is widely distributed in the bacterial kingdom (Dong et al., 2007). As *V. harveyi* HAI-1 is an AHL and as this signal is essential for full virulence towards giant river prawn larvae, the use of AHL-degrading bacteria might be an effective strategy to protect the larvae from the pathogen.

2 MATERIAL AND METHODS

2.1 Isolation of AHL-degrading bacteria from *Chaetoceros muelleri* culture

Open culture of microalgae *Chaetoceros muelleri* was grown in Guillard’s F/2 medium (with an addition of silicate) in sterile 250 ml Schott bottles provided with 0.22 μm filtered aeration. All culture parameters were kept constant (pH 7, continuous light of 100 μmol photons.m⁻².sec⁻¹, temperature of 24°C and 30 g.l⁻¹ salinity). The cell density was measured using a Bürker hemocytometer.

50 μl of the culture was transferred to sterile erlenmeyer flasks containing 5 ml of sterile NaCl solution (30 g.l⁻¹) containing 50 mg.l⁻¹ N-hexanoyl-L-homoserine lactone (HHL). The erlenmeyer flasks were covered with aluminium foil to prevent the growth of the microalgae and were incubated on a shaker (120 rpm) at 24°C. The isolation was performed in four consecutive cycles (72h for the first cycle and 48h for the second to fourth cycle). At the end of each cycle, 50 μl of the suspension was transferred to a new flask. At the end of the fourth cycle, the suspensions were spread-plated on Luria-Bertani agar containing 30 g.l⁻¹ NaCl (LB₃₀) and after 48h incubation at 24°C, colonies were picked, suspended in a 30 g.l⁻¹ NaCl solution and plated again. After 3 rounds of purification, isolates were grown in LB₃₀ broth for 24h at 24°C and grown culture were stored at -80°C in 40% glycerol. The isolate was used in further experiments.

2.2 Bacterial strains and culture conditions

*Vibrio harveyi* BB120 (Bassler et al., 1997), its mutant JAF548 (Freeman & Bassler, 1999) and the AHL-degrading isolate were stored at -80°C in 40% glycerol. The stocks were streaked onto LB agar and after 24 hours of incubation at 28°C, a single colony was picked and inoculated into 5 ml fresh LB broth and incubated overnight at 28°C under constant agitation (100 min⁻¹).

2.3 Quantification of N-hexanoyl-L-homoserine lactone (HHL)

A plate diffusion method was used for quantitative detection of HHL, using *Chromobacterium violaceum* CV026 as a reporter (Defoirdt et al., 2011). Briefly, CV026 was grown to an optical density of around 2 at 550 nm in buffered (2 g/l MOPS) LB
medium (pH 6.5) containing 20 mg.l\(^{-1}\) kanamycin and spread over buffered (pH 6.5) LB plates. Subsequently, 10 µl of sample solution was applied to the centre of the plates and the plates were incubated at 28°C for 24h and 48h. After the incubation, the zone of purple-pigmented CV026 was measured and the concentration of HHL in the sample was calculated based on a standard curve.

2.4 AHL degradation assay
AHL degradation by the isolate was studied as reported previously (Defoirdt et al., 2011). Briefly, the isolate was inoculated at 10\(^8\) CFU.ml\(^{-1}\) in buffered LB medium (pH 6.5) supplemented with 10 mg.l\(^{-1}\) HHL. At regular time intervals, 1 ml samples from each culture were taken and filtered over a 0.2 µm filter. The HHL concentration in the cell-free supernatants was determined as described above using C. violaceum CV026.

2.5 Giant freshwater prawn larvae challenge test
Giant freshwater prawn challenge tests were performed as described in Pande et al. (2013). Briefly, larvae were obtained from a single oviparous female breeder. A matured female which had just completed its pre-mating molt was mated with a hard-shelled male. 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 (6 g.l\(^{-1}\) salinity). The water temperature was maintained at 28°C by a thermostat heater. After hatching, the newly hatched larvae with yolk were left for 24 hours 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\(^{-1}\) salinity). The glass cones were placed in a rectangular tank containing water maintained at 28°C and was provided with aeration. The larvae were fed daily with 5 Artemia nauplii/larva and acclimatized to the experimental conditions for 24 hours.

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. Larvae were challenged by adding 10\(^6\) CFU.ml\(^{-1}\) of V. harveyi BB120 to the rearing water on the day after first feeding. The AHL-degrader isolate was added at 10\(^5\) CFU.ml\(^{-1}\). Survival was counted daily in the treatment receiving V. harveyi BB120 only, 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 determined according to Maddox and Manzi (1976) by randomly sampling 5 larvae from each treatment and calculated as:

\[ LSI = \Sigma \frac{Si}{N} \]

Si : stage of the larva (i = 1 to 12)
N : the number of larvae examined.

2.6 Statistical data analysis
Statistical analyses were performed using the SPSS software, version 20. Giant freshwater prawn survival data were arcsin transformed in order to satisfy normal distribution and homoscedasticity requirements. Data were analyzed by one way ANOVA, followed by Tukey multiple range tests with a significance level set at 0.05.
3 RESULTS

3.1 Isolation of AHL-degrading bacteria from Chaetoceros muelleri culture
AHL-degrading strain was isolated from open culture of Chaetoceros muelleri by sequentially culturing in a medium containing N-hexanoyl-L-homoserine lactone (HHL) as the sole carbon and nitrogen source. The isolate was inoculated at $10^8$ CFU.ml$^{-1}$ in buffered LB medium supplemented with 10 mg.l$^{-1}$ HHL in order to determine whether they were able to degrade AHL. The isolate was able to degrade HHL with degradation rate of 0.75 mg.l$^{-1}$.h$^{-1}$ (Fig. 1).

![Figure 1. N-hexanoyl-L-homoserine lactone (HHL) degradation by the isolate.](image)

3.2 Impact of the isolate on AHL quorum sensing in Vibrio harveyi
Because bioluminescence is one of the phenotypes that are regulated by quorum sensing in V. harveyi, we used bioluminescence as a read-out of quorum sensing activity and determined the impact of the isolates on bioluminescence of wild type V. harveyi BB120. In order to compensate for the competition for nutrients, we mixed BB120 with its mutant JAF548 as a control. JAF548 has a completely inactive quorum sensing system (and therefore is not luminescent; Freeman & Bassler, 1999). The results revealed that the isolate decreased quorum sensing-regulated luminescence of V. harveyi in co-culture (Fig. 2). Importantly, the isolates did not affect the growth of V. harveyi in co-culture (Table 1).
Figure 2. Quorum sensing-regulated bioluminescence of V. harveyi BB120 in coculture with the AHL-degrader isolate. A co-culture with the dark mutant of BB120, JAF548, served as control. Error bars represent the standard deviation of 6 replicates.

Table 1. Impact of the isolates on cell density of V. harveyi BB120 after 12 hours of coculture as determined by plate counting of luminescent cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell density (x 10^8 CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB120 + JAF548</td>
<td>4.7 ± 0.5^a</td>
</tr>
<tr>
<td>BB120 + Isolate</td>
<td>4.6 ± 0.2^a</td>
</tr>
</tbody>
</table>

Values in the same column with different superscript letters are significantly different (p<0.05).

3.3 Impact of the AHL-degrader strain on the survival and growth of giant freshwater prawn larvae challenged with Vibrio harveyi

The previous research showed that AHL quorum sensing is essential for full virulence of V. harveyi towards giant river prawn larvae (Pande et al., 2013). Hence, since the isolate was able to interfere with quorum sensing in V. harveyi, we went further to investigate whether this isolate was able to protect giant river prawn larvae from the pathogen. Addition of the isolate to the rearing water indeed resulted in a significantly improved survival of challenged prawn larvae (Table 2). Consistent with our previous work, we found no difference in growth (as assessed by determining the larval stage index) between the different treatments.

Table 2. Survival and growth (as expressed by the larval stage index -LSI) of giant freshwater prawn larvae after 6 days of challenge with V. harveyi BB120 (average ± standard deviation of 5 replicates). “Control” refers to unchallenged larvae that were otherwise treated in the same way as the other larvae.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival (%)</th>
<th>LSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>83 ± 7^c</td>
<td>4.4 ± 0.5^a</td>
</tr>
<tr>
<td>BB120</td>
<td>42 ± 8^a</td>
<td>4.4 ± 0.5^a</td>
</tr>
<tr>
<td>BB120 + Isolate</td>
<td>67 ± 5^b</td>
<td>4.6 ± 0.5^a</td>
</tr>
</tbody>
</table>

Values in the same column with different superscript letters are significantly different (p<0.05).
4 DISCUSSION

This study focused on quorum sensing disruption by acyl-homoserine lactone (AHL)-degrading bacteria isolated from microalgae culture. Microalgae are an important constituent in many aquaculture systems, especially in the so-called green-water systems, which are characterised by high levels of microalgae in the rearing water (Hargreaves, 2006). These green-water systems are currently used to culture various aquaculture animals and empirical evidence points to a better growth and survival in these systems when compared to the conventional clear water aquaculture (Muller-Feuga, 2000). However, the mechanisms by which green-water exert a beneficial effect are still poorly understood, especially the potential beneficial effects of bacteria associated with micro-algae remain largely unexplored (Natrah et al., 2013).

The AHL-degrader strain was isolated from open culture of Chaetoceros muelleri. The isolate showed HHL degradation rates that were similar to those of Bacillus sp. strains isolated from the intestinal tract of shrimp and fish (0.7-0.9 mg.l⁻¹.h⁻¹) (Defoirdt et al., 2011). HHL was used as test compound because it is relevant to aquaculture. Indeed, it is produced by pathogenic bacteria such as Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda and Vibrio salmonicida (Defoirdt et al., 2011). The AHL molecules produced by different bacteria all shares the homoserine lactone moiety, but they differ in the length and substitution of the acyl side chain (Dong & Zhang, 2005). The structural features of AHLs (Fig. 3) suggest that there may be at least four types of enzymes that could degrade AHL signals. Among them, lactonases and decarboxylases hydrolyze the lactone ring at the positions marked as 1 and 2, while acylases and deaminases separate the homoserine lactone moiety and acyl side chain at sites 3 and 4, respectively (Dong & Zhang, 2005).

**Figure 3. AHL structure and its potential cleavage sites by AHL inactivating enzymes**

To date, only two groups of AHL-degrading enzymes have been demonstrated enzymatically and structurally to inactivate AHL substrates, i.e., AHL lactonases and AHL acylases (Dong & Zhang, 2005). Several Bacillus species have been reported to produce AHL lactonases, which inactivate AHLs by hydrolysing the lactone ring (Dong & Zhang, 2005). Lactonases are intracellular enzymes capable of inactivating a wide range of AHLs, varying in acyl chain length and substitution (Dong et al., 2007). Meanwhile, Pseudomonas species have been reported to produce AHL acylases, which cleave AHLs by aminohydrolysis into homoserine lactone and fatty acid (Dong & Zhang, 2005).

Our *in vivo* challenge test revealed that the isolate significantly increased the survival of giant freshwater prawn larvae challenged with *V. harveyi*, whereas the isolate had no effect on larval growth. This is consistent with our previous work showing that AHL quorum sensing is essential for full virulence of *V. harveyi* towards giant freshwater prawn larvae (Pande et al. 2013) and previous reports documenting that AHL degraders are able to improve the survival of prawn larvae (Cam et al. 2009) and turbot larvae (*Scophthalmus*...
maximus L.) (Tinh et al. 2008) in the presence of exogenous AHL (which caused mortality in both species; probably by triggering pathogenicity mechanisms in pathogenic bacteria that were naturally present in the cultures). Hence, the use AHL-degrader bacteria molecules might be an interesting new type of probiotics for aquaculture with a defined mode of action. Along this line, AHL-degrading Bacillus sp. have been shown to inhibit the protease production, hemolytic activity and biofilm formation of A. hydrophila strain YJ-1, and to significantly improve the survival of zebrafish (*Danio rerio*) challenged with this pathogen (Chu et al. 2014). Further research is needed to reveal the efficacy of the isolate in different aquaculture host–pathogen systems.

REFERENCES


