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Enhancement of the immune response and protection against *Vibrio parahaemolyticus* by indigenous probiotic *Bacillus* strains in mud crab (*Scylla paramamosain*)

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ABSTRACT

In a previous study, bacterial communities of the intestine in three populations of crabs (wild crabs, pond-raised healthy crabs and diseased crabs) were probed by culture-independent methods. In this study, we examined the intestinal communities of the crabs by bacterial cultivation with a variety of media. A total of 135 bacterial strains were isolated from three populations of mud crabs. The strains were screened for antagonistic activity against *Vibrio parahaemolyticus* using an agar spot assay. Antagonistic strains were then identified by 16S rRNA gene sequence analysis. Three strains (*Bacillus subtilis* DCU, *Bacillus pumilus* BP, *Bacillus cereus* HL7) with the strongest antagonistic activity were further evaluated for their probiotic characteristics. The results showed that two (BP and DCU) of them were able to survive low pH and high bile concentrations, showed good adherence characteristics and a broad spectrum of antibiotic resistance. The probiotic effects were then tested by feeding juvenile mud crabs (*Scylla paramamosain*) with foods supplemented with 10^5 CFU/g of BP or DCU for 30 days before being subjected to an immersion challenge with *V. parahaemolyticus* for 48 h. The treated crabs showed significantly higher expression levels of immune related genes (CAT, proPO and SOD) and activities of respiratory burst than that in controlled groups. Crabs treated with BP and DCU supplemented diets exhibited survival rates of 76.67% and 78.33%, respectively, whereas survival rate was 54.88% in crabs not treated with the probiotics. The data showed that indigenous mud-associated microbiota, such as DCU and BP, have potential application in controlling pathogenic *Vibriosis* in mud crab aquaculture.

1. Introduction

Mud crab *Scylla paramamosain*, is one of the most important commercial maricultures in the southeast China. However, frequent outbreaks of aquatic diseases extremely discourage the development of a sustainable aquaculture of mud crabs [1]. For example, epizootic “milky disease” (MD) breaks out mainly in the fall when mud crab is near maturity, resulting in large economic losses in crab farming in southern China. Previous researches show that *Vibrio parahaemolyticus* is one of the most importantly pathogenic bacteria of MD [2,3]. Development of MD is a very rapid process in crab farms and could potentially cause up to 60% of mortality. Various efforts have been performed to overcome MD infection, enhancing the immune system through immunostimulant administration is currently believed to the most effective method [4].

Probiotics are live microbial or cultured product feed supplements that have beneficial effects on the health of the host when consumed in adequate doses [5]. Many beneficial effects have been attributed to probiotics, including the ability to produce adhesins, inhibitory substances like bacteriocins, antibacterial substances and siderophores, compete with pathogens for chemicals and energy, boost the immune response and improve the microbial balance [6]. The last decade has seen a growing interest in the application of probiotics through the use of beneficial microorganisms to prevent pathogenic microorganisms and reduce the incidence of diseases in fish [7–10], shrimp [11–14] and other aquatic animals [15–18]. A wide range of microalgae, yeasts, and

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gram-positive and -negative bacteria have been applied as probiotics to improve aquatic animal growth, survival, health, and disease prevention [19,20].

The gram-positive genus *Bacillus* is a widely used probiotic in aquaculture, as well as in human use [21,22]. Two probiotics, dominant gut *Bacillus* strains, with antagonistic activity have been reported to improve the growth and health of groupers, *Epinephelus coioides* [23]. In another similar study, the growth performance, immune response and disease resistance of *E. coioides* are enhanced after being treated with probiotic, *Bacillus subtilis* E20 [8]. *Bacillus* spores have been used as biocontrol agents to reduce *vibrios* and promote the growth through enhancing digestive enzyme activities and food absorption in shrimp culture facilities [24,25]. *Bacillus* spp. is often antagonistic and resistant against other micro-organisms, including fish and shellfish pathogenic bacteria [25,26].

In a previous study, bacterial communities of the intestine in three populations of crabs (wild crabs, pond-raised healthy crabs and diseased crabs) were probed by culture-independent methods and substantial diversities had been found [3]. Although much has been done to investigate the intestinal microbial composition and probiotics exploitation in shrimp [11–14], little is known for the culturable bacterial composition and probiotic application of the intestine of mud crabs.

Like shrimp, mud crab immune system is highly dependent on the innate mechanisms and incapable of producing antibody protection for disease prevention [27]. Therefore, other alternatives such as probiotic bacteria should be examined in order to control the infectious diseases in crab aquaculture [26,28,29]. The objective of this study was to identify and characterize the properties of microbiota in mud crab *S. paramamosain* against pathogenic *V. parahaemolyticus* and to determine their potential use as probiotics in crab farms. The results showed that crabs supplemented with two *Bacillus* strains, BP and DCU, exhibited significantly higher expression levels of immune related genes and survival rates after challenge with *V. parahaemolyticus* than that in the crabs not treated with the probiotics, indicating that indigenous intestine-associated microbiota of mud crab have potential as novel probiotics in *Viobiosis* disease prevention in crab farms.

2. Materials and methods

2.1. Microbial isolation

A total of 60 mud crabs (20 wild mud crabs, 20 pond-raised healthy mud crabs and 20 diseased mud crabs) weighing 80 ± 5 g were collected from locations described previously [3]. Three individual mud crabs of each group were euthanized, and the whole intestinal tracts were immediately removed with a sterile tweezer and clamped to prevent loss of samples, which were stored at 4 °C before further manipulations. The samples were homogenized and serially diluted, plated on LB, 2116E and TCBS plates, and incubated at 22 °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 22 °C. The isolates with different phenotypes were chosen for 16S rDNA sequencing.

2.2. Genotypic identification

Following DNA extraction, the 16S rDNA gene was amplified using bacterial universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5'-TAC GGC CTT GTT ACG ACT T-3′) in a MJ Mini Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The PCRs were conducted using 0.4 μl of *Taq* polymerase in a total volume of 25 μl. The samples underwent an initial denaturation of 10 min at 95 °C, and then 30 cycles of 15 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C, followed by 10 min at 72 °C. The PCR products were purified and were directly sequenced by Beijing Genomics Institute (Shenzhen, China). The sequences obtained were compared with the sequences available from the NCBI website using the Blastn program.

2.3. Antimicrobial activity assay

To assess the growth inhibition of a virulent strain of *V. parahaemolyticus*, all strains ($n = 135$) isolated from mud crabs were grown on media where they isolated at 22 °C for 24–48 h. After incubation, a loop of each strain was spotted onto the surface of agar plates previously inoculated with cultures of the target strain overnight. Clear zones after overnight incubation at 22 °C indicated the presence of antibacterial substances. Ten strains with strong inhibitory activities against *V. parahaemolyticus* were selected and stored at −80 °C in the medium by which they were isolated until used.

2.4. pH and bile tolerance

Three *Bacillus* strains (HL7, BP and DCU) were selected for further probiotic test. LB broth was adjusted to pH 1, 2, 3, 4, 5, 6, 7, 8 or 9, using 1 mol l^{-1} HCl or 1 mol l^{-1} NaOH before autoclaving. The media (100 mL) were inoculated with 1000 μL of overnight culture containing 10^8 CFU mL^{-1} of bacteria and incubated at 37 °C, and after 16 h, viable counts were determined by plate counting on triplicate LB agar (24 h at 37 °C).

The procedure used to determine the tolerance of the strains to bile has been reported [30]. Isolates in triplicate were tested for their ability to grow on LB agar containing increasing concentrations (w/v, 0%, 0.2%, 0.4%, 0.6%, 0.8% and 1.0%) of desiccated ox-bile (Oxoid, UK).

2.5. Sensitivity to antibiotics

Antibiotic susceptibilities were assessed by the disc diffusion test in 2216E agar. The antibiotics sensitivity discs included amoxicillin acid (30 μg), ampicillin (10 μg), tetracycline (30 μg), nitrofurantoin (300 μg), enrofloxacin (5 μg), vancomycin (30 μg), nalidixic acid (30 μg), doxycyclin (30 μg), streptomycin (10 μg), clindamycin (2 μg), cloramphenicol (10 μg), trimethoprim (1.25 μg), neomycin (5 μg), fortum (10 μg), minocycline (10 μg), cephaloridnum (30 μg), amikacin (10 μg), cefuroxime(5 μg), gentamicin(10 μg), erythromycin(10 μg). Agar plates were incubated at 22 °C for 48 h. The diameters of the growth inhibition zones were measured.

2.6. Hydrophobicity measurement

Cell surface hydrophobicity was determined by the bacterial adherence to hydrocarbons assay, which is based on the partitioning of cells in a two-phase system. In this study, the potential probiotics were harvested in the stationary phase by centrifugation at 5000 g for 5 min at 5 °C, washed twice and resuspended in a buffered salt solution (pH7.0) containing Na_2HPO_4 and NaH_2PO_4 (0.2 mol L^{-1}) to a final density of 10^9 CFU mL^{-1}. Optical density of these cell suspensions were measured by spectrophotometer, and 3 mL of bacterial suspension was put in contact with 600 μL of toluene and vortexed for 3 min. The two phases were allowed to separate for 1 h at 37 °C. The aqueous phase was carefully removed for optical density measurement. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H), which was calculated with the formula