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Differentially expressed genes in hemocytes of *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus* AHPND (VP_{AHPND}) and VP_{AHPND} toxin

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ABSTRACT

While toxin-harboring *Vibrio parahaemolyticus* has been previously established as the causative agent of early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) in shrimp, information on the mechanistic processes that happen in the host during infection is still lacking. Here, we examined the expression responses of the shrimp hemocyte transcriptome to *V. parahaemolyticus* AHPND (VP_{AHPND}) by RNA sequencing (RNA-seq). Using libraries (SRA accession number SRP137285) prepared from shrimp hemocytes under experimental conditions, a reference library was *de novo* assembled for gene expression analysis of VP_{AHPND}-challenged samples at 0, 3/6, and 48 h post infection (hpi). Using the library from 0-hpi as the control, 359 transcripts were found to be differentially expressed in the 3/6-hpi library, while 429 were differentially expressed in the 48-hpi library. The expression patterns reported in the RNA-seq of 9 representative genes such as anti-lipopolysaccharide factor (*Lv*ALF), crustin p (CRU), serpin 3 (SER), C-type lectin 3 (CTL), clottable protein 2 (CLO), mitogen-activated protein kinase kinase 4 (MKK4), P38 mitogen-activated protein kinase (P38), protein kinase A regulatory subunit 1 (PKA) and DNAJ homolog subfamily C member 1-like (DNJ) were validated by qRT-PCR. The expression of these genes was also analyzed in shrimp that were injected with the partially purified VP_{AHPND} toxin. A VP_{AHPND} toxin-responsive gene, *Lv*ALF was identified, and its function was characterized by RNA interference. *Lv*ALF knockdown resulted in significantly rapid increase of shrimp mortality caused by toxin injection. Protein-protein interaction analysis by molecular docking suggested that *Lv*ALF possibly neutralizes VP_{AHPND} toxin through its LPS-binding domain. The data generated in this study provide preliminary insights into the differences in the immune response of shrimp to the bacterial and toxic aspect of VP_{AHPND} as a disease.

1. Introduction

In 2013, the causative agent of acute hepatopancreatic necrosis disease (AHPND), earlier known as early mortality syndrome (EMS), was identified as *Vibrio parahaemolyticus* [49]. Being ubiquitous in the marine environment, *V. parahaemolyticus* is only considered as an opportunistic pathogen [20]. All AHPND-causing strains of *V. parahaemolyticus* (VP_{AHPND}) that are uniquely virulent to shrimp were found to have a large extra-chromosomal plasmid that is absent in non-AHPND strains [14,18,54]. This AHPND-causing plasmid contains 2 toxin genes named as PirA and PirB because they are homologous to the *Photorhabdus* insect-related (Pir) binary toxin [20,40]. Because the ability to cause disease is lost by experimental deletion or the natural absence of plasmid-encoded toxins, the toxins may be considered as the most important factors of AHPND pathogenesis.

In the early stage of VP_{AHPND} infection, sloughing of the hepatopancreas tubule epithelial cells can be observed in histological analysis, while atrophied pale hepatopancreas, the most common sign of AHPND

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in diseased shrimp, can be observed in the later stages of infection, when necrosis of the hepatopancreas tubule epithelial cells and massive hemocyte infiltration occurs [30,45]. Ref. [20] showed that VP_{AHPND} initially colonizes the stomach and secretes toxins at 6 h post infection (hpi), which would then allow both toxin proteins and, subsequently, bacteria to spread from the stomach to the hepatopancreas at 12-hpi. While this information describes the pathogenesis of the AHPND, the mechanistic processes of the shrimp immune system during infection especially the response to VP_{AHPND} toxins is not yet fully understood. Recent transcriptome analysis in hemocyte *Litopenaeus vannamei* challenged with non-AHPND strains of *V. parahaemolyticus* showed differential expression of immune-related genes induced by *Vibrio* infection [37]. Recently, a study showed that the hemocyanin (HMC) gene family was identified from *L. vannamei* hepatopancreas as up-regulated upon VP_{AHPND} infection. Among the hemocyanin isoforms found, hemocyanin subunit L3 (HMCL3) and hemocyanin subunit L4 (HMCL4) were induced in the hepatopancreas as a response to VP_{AHPND} toxin from 1 to 3 h after injection [6].

The aim of the study is to analyze the expression responses of the shrimp hemocyte transcriptome, which is a major immune tissue related to VP_{AHPND} infection, by RNA sequencing (RNA-seq). The effect of partially purified VP_{AHPND} toxin challenge on selected genes was also revealed by qRT-PCR. This provides preliminary insights into the differences in the immune response of shrimp to the bacterial and toxic aspect of AHPND as a disease. Likewise, the study aims to highlight various roles of genes in different stages of AHPND infection.

2. Materials and methods

2.1. Shrimp samples

Shrimp, weighing about 6–8 g, were obtained from a local shrimp farm. They were acclimatized in rearing tanks with ambient temperature of 30 ± 2 °C, water salinity of 20 parts per thousand, and constant aeration. They were fed with commercial pellets 2 times a day and kept in the rearing tanks for 2 weeks before any experiment or tissue collection.

2.2. VP_{AHPND} and VP_{AHPND} toxin challenge

After acclimatization, shrimp were challenged with VP_{AHPND} Thamai isolates by immersion in tanks that were inoculated by a bacterial suspension to a final concentration of 1.5×10^6 CFU/ml as described by Ref. [6]. VP_{AHPND} was grown on TCBS agar. Several colonies were inoculated in tryptic soy broth (TSB) containing 1.5% NaCl and incubated at 30 °C with 250 rpm shaking overnight. This culture was then inoculated to a fresh TSB with 1.5% NaCl medium in a 1:100 ratio and incubated at 30 °C with 250 rpm shaking until an optical density at 600 nm (OD_{600}) of 2 (approximately 10^8 CFU/mL) was achieved. The median lethal dose (LD_{50}) of bacterial inoculants at 48 h was determined in 10 shrimp.

The partially purified VP_{AHPND} toxin, used for knockdown and challenge experiments, was prepared from crude VP_{AHPND} toxin by ammonium sulfate precipitation and dialysis according to [6]. The partially purified VP_{AHPND} toxin was dissolved and dialyzed against 1 × PBS pH of 7.4, quantified by Bradford protein assay, and checked for the presence of PirAB toxin protein using 15% SDS-PAGE before use in any experiment. The partially purified VP_{AHPND} toxin challenge resulting in AHPND in shrimp was confirmed by observation of morphological changes in the hepatopancreas such as paling and atrophy as well as lethargy in surviving shrimp. The partially purified VP_{AHPND} toxin at the dosage that yields 100% mortality in two days which is 0.2 μg/g shrimp was prepared by diluting toxin in 1 × PBS mixed with a red food-grade dye. The red food-grade dye was used to visualize and make sure that injections were administered properly into the shrimp muscle during injection experiments.

2.3. Next generation sequencing and data analysis

For transcriptome analysis, hemocyte collection was done by drawing approximately 500 μl of hemolymph from the ventral sinus of the shrimp using a sterile syringe with an equal volume of filter sterilized and pre-cooled anticoagulant (MAS solution) as described by Ref. [43]. Hemocytes were immediately collected from the suspension by centrifugation in 800 × g for 10 min at 4 °C and kept in liquid nitrogen. Hemocyte from 10 individuals of VP_{AHPND}-challenged shrimp at each time point (0, 3, 6 and 48 h post challenge) was pooled in triplicates. The hemocyte pool for early phase of infection from the 3-h time point was further mixed with the 6-h time point. Total RNA was extracted using Favorgen Tissue Total RNA mini kit (Biotech Corp.).

Total RNA extracts were analyzed in EtBr-stained 1.2% agarose gel and by Agilent 2100 Bioanalyzer using RNA 6000 Pico Kit (Agilent) to determine the integrity and quality. After RNA concentrations were measured by Qubit® RNA HS Assay Kit (ThermoFisher Scientific) on Qubit® 2.0 flourometer, cDNA libraries from 4 μg total RNA were then constructed following the manufacturer's instruction for TruSeq® stranded mRNA LT sample prep kit (Illumina).

The 3 libraries, 0-h library, 3/6-h library and 48-h library, were sequenced using NextSeq 500 High Output v2 Sequencing Kit (Illumina) in a NextSeq 500 desktop sequencer (Illumina) along with 9 other indexed libraries, which were normalized and pooled with a 1% PhiX control spike-in. Using FastQ Toolkit available through the BaseSpace (Illumina) public app repository, adapter trimming and other quality control filtering of raw reads were performed. High quality reads from the 3 hemocyte libraries (SRR6942061, SRR6942062, SRR6942059), and an additional hepatopancreas library (SRR6942060), were assembled together to form a transcriptome reference in Trinity v2.06 software [13]; from which transcript abundance can be based and estimated using RSEM software that is wrapped by scripts included in Trinity. EdgeR software [38] was used to detect differentially expressed transcripts and genes using a dispersion parameter of 0.3.

Leveraging different software for functional annotation, such as BLAST [2]; PFAM [35]; KEGG [16]; Gene ontology [3] and eggNOG [34]; and then running GO-Seq [56]; the Trinotate workflow (http://trinotate.github.io/) was used to analyze gene ontology enrichment for differentially expressed features. UniProt accession numbers from the Trinotate workflow were mapped into Entrez GeneIDs using the UniProt Retrieve/ID mapping tool (http://www.uniprot.org/uploadlists/). The resulting Entrez GeneIDs were then used in KOBAS 2.0 (http://kobas.cbi.pku.edu.cn/home.do) to map KEGG Orthology or conduct enrichment analysis. The identified KEGG orthologies were used as inputs in KEGG Mapper - Search Pathway tool (http://www.kegg.jp/kegg/tool/map_pathway1.html) for mapping to the reference KEGG Pathways and determine distribution. BLAST2GO [10] was used for supplementary annotation. Supplementary tools from galaxy services [1] of National Center for Genome Analysis Support (https://galaxy.ncgas-trinity.indiana.edu/) and Galaxy Queensland (http://galaxy-qld.genome.edu.au/galaxy) such as Fasta tools, Trinity software, BLAST+ were also used.

2.4. Quantitative real-time PCR analysis

A total of 9 representative transcripts from the reference assembly including anti-lipopolysaccharide factor (*Lv*ALF), crustin p (CRU), serpin 3 (SER), C-type lectin 3 (CTL), clottable protein 2 (CLO), mitogen-activated protein kinase kinase 4 (MKK4), P38 mitogen-activated protein kinase (P38), protein kinase A regulatory subunit 1 (PKA) and DNAJ homolog subfamily C member 1-like (DNJ) were analyzed using quantitative real-time PCR (qRT-PCR) to evaluate and confirm the differential expression profiles reported by RNA-Seq analysis. The shrimp EF-1α gene was used as an internal control. Twelve shrimp were challenged with VP_{AHPND} as previously described. Hemocyte of three