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A probiotic *Bacillus* strain containing amorphous poly-beta-hydroxybutyrate (PHB) stimulates the innate immune response of *Penaeus monodon* postlarvae

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ABSTRACT

In this study, the PHB-accumulating *Bacillus* sp. JL47 strain (capable of accumulating 55% PHB on cell dry weight) was investigated for its effects on the immune response of giant tiger shrimp (*Penaeus monodon*) postlarvae (PL) before and after the *Vibrio campbellii* challenge. Briefly, shrimp PL were cultured and fed with *Artemia* nauplii enriched with *Bacillus* sp. JL47. Shrimp receiving the *Artemia* nauplii without JL47 enrichment were used as control. After 15 days of feeding, the shrimp were challenged with pathogenic *V. campbellii* LMG 21363 at 10^6 cells mL^{-1} by immersion. Relative expression of the immune related genes encoding for prophenoloxidase (proPO), transglutaminase (TGase) and heat shock protein 70 (Hsp70) in the shrimp were measured before (0 h) and after (3, 6, 9, 12, 24 h) the *Vibrio* challenge by quantitative real-time PCR using β-actin as the reference gene. The expressions of TGase and proPO were significantly up-regulated ($p < 0.05$) within 9 h and 12 h, respectively after challenge in shrimp receiving the *Bacillus* sp. JL47 as compared to the challenged and non-challenged controls. Hsp70 expression was significantly increased ($p < 0.05$) at 3 h post-challenge in all challenged shrimp. Interestingly, proPO and TGase genes were significantly up-regulated ($p < 0.05$) in *Bacillus* sp. JL47 treated shrimp even before the *Vibrio* challenge was applied. No up-regulation in the Hsp70 gene, however, was observed under these conditions. The data suggest that the protective effect of the PHB-accumulating *Bacillus* sp. JL47 in shrimp was due to its capacity to stimulate the innate immune related genes of the shrimp, specifically the proPO and TGase genes. The application of probiotic *Bacillus* species, capable of accumulating a significant amount of PHB, is suggested as potential immunostimulatory strategy for aquaculture.

1. Introduction

Black or giant tiger shrimp *P. monodon* is one of the most economically important crustacean species in the aquaculture industry. However, the culture of this species has consistently been hampered by disease outbreaks, most often caused by bacterial and viral pathogens [1,2]. For example, mass mortality at the early larval stages of the animal was reported to result from luminescent vibriosis [3,4]. Hence, managing their health by enhancing their immunity is of vital importance.

Invertebrates such as shrimp do not have adaptive immunity but have to rely on their innate immune system as the major defense mechanisms to fight invading pathogens. The activation of this innate immune system is initiated upon the recognition of non-self-molecules associated with pathogens, also known as pathogen-associated molecular patterns (PAMPs), by well-defined receptors referred to as pathogen/pattern recognition receptors (PRRs) [5,6]. Among the diverse array of immune responses, melanisation (prophenoloxidase (proPO) activation system) has been suggested as one of the most important immune mechanism in many invertebrates [7]. In this immune reaction, the recognition of microbial PAMPs by appropriate PRRs leads to the activation of a series of serine proteinases and eventually culminates in the

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proteolytic cleavage of the proPO zymogen to the active phenoloxidase (PO) enzyme. The activation of PO results in the production of quinones and other short-lived intermediates which possess cytotoxic activity towards microorganisms, restrain the invasion of microbial pathogens into the host body cavity as well as participate in wound healing process in damaged tissues (i.e. sclerotisation) [7–11]. Finally, in this immune response the production of the polymeric melanin as a more long-lived product is deposited precisely at the site of infections or around the surface of foreign microorganisms to physically encapsulates foreign microorganisms [7]. Furthermore, clot formation is also another first line of defense in shrimp that is being activated to prevent hemolymph loss and microbial spread at sites of injury. Considering its quickness and efficiency, it is considered to be an integral part of the overall immune response in crustaceans for survival. In the clotting process, transglutaminase (TGase) is the central enzyme involved in the final step for the stabilization of the hemolymph clot. In shrimp, TGase and clotting proteins have been suggested to be essential molecules in shrimp hemolymph coagulation and immunity [12]. Moreover, heat shock proteins (Hsps) are considered to be crucial mediators in conferring tolerance towards environmental stress [13]. Cells inducing Hsps as a result of stress show resistance to a subsequent stress - a phenomenon called “stress tolerance” [14]. Molecular chaperones like heat shock protein 70 (Hsp70) were reported to repair partially denatured proteins, facilitate the degradation of irreversibly denatured proteins and inhibit protein aggregation and therefore protecting cells from harmful environmental stresses [15]. In addition, Hsp70 facilitates immune responses against many diseases as demonstrated in a wide variety of experimental models *in vitro* and *in vivo* [16–18].

Stimulation of the immune-related genes in the host has been an effective strategy to protect shrimp from diseases. Probiotic bacteria like *Bacillus* species were reported to have immunostimulatory effects in shrimps resulting in improved resistance towards pathogenic diseases [19–21]. Likewise, many products of bacterial origin were also reported to have immunostimulatory effects such as peptidoglycan (PG), lipopolysaccharide (LPS), β-glucans, and other bacterial cell wall components [22–24]. A recent report shows that the biopolymer PHB-hydroxyvalerate (PHB-HV) extracted from *Bacillus thuringiensis* increased both the specific and non-specific immune mechanisms in fish [25]. Hence, the idea of using *Bacillus* species carrying significant amount of amorphous PHB is suggested in this study. In the previous work [26], we demonstrated the protective effects of the PHB-accumulating *Bacillus* sp. JL47 (containing 55% PHB) in *P. monodon* postlarvae against pathogenic *V. campbellii* LMG 21363. In this experiment we aimed at investigating the effects of the PHB-accumulating *Bacillus* isolate JL47 at a molecular level by looking at the *in vivo* expressions of proPO, TGase and HSp70 genes of the *P. monodon* postlarvae before and after a *Vibrio campbellii* challenge.

2. Materials and methods

2.1. Bacterial strains

The PHB-accumulating *Bacillus* sp. JL47 (GenBank: KJ496325.1), was cultured following the procedures described in the previous work for optimum PHB production [26]. In brief, JL47 was activated in LB (tryptone (Himedia, India; 10 g L^{-1}), yeast extract (Himedia, India; 5 g L^{-1}) and NaCl (Sigma-Aldrich, Singapore; 20 g L^{-1}) medium for 16 h and subsequently inoculated at 1% (v/v) in LB supplemented with 20 g L^{-1} glucose (Sigma-Aldrich, Singapore). The culture was grown at 30 °C and 100 rpm agitation for 48 h. After culture, the bacterial cells were harvested by centrifugation at 5000 rpm for 5 min, discarding the supernatant and washing with sterile saline (8.5 g L^{-1} NaCl) (repeated 2 times). The bacterial cells were kept at −80 °C until used for *Artemia* enrichment.

Pathogenic *Vibrio campbellii* LMG 21363 was used in the challenge experiment. The bacterial preparation and the challenge with pathogenic *V. campbellii* LMG 21363 (except for the addition of sub-lethal dose of ammonium chloride in the water) in shrimp were conducted according to the procedures described in the previous work [26].

2.2. Artemia enrichment

High 5 *Artemia* cysts (INVE Aquaculture, Thailand) were hatched daily in filtered seawater at 2 g cysts L^{-1} for 30 h in a 30-L hatching tank. Vigorous aeration was provided with illumination set at approximately 27 μE/(m^2.sec)). After 30-h incubation, *Artemia* nauplii (*Artemia* instar II) were harvested, washed with filtered seawater and restocked in enrichment tanks at ~5000 individuals L^{-1} seawater. *Artemia* instar II nauplii were then enriched with *Bacillus* sp. JL47 (containing ~55% amorphous PHB on cell dry weight) at 0.5 g (wet weight) L^{-1}. The enrichment tank was provided with vigorous aeration. The enrichment was done for 6 h, after which the enriched *Artemia* were harvested and rinsed with seawater prior to feeding. Non-enriched *Artemia* nauplii were also maintained and harvested prior to feeding.

2.3. Shrimp culture and sampling

P. monodon postlarvae (PL5) were obtained from the Tigbauan Main Station shrimp hatchery of the Southeast Asian Fisheries Development Center-Aquaculture Department (SEAFDEC/AQD), Tigbauan, Iloilo Philippines and acclimatized to the experimental conditions for five days by stocking the animals at 15 shrimps L^{-1} in ~13 L capacity round containers (13 cm radius; 25 cm height) containing 10 L filtered and UV-treated seawater with moderate aeration. The overall status of the shrimps was also examined by randomly collecting shrimp samples from the same batch and the shrimp samples were submitted to the Fish Health Section of SEAFDEC/AQD for standard shrimp pathogen analyses to ensure that the shrimps are free from diseases (i.e. white-spot syndrome virus (WSSV), monodon-type baculovirus (MBV) and infectious hypodermal and hematopoietic nicrosis virus (IHHNV)). During the acclimation period, all shrimps were fed with non-enriched *Artemia* to satiation. On a daily basis, the tanks were siphoned and ~80% of the water was replaced with filtered and UV-treated seawater. After the acclimation period, the experiment was started and the shrimp from each tank were recounted to ensure uniformity of stocking density at 15 shrimps L^{-1}. Shrimps were fed with *Bacillus* sp. JL47-enriched *Artemia* as treatment (n = 3). Shrimps fed non-enriched *Artemia* were used as control (n = 3; wherein 3 tanks were used for the challenge test (negative control) while the remaining 3 tanks were not challenged (positive control)) (see Fig. 1 for the schematic diagram). Feeding of enriched and non-enriched *Artemia* was done twice daily (morning and afternoon) to satiation. Water was changed daily at 80% of the water volume with fresh filtered and UV- treated sea water. Excreta were siphoned out every morning prior to feeding. The immersion challenge with *V. campbellii* LMG 21363 was performed after 15 days of culture by the addition of 10^6 cells mL^1 in the culture water. Five shrimps were sampled per tank just before the challenge (0 h) and 3, 6, 9, 12 and 24 h after the challenge. Shrimp sampled at each time from the same tank were pooled and treated as one replicate in a given treatment. This yielded three biological replicates for each treatment. During the sampling, shrimp were immediately put in eppendorf tubes with RNA later (Ambion, USA) and kept cooled in ice all throughout the sampling. In the lab, shrimps were kept at −80 °C until RNA isolation.