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Pseudoalteromonas probiotics as potential biocontrol agents improve the survival of *Penaeus vannamei* challenged with acute hepatopancreatic necrosis disease (AHPND)-causing *Vibrio parahaemolyticus*

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ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) has caused a severe decline of global shrimp production and economic losses. One of the causative agents for AHPND is *Vibrio parahaemolyticus* (Vp_{AHPND}), bearing toxin genes $pirAB^{vp}$. In this study, potential probiotic bacteria CDM8 and CDA22, isolated from the hindgut of healthy *Penaeus vannamei*, were rod-shaped and motile with a single polar flagellum, and they can form yellow colonies, produce oxidase and hydrolyze gelatin. The possibility of their application against Vp_{AHPND} was evaluated. Both strains were found to display antagonistic activity against $Vp_{AHPND,}$ which could be inhibited by catalase, and they were identified as *Pseudoalteromonas* spp. based on phylogenetic analysis. These two bacteria produced extracellular antibacterial compounds, according to the disc diffusion assay. Furthermore, CDM8 or CDA22 as a feed additive could (i) significantly decrease the presumptive *Vibrio* counts in the hindgut of shrimp (*P. vannamei*) after being fed for 21 days, compared to that in shrimp fed with the mixture of CDM8 and CDA22 and the commercial feed, and (ii) strikingly reduce the cumulative mortality (vs the control group: 36.7% or 76.7% vs 96.7%) of shrimp when challenged with Vp_{AHPND}. In addition, they could decrease copy numbers of $pirA^{vp}$ gene in shrimp, according to the quantitative PCR results. However, there is no synergistic relationship between CDM8 and CDA22 when used together as feed additives. All these results suggested that bacteria CDM8 and CDA22 were anti-Vp_{AHPND} probiotic candidates and potential biological control agents against AHPND in shrimp aquaculture.

1. Introduction

Penaeus vannamei is one of the most important commercial species of shrimp in the world, and the production of aquacultured *P. vannamei* accounts for > 60% that of all farm-raised shrimp (http://www.cport.net/product/view/white-shrimp). Viral and bacterial diseases are major factors in hindering the sustainable development of shrimp farming and are among the cases of market volatility. Acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome (EMS), is one of the emerging shrimp diseases, which was first reported in China and successively occurred in other Asian and Latin American countries including Vietnam (2010), Malaysia (2011), Thailand (2012), Mexico (2013), and Philippines (2015) (de la Pena et al., 2015; Hong et al., 2016). This disease has decreased shrimp production and caused serious economic losses in these affected countries.

AHPND is characterized by severe atrophy of shrimp hepatopancreas (HP) which exhibits a unique histopathology at the acute stage, consisting of massive sloughing of HP epithelial cells in the absence of bacterial or other pathogens (Joshi et al., 2014). One causative agent for AHPND is *Vibrio parahaemolyticus* (Vp_{AHPND}), harboring toxin genes named $pirA^{vp}$ and $pirB^{vp}$ (Tran et al., 2013; Lee et al., 2015). Latest studies show that Vp_{AHPND} can infect and be detected in various tissues of shrimp, such as hepatopancreas, stomachs, intestines and gills (Chen et al., 2017; Khimmakthong and Sukkarun, 2017).

As antibiotics are considered the standard treatment to prevent and control diseases, they have frequently been applied as a therapy for AHPND/EMS. Tetracycline is one of the most popular antibiotics (Neela et al., 2007). However, the imprudent use of antibiotics likely contributes to the development of antibiotic-resistant pathogens and also to

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the dissemination of resistance determinants and antibiotic contamination (Cabello, 2006; Thuy et al., 2011). According to a previous report, Vp_{AHPND} strains isolated from Mexico are resistant to oxytetracycline and tetracycline and exhibit a high level of resistance to tetracycline (Han et al., 2015a). In recent years, beneficial bacteria called probiotics are extensively applied in shrimp farming, which are environment-friendly biological control agents and used as an alternative to antibiotics to prevent diseases (Leyvamadrigal et al., 2011; Sapcharoen and Rengpipat, 2013; Pham et al., 2014). Probiotics can participant in the inhibitory activity against pathogens, establishing a balance of gastrointestinal microbial flora, improving digestive functions or immune system responses of marine animals, and improvement of water quality (Verschuere et al., 2000; Balcázar et al., 2006; Nimrat et al., 2012). Creation of a hostile environment for pathogens by production of inhibitory compounds (e.g., bacteriocins and hydrogen peroxide) was one of the mechanisms of action of probiotics (Loh, 2017).

The present study was aimed at identifying the candidate bacteria from hindgut of healthy *P. vannamei* and assessing their probiotic effects on shrimp against Vp_{AHPND}.

2. Materials and methods

2.1. Bacterial isolation and screening

Shrimp were washed with 75% ethanol before sampling the hindguts with sterile tweezers. Hindgut samples were homogenized using tissue grinders and vortexed in sterile saline solution (0.85% (w/v) NaCl). A tenfold dilution series of homogenates was prepared and plated on marine 2216 agar (1.5% (w/v), MA, pH 8.0) plates. The plates were incubated at 28 °C for 24 h. After primary isolation and purification on MA, the strains were cultured in the same medium or in marine 2216 broth (MB) and stored as glycerol suspensions (20%, v/v) at −80 °C.

2.2. Bacterial antagonistic activity against Vp_{AHPND}

The bacterial isolates were tested for antagonistic activity against a Vp_{AHPND} strain 20130629002S01, which was isolated by our lab in 2013 from diseased *P. vannamei* originated from Guangxi, China, using the agar-well diffusion method as described by Zhang et al. (2017) with minor modifications. One hundred microliters of overnight culture (at the concentration of ~10^6 CFU/ml) of the Vp_{AHPND} strain at 28 °C were plated on MA plates, followed by adding sterile filter paper disks impregnated with 5 μl of the extracellular supernatants (ECSs) or the overnight culture of the bacteria isolated from shrimp. After incubation of the MA plates at 28 °C for 24 h, the diameters of inhibitory zones were measured with a vernier caliper. The disks impregnated with 5 μl of sterile PBS (0.01 M, pH 7.2) were used as the control.

2.3. Identification and characterization of the potential probiotics

The 16S rRNA genes of candidate probiotic bacteria were amplified and sequenced as described previously (Labreuche et al., 2010). The sequences were compared against the database contained in National Center for Biotechnology Information using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) and to calculate the sequence similarities (Pham et al., 2014). After multiple sequence alignment, phylogenetic analysis was performed using MEGA 5 to determine bacterial taxonomic position. The related sequences were obtained from GenBank.

Gram reaction was carried out using a Gram Staining Kit (Luqiao, Beijing, China) according to the instruction of the manufacturer. Cell morphology was examined with a transmission electron microscopy (TEM). For TEM, bacteria were cultured on MA at 28 °C for 2 days, and cells were negatively stained with 1% (w/v) phosphotungstic acid. Bacterial biochemical tests were performed with the API 20NE system (bioMérieux). Bacterial cells were collected (at 5000 × *g* for 10 min) from the overnight culture of CDM8 and CDA22 and suspended in PBS. Fifteen microliters of the suspensions of CDM8 and CDA22 (at the concentration of 1×10^9 CFU/ml), using PBS as the control, added onto blood agar plates (Haibo, Qingdao, China), which were incubated for 36 h at 28 °C to determine their hemolytic activities.

2.4. Effect of catalase on bacterial antagonistic activity

Extracellular products (ECPs) of the potential probiotic bacteria were prepared using unplasticized cellophane as described by Gauthier and Flatau (1976) with some modifications. Briefly, bacterial overnight broth culture (100 μl) was spread onto the surface of the cellophane on a MA plate. After incubation at 28 °C for 12 h, the lawn was scraped off and suspended in sterile PBS (0.01 M, pH 7.2). The cells were removed by centrifugation at 12000 × *g* at 4 °C for 30 min, and the supernatant was filtered through a 0.22 μm porosity filter before further use. Filter paper disks impregnated with varied concentrations of catalase (i.e., 0.1 mg/ml, 0.2 mg/ml, 0.5 mg/ml) were added onto MA plates to assess the inhibition effect on antagonistic activity of bacterial ECSs against Vp_{AHPND}.

2.5. SDS-PAGE and in-gel antibacterial assay

The in-gel antibacterial activity of potential probiotic bacteria was assessed according to the method previously described (Yu et al., 2013). The total proteins in ECPs were quantified by using a Pierce BCA Protein Assay Kit (Thermo, USA). ECPs were mixed (4:1) with sample buffer (0.25 M Tris/HCl, pH 6.8, 10% SDS, 5% β-mercaptoethanol, 50% glycerol and 0.5% bromophenol blue). The mixture was loaded into the gel with or without heating and separated by SDS-PAGE. Duplicate lanes loaded with the identical mixture were treated separately after gel electrophoresis. One slice was stained with Coomassie brilliant blue. The other slice was washed three times in distilled water and then placed on a plate. The overnight broth culture of Vp_{AHPND} was inoculated into semi-solid MA and spread over the gel surface in order to examine antibacterial activity of proteins. The plate was incubated at 28 °C for 24 h to observe the inhibition zone. The Coomassie brilliant blue stained bands corresponding to the antibacterial protein were identified using MALDI-TOF/TOF mass spectrometry in Fudan University (Shanghai, China).

2.6. Experimental shrimp

Shrimp were purchased from a company in Qingdao, China, and acclimated indoors in 40-liter plastic tanks for 7 days. The rearing conditions were as follows: water temperature 28 ± 2 °C, salinity 34 ± 2 g/l, pH 7.8 ± 0.5. There were no health issues during the acclimation period.

2.7. Probiotic supplementation

Two probiotic candidates CDM8 and CDA22 were cultured in MB at 28 °C for 24 h. Bacterial cells suspensions were harvested by centrifugation at 8000 × *g* at 4 °C for 10 min (Boonthai et al., 2011; Wu et al., 2014).

Shrimp were randomly distributed into four treatment groups (i.e., Groups A-D). Each group contained four parallels, and every parallel contained 35 shrimp. Shrimp in the four groups were fed with specific diets as follows: Group A, diet supplemented with CDM8 dosed at 10^7 CFU/kg; Group B, diet supplemented with CDA22 dosed at 10^7 CFU/kg; Group C, diet supplemented with the mixture of CDM8 and CDA22 (at a ratio of 1:1) dosed at 10^7 CFU/kg; Group D, commercial feed. In the 28-day feeding trial, shrimp in every group were fed three times (i.e., at 08:00, 12:00, and 18:00) a day with the diet of 5% of body