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Beneficial effects of four *Bacillus* strains on the larval cultivation of *Litopenaeus vannamei*

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ABSTRACT

We studied the effects of four strains of *Bacillus* isolated from the guts of healthy wild adult shrimp on survival and rate of development of whiteleg shrimp *Litopenaeus vannamei* larvae to understand how endemic *Bacillus* probiotic strains improve the health of larvae. This included bacterial isolation, assays of hemolytic activity, antagonism against pathogenic *Vibrio* species, growth, and adhesion to shrimp intestinal mucus, and molecular identification. *Bacillus* strains were tested on larval shrimp using a daily concentration of 1×10^5 CFU mL^{-1}, starting each bioassay at nauplii V at a density of 225 nauplii L^{-1}. Inoculations of four natural, commercial products and antibiotic oxytetracycline were added directly to the water. All treatments induced a significant increase in survival compared to the control, with strain YC5-2 showing the highest survival (~67%), followed by Alibio^{MR} (~57%). A mix of two strains induced the highest rate of development (7.00), followed by Alibio^{MR} (6.35). The results showed remarkable antagonistic activity by the four non-hemolytic *Bacillus* strains against *Vibrio campbelli*, *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus* and the potential and efficiency of probiotics isolated from shrimp gut to improve survival of shrimp larvae.

1. Introduction

Intensive shrimp culture practices involve an indiscriminate use of chemicals and antibiotics to prevent mortality of larvae and juvenile marine species (Peraza-Gómez et al., 2009). Probiotic organisms are an alternative to antibiotics because antibiotics have led to resistance of bacterial pathogens (Gatesoupe, 1999). Previous applications of probiotic organisms have shown beneficial host effects, including improved growth, survival, and health (Moriarty, 1998; Skjermo and Vadstein, 1999). Various mechanisms have been proposed to explain their beneficial effects, including competition for adhesion sites, competition for nutrients, enzymatic contribution to digestion, improved water quality, and stimulation of the host immune response (Kumar-Sahu et al., 2008). Selection of probiotics in aquacultural enterprises is usually based on results of tests showing antagonism toward the pathogens, an ability to survive and colonize the intestine, and a capacity to increase an immune response in the host.

Previous studies show that inoculation with a probiotic strain during cultivation of whiteleg shrimp *Litopenaeus vannamei* larvae (nauplii stage V) prevents colonization by a pathogenic strain, because the probiotic succeeds in colonizing the gut of the larvae (Gómez-Gil et al., 2000; Zherdmant et al., 1997). Nevertheless, studies of probiotics to improve growth or survival in crustacean larvae are scarce. Recently, methods for improving water quality of hatcheries and application of probiotics has gained momentum (Balcázar et al., 2007a; Gómez et al., 2008; Guo et al., 2009; Van Hai et al., 2009). Daily administration of probiotics based on *Bacillus* spp. during hatchery and farming stages leads to higher feed conversion ratios, improved specific growth rates, and higher final shrimp biomass than controls (Guo et al., 2009; Liu et al., 2009a). Metamorphosis improved with administration of the probiotic *B. fusiformis* (Guo, et al., 2009). Zhou et al. (2009) found that *B. coagulans* SC8168, as a water additive at certain concentrations, significantly increased survival and some digestive enzyme activities of shrimp larvae. *Bacillus* spp. possesses adhesion abilities, produce bacteriocins, and provide immunostimulation (Ravi et al., 2007).

Nguyen et al. (2007) suggest that the beneficial effect of the probiotics on the host has been wrongly attributed to what is found during in vitro observations, that in vivo physiology might be different from in vitro metabolic processes. Development of suitable probiotics is not a simple task and requires full-scale trials, as well as development of appropriate monitoring tools and controlled production (Decamp et al., 2008). In vitro and in vivo studies are needed to demonstrate antagonisms to pathogens and their effect on survival and growth of the host.

Bacillus spp. is often used as benefic microorganisms in aquaculture systems (Nakayama et al., 2009). However, the mechanism of

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action is not completely understood. In this study, the effect of probiotics isolated from *L. vannamei*, two commercial probiotics, and a commercial antibiotic were evaluated on survival and development of shrimp larvae. Additionally, the capacity to adhere and grow on the intestinal mucosa were studied to understand how endemic probiotics improve health in larvae.

2. Materials and methods

2.1. Bacterial isolates

Bacterial strains were isolated from the intestinal tract of adult shrimp. The contents of the foregut (stomodeum) were aseptically removed from live, healthy shrimp. The samples were homogenized and serially diluted, plated on marine agar (#2216, Difco; Becton, Dickenson and Company, Franklin Lakes, NJ) and incubated at 37 °C for 24 − 48 h. Colonies of single, dominant types were selected and re-streaked onto marine agar to obtain pure cultures after incubation for 24 h at 37 °C; 20 colonies from the intestine were also characterized and selected if determined to be rod-shaped and Gram-positive. Of these, nine strains were selected and stored at − 85 °C in tryptic soy broth (TSB) containing 2.5% (w/v) NaCl and 15% (v/v) glycerol until used.

2.2. Hemolytic activity of Bacillus strains

To measure hemolytic activity of the various *Bacillus* strains on erythrocytes, nine isolated *Bacillus* probiotic strains were inoculated by streaking on plates containing blood-based agar (#211728, Difco) supplemented with 5% (w/v) human sterile blood and 3% (w/v) NaCl. Plates were incubated at 37 °C for 24 h and results were determined, as described by Koneman et al. (2001), as: α-hemolysis (slight destruction of hemocytes and erythrocytes with a green zone around the bacterial colonies); β-hemolysis (hemolysin that causes a clean hemolysis zone around the bacterial colonies); and γ-hemolysis (without any change in the agar around the bacterial colonies.

Hemolytic activity in shrimp hemocytes was tested, as described by Chin-I et al. (2000). Briefly, a 1-mL syringe was rinsed with EDTA buffer (450 mmol L^{-1} NaCl, 10 mmol L^{-1} KCl, 10 mmol L^{-1} EDTA-Na_2, and 10 mmol L^{-1} HEPES at pH 7.3). After disinfecting the surface of the shrimp weighing ~20 g with 70% ethanol; hemolymph was drawn with a sterile needle from between the fifth pair of pereiopods; 1 mL hemolymph was immediately transferred to a sterilized tube containing 0.2 mL EDTA buffer and stained with 133 μL 3% (w/v) Rose Bengal dye (#R4507, Sigma St. Louis, MO) dissolved in EDTA buffer with gentle shaking to achieve complete mixing. Aseptically, 1 mL of the stained hemolymph preparation was added to 15 mL sterile basal agar medium containing (10 g L^{-1} Bacto peptone (#211677, Difco), 5 g L^{-1} HCl, and 15 g L^{-1} Bacto agar (#214050, Difco) at pH 6.8) cooled to 45–50 °C, followed by gentle mixing and poured into Petri dishes. Shrimp blood agar plates with a rose red color were considered satisfactory because of the homogenously distributed stained hemocytes. When the hemocytes were destroyed by hemolytic bacteria, a clear zone (>4 mm) appeared around the colonies.

2.3. Pathogenic strains

Pathogenic bacterial strains *Vibrio harveyi* CAIM 1793, *V. parahaemolyticus* CAIM 170, *V. campbelli* CAIM 333, *V. alginolyticus* CAIM 57, and *V. vulnificus* CAIM 157 were obtained from the Colección de Microorganismos de Importancia (CAIM, www.ciad.mx/caim). Strains were maintained in trypticase soy broth (#236950, Difco) containing 3% (w/v) NaCl and 15% (v/v) of glycerol at − 80 °C until used.

2.4. Antagonism test

The nine potential probiotic strains and five pathogenic strains were thawed in an ice bath. Each of the bacterial isolates was grown in 10 mL TSB at 30 °C for 24 h. Each sample was centrifuged at 5000 *g* for 10 min; each pellet was suspended in a sterile saline solution containing 3% (w/v) NaCl. Density of bacteria was measured by spectrophotometry (DU 640, Beckman Coulter, Brea, CA) at 600 nm. The optical density was adjusted to 1.0 nm to obtain a final density of 1×10^9 cells mL^{-1}; this inoculum was serially diluted to a density of 1×10^6 cells mL^{-1} for in vitro antagonist tests, according to Dopazo et al. (1988). For this test, 10 μL of each suspension of bacteria were blotted on the surface of trypticase soy agar (TSA) + NaCl medium (TSA containing 3% (w/v) NaCl (S-7653, Sigma) and then incubated for 24 h at 37 °C. The plates were then placed in a closed chamber and exposed to chloroform vapors for 45 min to kill the bacteria. Each plate was covered with 6 ml TSA + NaCl medium containing a 0.1 mL suspension of either *V. harveyi* CAIM 1793, *V. parahaemolyticus* CAIM 170, *V. campbelli* CAIM 333, *V. alginolyticus* CAIM 57, or *V. vulnificus* CAIM 157. Plates were examined after incubation at 30 °C for 24 h. Strains showing a >5 mm diameter inhibition halo were considered positive for the test, strains producing smaller inhibition zones were considered as sensitive without total inhibition.

An additional inhibition test was performed (Balcázar et al., 2007a), where the pathogenic strains of bacteria were inoculated in trypticase soy agar (TSA) supplemented with 2.5% (w/v) NaCl and placed in Petri dishes. Wells of 3 mm were made on solidified agar; wells were then filled with 10 μL of overnight bacterial culture. Trypticase soy broth (TSB) containing 2% (w/v) NaCl, was added as the negative control. The plates were incubated at 30 °C and zones of inhibition around the wells were measured after 24–48 h.

2.5. Adhesiveness

2.5.1. Preparation of mucin and shrimp mucus

Partially purified mucin type III derived from pig stomach (M1778, Sigma) was used, hereafter called gastric mucin. Crude mucus from the intestine of adult shrimp was obtained by gentle scraping and suspending it in cold HEPES (N-[2-hydroxyethyl]piperazine-N-[2-ethane-sulfonic acid]) plus Hank's balanced salt solution H-H Buffer (136.89 mmol L^{-1} NaCl, 5.37 mmol L^{-1} KCl, 1.26 mmol L^{-1} $CaCl_2\cdot2H_2O$, 0.81 mmol L^{-1} $MgSO_4\bullet7H_2O$, 0.35 mmol L^{-1} Na_2HPO_4, 2.57 mmol L^{-1} KH_2PO_4, and 9.98 mmol L^{-1} HEPES at pH 7.4). Shrimp mucus and porcine gastric mucin were conjugated with horseradish peroxidase (P8375, Sigma), as described by Hudson and Hay (1989), and stored at − 20 °C.

2.5.2. Adhesion assay of shrimp mucus and porcine gastric mucin

The seven isolated strains were grown in LDM medium, described in Conway and Henriksson (1989). The adhesion assay was described by Rojas and Conway (2001). Bacteria cells were harvested, washed, and suspended in H-H buffer and adjusted to 0.9 − 1.0 OD at 595 nm; 10 μL of the bacterial suspensions were immobilized in Inmobilon-P polyvinylidene difluoride membranes (P-15552, EMD Millipore, Billerica, MA) and a 3% bovine serum albumin (BSA) solution (AP-4500-80, SeraCare Life Sciences, Milford, MA) was used as a blocking agent at non-specific adhesion sites. The membranes were washed three times for 10 min with 10 mL H-H buffer and then incubated in 100 μL HRP-mucus or HRP-mucin (1:1000 solution) in 10 mL H-H buffer at room temperature for 2 h. Membranes were washed three times in H-H buffer (20 min each wash) and rinsed with 0.1 M sodium acetate buffer at pH 5.0 prior to development with the substrate diaminobenzidine (2.5 mg diaminobenzidine, 2.5 μL 30% hydrogen peroxide solution, and 10 mL 0.1 M sodium acetate at pH 5.0). The reaction was stopped after 5 min by rinsing the membrane in 0.1 M sodium metabisulfite.