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GFP tagged *Vibrio parahaemolyticus* Dahv2 infection and the protective effects of the probiotic *Bacillus licheniformis* Dahb1 on the growth, immune and antioxidant responses in *Pangasius hypophthalmus*

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ABSTRACT

In this study, the pathogenicity of GFP tagged *Vibrio parahaemolyticus* Dahv2 and the protective effect of the probiotic strain, *Bacillus licheniformis* Dahb1was studied on the Asian catfish, *Pangasius hypophthalmus*. The experiment was carried out for 24 days with three groups and one group served as the control (without treatment). In the first group, *P. hypophthalmus* was orally infected with 1 mL of GFP tagged *V. parahaemolyticus* Dahv2 at two different doses (10^5 and 10^7 cfu mL^{-1}). In the second group, *P. hypophthalmus* was orally administrated with 1 ml of the probiotic *B. licheniformis* Dahb1 at two different doses (10^5 and 10^7 cfu mL^{-1}). In the third group, *P. hypophthalmus* was orally infected first with 1 mL of GFP tagged *V. parahaemolyticus* Dahv2 followed by the administration of 1 mL of *B. licheniformis* Dahb1 (combined treatment) at two different doses (10^5 and 10^7 cfu mL^{-1}). The growth, immune (myeloperoxidase, respiratory burst, natural complement haemolytic and lysozyme activity) and antioxidant (glutathione-S-transferase, reduced glutathione and total glutathione) responses of *P. hypophthalmus* were reduced after post infection of GFP tagged *V. parahaemolyticus* Dahv2 compared to control. However, after administration with the probiotic *B. licheniformis* Dahb1 at 10^5 cfu mL^{-1}, *P. hypophthalmus* showed significant increase in the growth, immune and antioxidant responses compared to 10^7 cfu mL^{-1}. On the otherhand, the growth, immune and antioxidant responses of *P. hypophthalmus* infected and administrated with combined GFP tagged *Vibrio* + *Bacillus* at 10^5 cfu mL^{-1} were relatively higher than that of GFP tagged *V. parahaemolyticus* Dahv2 and control groups but lower than that of probiotic *B. licheniformis* Dahb1 groups. The results of the present study conclude that the probiotic *B. licheniformis* Dahb1 at 10^5 cfu mL^{-1} has the potential to protect the *P. hypophthalmus* against *V. parahaemolyticus* Dahv2 infection by enhancing the growth, immune and antioxidant responses. The probiotic *B. licheniformis* Dahb1 would be effectively used in the treatment of aquatic diseases for improvement of aquaculture industry.

1. Introduction

Aquaculture is emerging as one of the most viable and promising sector for providing nutritional and food security to humans [1]. Disease occurrences in aquaculture practice are problematic and lead to heavy economic losses for the aquaculture industry. Bacterial infections are one of the most important causes of disease problems in Indian aquaculture [2]. Vibriosis, caused by *Vibrio* spp., is a common bacterial disease of economically important both marine and freshwater fishes [3,4].

To overcome the bacterial disease problem in aquaculture, researchers have developed different ways to control the disease causing organisms using chemotherapeutics, vitamins, probiotics, plant-based compounds and recombinant vaccines. Traditional disease control strategies employ antibiotics and chemical disinfectants, but these are no longer recommended practices due to the emergence of bacterial resistance, and also to concerns over environmental impacts and wildlife protection. Although vaccinations

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have been suggested as an effective prophylactic method for use in the disease control of fish [5], there are some methodological problems for vaccine administration in so far as they may be very expensive and stressful to fish [6]. Already, some success has been achieved with immunostimulants as a more environment friendly approach to disease management [7,8]. From a scientific point of view, the use of probiotic bacteria has been suggested to become an alternative method for the prevention and control of various finfish and shellfish diseases in aquaculture [9]. A wide range of microalgae (*Tetraselmis*), yeasts (*Debaryomyces*, *Phaffia*, and *Saccharomyces*), Gram-positive (*Bacillus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Streptococcus*, and *Weissella*) and Gram-negative bacteria (*Aeromonas*, *Alteromonas*, *Photorhodobacterium*, *Pseudomonas*, and *Vibrio*) have been applied as probiotics to improve aquatic animal growth, survival, health and disease prevention [9–13]. *Bacillus* species is widely used as a probiotic in aquaculture for improving the growth performance, disease resistance and immunomodulation in fish [12–15].

Green fluorescent protein (GFP) has been applied to investigate the interaction between microbes and their hosts [16]. Bacteria tagged with the green flourescent protein gene can be easily identified as fluorescent green colonies. GFP-marked fish pathogens have been constructed to study the invasion pathways both *in vivo* and *in vitro* in fish models [17]. Apart from traditional techniques, recent techniques such as confocal laser scanning microscopy, transmission and scanning electron microscopy were used to validate colonization of the pathogens in aquatic animals [18,19]. However, the use of green fluorescent protein to monitor the host -pathogen interaction studies of aquatic organisms are limited.

In this study, the pathogenicity of GFP tagged *Vibrio parahaemolyticus* Dahv2 was visualized through the localization and colonization of green fluorescent colonies in the tissues of *P. hypophthalmus* using confocal laser scanning microscopy (CLSM), which will facilitate the monitoring of host–pathogen interactions for development of effective therapeutics. In addition, the protective effects of the probiotic *Bacillus licheniformis* Dahb1on the growth, innate immune and antioxidant defenses in the Asian catfish, *Pangasius hypophthalmus* experimentally infected with GFP tagged *V. parahaemolyticus* Dahv2 were determined.

2. Material and methods

2.1. Collection and maintenance of Pangasius hypophthalmus

Healthy Asian catfish, *Pangasius hypophthalmus* showing no signs of disease (examined through gross examination of skin, fins and gills), with no previous history of parasitic infections, and having a mean body weight of 15 ± 2.5 g were obtained from local fish farm and maintained in aerated fresh water for 1–2 weeks in 300 L FRP tanks at 29 ± 2.0 °C. Prior to start of the experiment, all the fishes were fed commercial diet (Tairoun Feed Company, Taipei, Taiwan) for twice a day at the rate of 2% of their body weight during the acclimatization period. During acclimatization, renewal of water was performed once in a day corresponding to 85% of tank water. Faeces and uneaten feed were siphoned out regularly. Aeration was provided throughout the acclimatization period to avoid stress.

2.2. Bacterial culture conditions

B. licheniformis Dahb1 (HM235407.1) and *V. parahaemolyticus* Dahv2 (HQ693275.1) used in this study were taken from the stock culture collection previously maintained in our laboratory and stored in 20% (v/v) glycerol at −86 °C. The isolation and identification of the bacterial strains were reported earlier [20]. Briefly, *B. licheniformis* Dahb1 was grown in shaker incubator for 24 h at 30 °C in nutrient broth. *V. parahaemolyticus* Dahv2 was grown for 24 h at 28 °C in tryptic soy broth. Both the cultures were then centrifuged at 10,000g for 20 min at 4 °C. The supernatant was removed and the bacterial pellets were resuspended in PBS at 10^5 and 10^7 Cfu ml^{-1}. The bacterial suspension was obtained based on a standard curve created from a series of different bacterial concentrations and optical densities at 600 nm using a spectrophotometer.

2.3. Tagging of green fluorescent protein (GFP) in V. parahaemolyticus Dahv2

The donor strain pVSV102 plasmid harbouring GFP and kanamycin-resistance expression cassettes were transferred from *Escherichia coli* to the receptor *V. parahaemolyticus* Dahv2 by triparental mating using the conjugative helper strain CC118 Ipir [21,33]. Green fluorescent colonies were detected under the confocal laser scanning microscope (CLSM) (Carl Zeiss, Germany). After two days, each fluorescent colony was tested to verify whether the GFP expressing strain was genuine and active.

2.4. Screening of B. licheniformis Dahb1 for their ability to inhibit in vitro biofilm formation of V. parahaemolyticus Dahv2 using the microtitre plate (MTP) assay

Cell free extracts of *B. licheniformis* Dahb1were tested for their ability to inhibit *V. parahaemolyticus* Dahv2 biofilm formation in microtitre plates [22]. *B. licheniformis* Dahb1were cultured in nutrient broth for 24 h at 30 °C. l mL of culture were sonicated using a UP100H ultrasonic processor (Hielscher, Germany) for 1 min at 100 W and then centrifuged at 800g for 10 min before the supernatant was passed through a 0.2-μm pore-sized filter and storage at −20 °C. An overnight culture of *V. parahaemolyticus* Dahv2 (10 μl, ~10^6 cells ml^{-1}) was inoculated into 96- well polystyrene microtitre plates containing 100 μl of Luria- Bertani (LB) medium and 100 μl of different concentrations of *B. licheniformis* Dahb1 (20–100 $\mu g ml^{-1}$) cell free extract were then incubated statically for 24 h at 30 °C. The cultures were discarded and the wells were gently rinsed twice with deionised water and allowed to air dry before staining with crystal violet (CV). The wells were stained with 210 μl of 0.1% (w/v) CV for 10 min before rinsing twice with deionised water and air drying. The slides were observed under light microscope. Another piece of biofilm grown as above were stained with acridine orange and was then visualized under confocal laser scanning microscope (CLSM).

2.5. Experimental design and infection treatment

The experiment consists of three treatment groups and one group served as the control. Each group consists of 12 *P. hypophthalmus*, each weighing approximately 15 ± 2.5 g body weight. The experiment was carried out for a period of 24 days and triplicates were maintained for each group. Both the control and experimental groups were fed with commercial diet (Tairoun Feed Company, Taipei, Taiwan) twice a day at the rate of 2% of their body weight. Among the treatment groups, the first group was orally infected with the pathogenic GFP tagged *V. parahaemolyticus* Dahv2 (1 mL) at two different doses (1×10^5 and 1×10^7 CFU mL^{-1}). The second group was orally administered with the probiotic *B. licheniformis* Dahb1 (1 mL) at two different doses (1×10^5 and 1×10^7 CFU mL^{-1}). The third group was first orally infected with 1 mL of GFP tagged *V. parahaemolyticus* Dahv2 followed by the administration of 1 mL of *B. licheniformis* Dahb1 (combined treatment) at two different doses (10^5 and 10^7 cfu mL^{-1}). During the experimental period, the water in each tank was renewed and the