Fish and Shellfish Immunology 84 (2019) 178–188

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Identification of an anti-lipopolysaccharide factor AV-R isoform (*Lv*ALF AV-R) related to Vp_PirAB-like toxin resistance in *Litopenaeus vannamei*

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ARTICLE INFO

Keywords:
AHPND
Penaeid shrimp
Vibrio parahaemolyticus
Immune
Resistance

ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) is a shrimp farming disease, caused by the pathogenic *Vibrio parahaemolyticus* carrying a plasmid encoding Vp_PirAB-like toxins. Formalin-killed cells of *V. parahaemolyticus* AHPND-causing strain D6 (FKC-VpD6) were used to select Vp_PirAB-like toxin-resistant *Litopenaeus vannamei* by oral administration. Stomach and hepatopancreas tissues of shrimps that survived for one week were subjected to RNA sequencing. Differentially expressed genes (DEGs) between surviving shrimp, AHPND-infected shrimp, and normal shrimp were identified. The expressions of 10 DEGs were validated by qPCR. Only one gene (a gene homologous to *L. vannamei* anti-lipopolysaccharide factor AV-R isoform (*Lv*ALF AV-R)) was expressed significantly more strongly in the hepatopancreas of surviving shrimp than in the other groups. Significantly higher expression of *Lv*ALF AV-R was also observed in shrimp that survived two other trials of FKC-VpD6 selection. Recombinant ALF AV-R bound to LPS, PGN, Gram-negative bacteria, and some Gram-positive bacteria in ELISAs. ALF AV-R recombinant protein did not interact with native Vp_PirAB-like toxin in an ELISA or a Far-Western blot. For *L. vannamei* orally fed ALF AV-R protein for 3 days, the survival rate following challenge with VpD6-immersion was not significantly different from that of shrimp fed two control diets. These results suggest that *Lv*ALF AV-R expression was induced in the hepatopancreas of shrimp in response to the presence of Vp_PirAB-like toxin, although other factors might also be involved in the resistance mechanism.

1. Introduction

Whiteleg shrimp, *Litopenaeus vannamei* account for about 72% of global farmed shrimp production [1]. They are vulnerable to viral and bacterial diseases that cause mass mortality in shrimp farms [2,3].

Acute hepatopancreatic necrosis disease (AHPND), also called early mortality syndrome (EMS), was first reported in China in 2009 [4]. In 2010–2015, AHPND spread to several countries in Asia and South America, resulting in a great reduction of production [5–8]. The causative agent of AHPND is a unique strain of *Vibrio parahaemolyticus* carrying a plasmid that encodes the Vp_PirAB-like toxin gene [9,10]. Vp_PirA-like and Vp_PirB-like proteins have been identified as the virulence factors of AHPND [11,12]. Recently, the plasmid encoding Vp_PirAB-like toxin was found in other three AHPND-causing Vibrio species, but not *V. parahaemolyticus* [13–15]. The protein structure of Vp_PirAB-like toxin is very similar to that of delta-endotoxin or Cry toxin [11]. Cry toxins are produced by *Bacillus thuringiensis*, which have been widely used as pesticides to control insect pests [16].

The target organ of AHPND is the hepatopancreas, which displays hemolytic infiltration and cell sloughing post infection [17]. However, Vp_PirA-like and Vp_PirB-like were detected in the hepatopancreas, stomach and hemolymph of *L. vannamei* that had been immersed in water containing an AHPND-causing strain of *V. parahaemolyticus* [18].

We previously reported that the virulence of Vp_PirAB-like toxin, unlike the virulence of normal bacterial exotoxins, remained in non-living bacterial cells after the cells were treated with formalin and incubated at 60 °C for 2 h [12]. Moreover, most of the toxin proteins are accumulated in bacterial cells, although some are secreted into the extracellular environment.

Here, we supplemented a commercial diet with a formalin-killed AHPND strain of *V. parahaemolyticus* to select for resistance to Vp_PirAB-like toxin in *L. vannamei*. To identify genes involved in conferring toxin resistance, the hepatopancreas and stomach mRNAs of surviving shrimp were sequenced and analyzed together with non-treated shrimp and AHPND-infected shrimp. A gene homologous to anti-lipopolysaccharide factor AV-R isoform of *L. vannamei*, was

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https://doi.org/10.1016/j.fsi.2018.10.005
Received 19 July 2018; Received in revised form 27 September 2018; Accepted 3 October 2018
Available online 04 October 2018
1050-4648/

expressed in the hepatopancreas of resistant shrimps significantly more strongly than it was expressed in the other shrimps.

2. Materials and methods

2.1. Experimental shrimp and resistant shrimp selection

Juvenile *L. vannamei* were purchased from IMT engineering Inc. (Niigata prefecture, Japan) and stocked in a 500-L tank with aerated, recirculating artificial seawater (28–32 ppt) at 28 °C prior use. Two hundred healthy shrimps (approximately 1–2 g body weight) were reared in a 100-L plastic tank with aerated, recirculating artificial seawater (28 ppt) at 28 °C. Shrimps were fed commercial diet containing 5% (w/w) of formalin-killed cells of *V. parahaemolyticus* AHPND-causing strain D6 (FKC-VpD6) [9]. The FKC diet was prepared as previously reported [12]. Shrimps were fed twice per day (a total of 5% of body weight per day) and checked daily to remove dead shrimp from the tank. After 2 weeks of feeding, the surviving shrimps were collected, and their hepatopancreas and stomach were dissected.

AHPND-infected shrimps were prepared using five *L. vannamei* (approximately 4–6 g body weight) immersed with the *V. parahaemolyticus* AHPND-causing strain FP11_PirAB-like [12] at a final concentration of 1×10^6 CFU/ml. Three shrimps were collected at 24 h after immersion, and their hepatopancreas and stomach were dissected individually. The hepatopancreas and stomach were also individually collected from three apparently healthy shrimps (approximately 4–6 g body weight) as a control group. All the tissue samples were temporarily stored at −80 °C for later total RNA isolation.

2.2. RNA sequencing and differentially expressed genes analysis

The pooled total RNA from hepatopancreas or stomach of surviving shrimps (sur-FKC) was used to prepare cDNA libraries following the protocols of Illumina Truseq Stranded mRNA Library Prep Kit (Illumina, USA). The cDNA libraries of sur-FKC were sequenced by Illumina Miseq sequencer (USA). The raw reads of sur-FKC were *de novo* assembled with the raw reads of non-treated shrimps (control) and shrimp immersed with FP11_PirAB-like for 24 h (Vp-inf), which were previously sequenced using Trinity software to obtain a single RNA-Seq data set [19].

To identify differentially expressed genes, all samples were pairwise compared. Genes that were differentially expressed at *P*-values < 1e-5 and expression level greater than 4-fold were selected. The differentially expressed genes at FDR (false discovery rate) value of less than 1e-5 were considered as significantly differentially expressed genes (DEGs). The RNA-Seq files have been submitted to the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) under GEO Series accessions number GSE104715.

The DEGs were analyzed using BLAST2GO software [20]. Matches to the sequences were searched for in the NCBI non-redundant protein database with BLASTX using the default parameters and an E-value of 1e-5). Matches from microorganisms and vertebrates were discarded. Gene ontology was annotated with BLAST2GO with default parameters in BLAST (E value cutoff 1e-3). The genes were then grouped as described previously [21].

2.3. Validation of DEGs by quantitative PCR (qPCR)

One μg of total RNA from an individual shrimp (the same sample used for RNA-Seq) was used to synthesize cDNA by using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). To validate the DEGs, we selected 10 genes whose expression levels were more than 16-fold (either low- or high-expressed) to perform qPCR by StepOnePlus (Applied Biosystems, USA) using Thunderbird™ SYBR® qPCR Mix. The primers were designed using Primer Express 3.0.1 (Applied Biosystems, USA) and are listed in Table 1. The qPCR conditions were 95 °C for 60 s followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s and additional of dissociation curve analysis.

Table 1
List of primers used in this study.

Target gene		Sequences (5′-3′)	Length (bp)
DN3154	Fw	AGGTGGATACGGCGGAAAC	19
	Rv	CGAAGGATCGCCTGTACGA	19
DN6348	Fw	GGACCTTCACCTTACCGATTTG	22
	Rv	GAAGAAGCCCAGGAGGAGGAT	21
DN13245	Fw	GGGCACGCTCCTCTCCAT	18
	Rv	TTTACCCCGCGTGACTTCTT	20
DN13839	Fw	ACGGCAACTTCAACTACGACTTC	23
	Rv	GAGCGCCGACAGCTGAGA	18
DN13910	Fw	GGAGCTGTTAGGACACTACTGCAA	24
	Rv	ACATGCGACCCCTGAAATACA	21
DN18811	Fw	CCGGCAGGAAGGCAGAA	17
	Rv	GGGCGTGGACGTATCCTATG	20
DN20905	Fw	GCTGCAGGCCCTCTTCTTCT	20
	Rv	TTGCCTCCTTCCCACATCTT	20
DN21485	Fw	TGGCGGTGTTCCTGGTGG	18
	Rv	GTTCCTCCACAGCCCAACG	19
DN21632	Fw	GCGACGACCTTCCCTTCA	18
	Rv	AGACATAGCGCTGGCACACA	20
DN22624	Fw	CGGAGACAAGAGCAACTTCATATG	24
	Rv	GGGCTGGGTCTCGCTCAT	18
ALF AV-R	Fw	TGACAAGCCTGGTGGTGGC	19
	Rv	GTGTCCTGGCTTCCCCTC	18
EF-1α	Fw	TGGCTACTCACCTGTGCTTG	20
	Rv	CCAGCTCCTTACCAGTACGC	20
rALF AV-R/no SP	Fw	GCGGCCGCGTTCAGCCACCGCTTAG	25
	Rv	GAATTCGCAAGGATGGCAGGCTGTGG	26

The expression levels of the selected genes were analyzed by the comparative CT method (ΔΔCt) [22]. The elongation factor-1 alpha (EF-1α) was used as a reference gene for relative quantifications.

2.4. Expression level of LvALF AV-R

2.4.1. Tissues distribution

LvALF AV-R gene which was highly expressed in the survived shrimp, was examined in three apparently healthy *L. vannamei* (body weight about 7 g). Total RNA was extracted from hemocytes and 9 other tissues (gills, muscle, heart, hepatopancreas, stomach, intestine, eye, lymphoid organ and nerve).

2.4.2. Bacterial stimulations

L. vannamei (body weight about 3 g) were divided into two groups (15 shrimp per group), and reared in 15-L plastic tanks with aerated artificial seawater (28 ppt) at 28 °C. One group was daily fed a commercial diet containing 5% (w/w) of formalin-killed cells of *V. parahaemolyticus* non-AHPND causing strain N7 (FKC-VpN7) [23], whereas the other group was immersed with *V. parahaemolyticus* AHPND-causing strain D6 (VpD6) at a bacterial concentration of 2×10^5 CFU/ml and fed with a commercial pellet. The hepatopancreas was collected and total RNA was extracted from three shrimps individually at 6 h, 24 h, 3 d and 7 d.

One μg of total RNA of each sample was used for cDNA synthesis with High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, USA). The mRNA level of *Lv*ALF AV-R was examined with specific primer set of ALF AV-R (Table 1) by qPCR. qPCR conditions were 95 °C for 60 s followed by 40 cycles of 95 °C for 15 s, 67 °C for 60 s and additional of dissociation curve analysis. The annealing temperature used was optimized to discriminate an expression of ALF AV-R and other ALF isoforms.

2.5. Repetitions of FKC-VpD6 feeding

To confirm the relation between *Lv*ALF AV-R and toxin resistance,

two other trials of FKC-VpD6 feeding were performed. In the first repetition, 500 *L. vannamei* (body weight 3–5 g) were reared in two 500-L plastic tanks (250 shrimps each) with aerated seawater (20 ppt) at 28 °C. In the second repetition, 540 *L. vannamei* (body weight 2–3 g) were reared in two 500-L plastic tanks (270 shrimps each). Both groups were fed the diet supplemented with FKC-VpD6 equivalent to approximately 5% of body weight per day for one week. In addition, while performing the second repetition, moribund shrimps were collected at 24, 48, and 72 h for histological analysis. After one week feeding, all surviving shrimps from the first repetition and a part of surviving shrimp from the second repetition were collected and total RNA was extracted from the hepatopancreases. One μg of total RNA from individual shrimp was used for cDNA synthesis. The expression of *Lv*ALF AV-R was quantified by qPCR as described above.

2.6. Histopathology of the hepatopancreas

L. vannamei from the second repetition were used. Shrimp were collected after feeding FKC-VpD6 for 24, 48, and 72 h. After feeding for one week, some surviving shrimps were also collected for histological examination. The hepatopancreases were fixed in Davidson's fixative solution for 24 h, transferred to 70% ethanol, dehydrated with 70%–100% ethanol, embedded in paraffin, and sectioned at 5 μm thickness. The sections were stained with hematoxylin and eosin (H&E) following the standard methods [24].

2.7. Production of LvALF AV-R recombinant protein

*Lv*ALF AV-R without its signal peptide was amplified from the hepatopancreas of sur-FKC shrimp using the rALF AV-R/no SP primer set, where *Eco*RI and *Not*I were added to the forward (5′ end) and reverse (3′ end) primers, respectively (Table 1). The *Lv*ALF AV-R amplicon was cloned into pGEM®-T easy vector, which was then transformed into *E. coli* JM109 (Nippon gene, Japan). A plasmid carrying the correct sequence was digested with *Eco*RI and *Not*I and ligated to pMT/BiP/V5-His C Drosophila Expression Vectors (Invitrogen, USA). The ligated vector and pCoBlast vector were co-transfected into *Drosophila* S2 cells with Effectene Transfection Reagent (Qiagen, Germany) following the manufacturer's instructions. Stable cell lines were selected by passaging the cells several times in Schneider *Drosophila* medium (SDM) containing 25 μg/ml blasticidin.

Following large-scale culture of the stable cell lines, protein expression was induced with 500 mM $CuSO_4$. Five days after induction, the cultured cells were centrifuged at 300 × g and the supernatant was collected. The supernatant was filtered through a 0.22 μm filter (Merck Millipore Ltd., Ireland). The recombinant protein was then purified with a polypropylene column containing Ni-NTA agarose (Qiagen, Germany), following the manufacturer's protocol. The concentration of recombinant protein was measured with a Qubit™ Protein Assay Kit (ThermoFisher Scientific, USA). A GFP recombinant protein was also constructed as a negative control in further experiments.

2.8. SDS-PAGE and Western blot analysis of recombinant proteins

The purified recombinant proteins of *Lv*ALF AV-R (rALF AV-R) and GFP (rGFP) were mixed with an equal volume of 2xSDS sample buffer and boiled for 5 min. Recombinant proteins were separated by 15% SDS-PAGE. The electrophoresed gel was stained with CGP [25] or blotted onto 0.2 μm PVDF membrane (Atto, Japan) for Western blot analysis. The blotted membrane was blocked with 2% skimmed milk in Tris-buffered saline (TBS) with 0.05% Tween 20 for 1 h, incubated with V5 tag monoclonal antibody (Invitrogen, USA) as primary antibody (1:5000 dilution in blocking buffer) for 1 h, washed 4 times with TBS - 0.05% Tween 20 (washing buffer), incubated with anti-mouse IgG (H + L), AP conjugate (Promega, Japan) as secondary antibody (1:5000 dilution in blocking buffer) for 30 min, washed 3 times with washing buffer, and stained with BCIP/NBT alkaline phosphatase substrate (Sigma-Aldrich, Japan) to visualize the detected proteins.

2.9. Bacterial polysaccharides binding of rALF AV-R

Bacterial polysaccharides, including lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (SIGMA, Germany) and peptidoglycan (PGN) derived from *Bifidobacterium thermophilum* were used for enzyme-linked immunosorbent assay (ELISA). LPS and PGN were dissolved in phosphate-buffered saline (PBS) at a concentration of 50 μg/ml. One hundred μl (5 μg) of polysaccharide solution was used to coat each well of a Clear Flat-Bottom Immuno Nonsterile 96-Well Plate (Thermo Scientific, USA). The plate was incubated at 4 °C for 12–16 h, washed with 100 μl of PBS with 0.5% tween 20 (washing buffer) three times, and blocked with 1% bovine serum albumin (BSA) in PBS (blocking buffer) at room temperature (RT) for 2 h. The rALF AV-R or rGFP (control) was diluted with PBS to four different concentrations (2.5, 5, 10, and 20 μg/ml). After blocking, the plate was washed three times, incubated with 100 μl of diluted recombinants (0.25, 0.5, 1, and 2 μg) at RT for 1 h, washed three times, incubated with 100 μl of monoclonal anti-V5 antibody (1:5000 dilution in PBS) at RT for 1 h, washed three times, incubated with 100 μl of anti-mouse IgG (H + L), AP conjugate at RT for 1 h, washed three times, mixed with 100 μl of phosphatase substrate solution (Sigma-Aldrich, Japan), incubated in the dark for 15 min, mixed with 50 μl of 3 N NaOH solution to stop reaction. The absorbance was read at 405 nm. The average test values were deducted with average value of negative control wells, which coated with PBS.

2.10. Bacterial binding activity of rALF AV-R

Four bacterial species including two Gram-negative bacteria (VpD6 and *E. coli*) and two Gram-positive bacteria (*Streptococcus agalactiae* and *Bacillus amyloliquefaciens*) were used in the ELISAs. *V. parahaemolyticus* was cultured in heart infusion broth at 30 °C, *S. agalactiae* was cultured in Todd Hewitt broth at 37 °C, and *E. coli* and *B. amyloliquefaciens* were cultured in LB broth at 37 °C for 12–16 h. The cells were pelleted and resuspended in 1xPBS. The ELISA 96-well plate was coated with 100 μl of bacterial suspension (10^7–10^8 CFU/ml), incubated at 4 °C for 12–16 h and blocked with blocking buffer at RT. rALF AV-R or rGFP was then added to each well as described in bacterial polysaccharides binding assay.

2.11. Binding property of rALF AV-R and Vp_PirAB-like toxin

2.11.1. ELISA

Recombinant Vp_PirA-like and Vp_PirB-like were prepared as previously described [12]. Two μg of recombinant toxin was coated on an ELISA 96-well plate. The plate was incubated at 4 °C for 12–16 h and blocked with blocking buffer. rALF AV-R or rGFP was added to each well to determine the binding as described above. In addition, the native Vp_PirAB-like toxin was isolated from cell-free supernatant of VpD6 with 65% ammonium sulfate (modified from Sirikharin et al. [26]). Twenty-five μg of cell-free supernatant (suspended in PBS) was used in the binding assay with rALF AV-R, whereas, the cell-free supernatant protein of VpN7 was used as control.

2.11.2. Far-Western blotting

The cell extract or cell-free supernatant of VpD6 and VpN7 and recombinant Vp_PirA- and B-like proteins were used as bait protein. The cell extract was prepared by sonication of bacterial cells in cold 1xPBS. The soluble fraction was filtered through a 0.22 μm filter (Merck Millipore Ltd., Ireland) and its concentration was measured with a Qubit™ Protein Assay Kit (ThermoFisher Scientific, USA). Six μg of cell extract, 12 μg of supernatant, and 500 ng of recombinant toxin proteins were separated by 18% SDS-PAGE and transferred onto PVDF membranes. In addition, 6 μg of BSA was loaded as a negative control. The

blotted membranes were blocked and probed with rALF AV-R or rGFP (control) for 1 h. The binding complex was detected with V5 tag antibody and anti-mouse IgG (H + L), AP conjugate as primary and secondary antibodies, respectively. The interaction was visualized by adding BCIP/NBT alkaline phosphatase substrate.

A Western blot analysis was performed to detect the Vp_PirA- and B-like toxin proteins in the samples. Specific antisera against Vp_PirA- and B-like peptides [12] were used as primary antibodies (1:5000), followed by anti-rabbit IgG(Fc), AP conjugate (Promega, Japan) as secondary antibody and BCIP/NBT alkaline phosphatase substrate.

2.12. Oral administration of rALF AV-R and challenge test

The rALF AV-R-supplemented diet was prepared by mixing recombinant protein (suspended in PBS) in commercial feed powder (about 65 μg/g). The mixture was pelleted, and the pellets were dried at 60 °C for 2 h. rGFP- and PBS-supplemented diets were also prepared for controls.

L. vannamei (body weight 2–3 g) were reared in three 50-L tanks (50 shrimps each) with artificial seawater (28–32 ppt.) at 28 °C. The shrimps were fed rALF AV-R at a rate of 10 μg/shrimp/day (corresponding to 5% of body weight per day). After three days of feeding, shrimp from each group were divided into two groups (18 shrimps each) for immersion challenge with VpD6 at final concentrations of 2.5×10^5 and 5×10^5 CFU/ml. The survival rate was observed at 12, 24, 36, 48, 60 and 72 h post challenge. During the challenge tests, the shrimps were continually fed the supplemented diets until the end of the experiment. The survival rates were plotted as Kaplan-Meier curves.

2.13. Statistical analysis

mRNA expressions (log-values) as determined by qPCR were analyzed by one-way ANOVA with Dunnett's multiple comparison test at a P value < 0.05. The survival rate was analyzed using the Kaplan-Meier method and compared using the log-rank test at a P value < 0.05. Statistical analyses and graphs were performed using GraphPad Prism 7.04 (GraphPad Software Inc., USA).

3. Results

3.1. Illumina sequencing and de novo assembly

Of a total of 200 *L. vannamei*, only 4 survived after feeding with FKC-VpD6 for 2 weeks. Raw reads were obtained from stomach and hepatopancreas: 21,485,676 from the control group, 24,930,246 from the Vp-inf group, and 18,507,162 from the sur-FKC group. The assembled transcriptome generated 94,135 transcripts with an average size of 905 bp, representing 79,591 genes (Table 2).

3.2. Identification of DEGs

The pairwise comparison of DEGs between the three groups of control, Vp-inf and sur-FKC from each tissue were analyzed and displayed with Venn diagrams. In the hepatopancreas, a total of 319 DEGs were detected and 83 genes in sur-FKC showed significantly differential expression with two other groups (Fig. 1A). In the stomach, a total of 250 DEGs were detected and 18 genes in sur-FKC showed significantly differential expression with two other groups (Fig. 1A). The comparisons of sur-FKC and other groups showed that most DEGs found in the hepatopancreas were low-expressed genes, whereas most DEGs found in the stomach were high-expressed genes (Fig. 1B).

Of the 218 annotated DEGs in the hepatopancreas, 161 encoded known proteins (Table S1), whereas of the 187 annotated DEGS in the stomach, 91 encoded known proteins (Table S2). The immune-related genes found in the DEGs were manually grouped into proteins of six functional types for the hepatopancreas (Table S3) and into proteins of four functional types for the stomach (Table S4). The main gene ontology (GO) categories were catalytic activity in the hepatopancreas and structural molecular activity in the stomach (Fig. 1C).

3.3. Validation of mRNA expression from RNA-Seq

Of the 10 selected DEGs, the expression level of 8 genes were examined in the hepatopancreas. The expression of five of these genes (DN13910, DN21485, DN20905, DN13245 and DN21632) were consistent with the RNA sequence data in all comparisons (Fig. 2A). Of the three genes that were examined in the stomach, only one of them (DN3154) was expressed at a level similar to that in the RNA sequence data (Fig. 2B). Of all the genes validated by qPCR, only one gene (DN21485) was significantly expressed in the hepatopancreas of surviving shrimp. Therefore, gene DN21485 which encodes a protein identical to *L. vannamei* anti-lipopolysaccharide factor AV-R isoform (*Lv*ALF AV-R), was furthered examined.

3.4. Expression of LvALF AV-R mRNA

In healthy *L. vannamei*, *Lv*ALF AV-R was highly expressed in hemocytes, heart and lymphoid organ but very weakly expressed in nerve, muscle, eye, and hepatopancreas (Fig. 3A). *Lv*ALF AV-R was also expressed in the hepatopancreas of *L. vannamei* fed FKC-N7 and upon infection with *V. parahaemolyticus* AHPND-causing strain D6 (VpD6). After treatment, expression of *Lv*ALF AV-R slightly increased at 3 d of VpD6 treatment and at 7 d of both treatments. Nevertheless, the two treated groups did not significantly differ from the control at any of the sampling times (Fig. 3B).

FKC-D6 feeding was repeated in the second and third trials to obtain AHPND-toxin resistant shrimp. After one week feeding, 16 shrimps (3.2%) survived in the second trial and 32 shrimps (6.4%) survived in the third trial. All surviving shrimps were collected and named as sur-

Table 2
Summary of reads in control, Vp-inf and sur-FKC transcriptomes.

Trinity Outputs	control		Vp-inf		sur-FKC	
	St*1	HP*2	St	HP	St	HP
Number of raw reads	13,516,128	7,969,548	10,328,124	14,602,122	9,564,080	8,943,082
Total trinity 'genes'	79,591					
Total trinity 'transcripts'	94,135					
Percent GC	44.72					
Transcript contig N50	1777					
Median contig length (base)	422					
Average contig (base)	905.37					
Total assembled bases	85,226,619					

(*1 St = stomach and *2 HP = hepatopancreas).

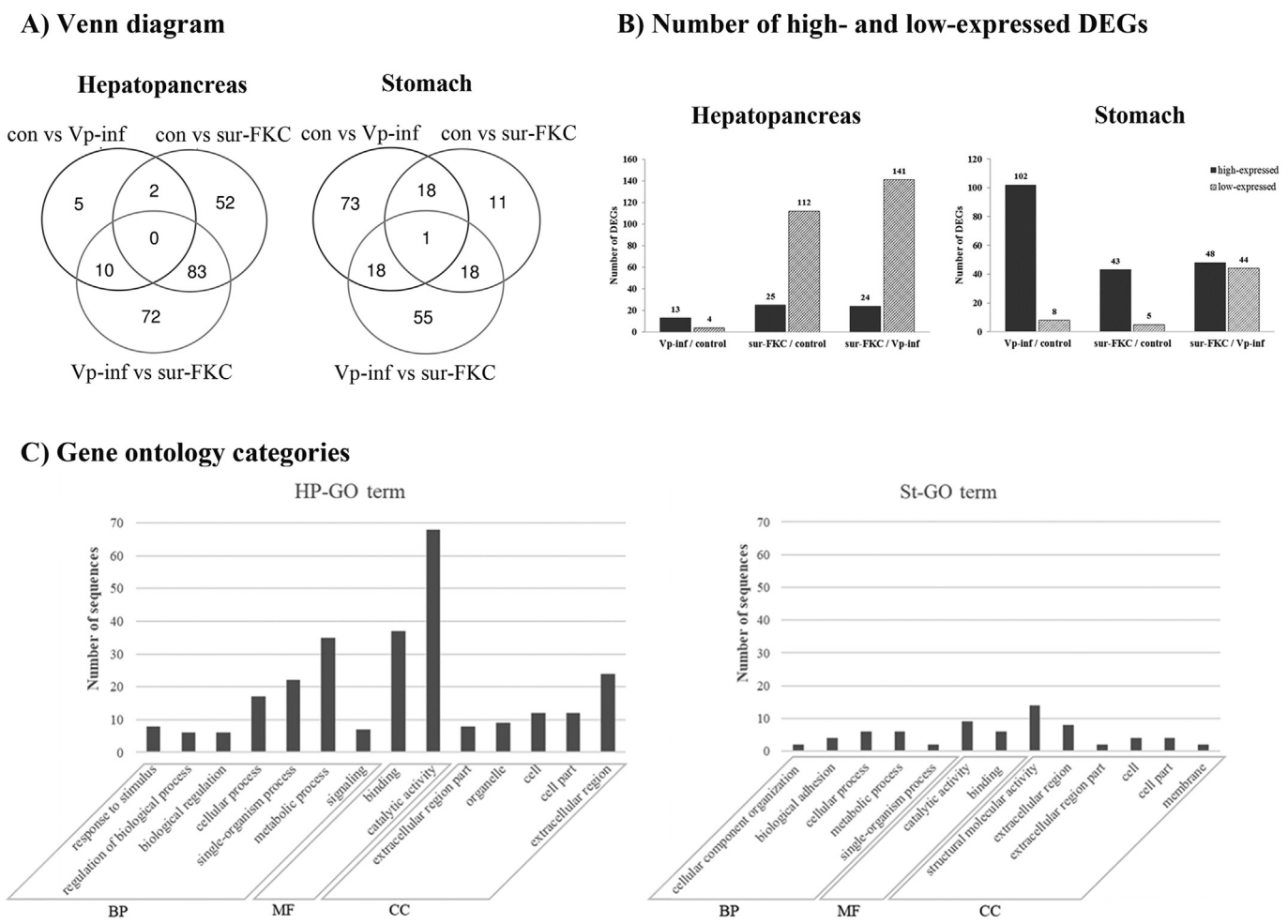

Fig. 1. Identification of differentially expressed genes (DEGs). **A)** Venn diagram showing the number of differentially expressed genes by pairwise comparison among 3 groups of control, Vp-inf and sur-FKC in the hepatopancreas and stomach. Venn diagrams were drawn by http://bioinformatics.psb.ugent.be/webtools/Venn/. **B)** Numbers of high-expressed and low-expressed genes of DEGs in Vp-inf and sur-FKC compared to control and sur-FKC compared to Vp-inf. **C)** Gene ontology (GO) classification of differentially expressed genes in the hepatopancreas and stomach. Categories of GO are classified into three group of biological process (BP), molecular function (MF) and cellular component (CC).

FKC_2 and sur-FKC_3. In the latter case, mRNA expression was examined in 24 of 32 shrimps. *Lv*ALF AV-R mRNA was highly expressed in the hepatopancreas of surviving shrimp in each of the three trials. Its expression in the surviving shrimp of trial 2 was also significantly higher than it was in the control and Vp-inf shrimp, whereas surviving shrimp of trial 3 was significantly higher than control group (Fig. 3C).

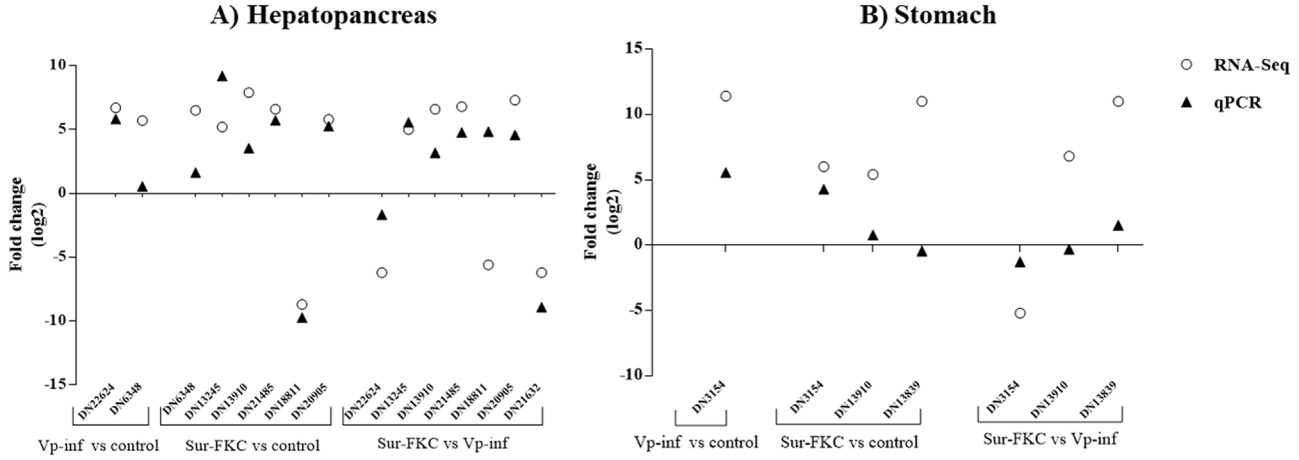

Fig. 2. Expression levels of selected DEGs measured by RNA sequencing and qPCR in the hepatopancreas (**A**) and stomach (**B**). In each tissue, pairwise comparisons are made among the three groups, Vp-inf, sur-FKC and control. qPCR results are presented as mean from three individual shrimp of control or Vp-inf and four individual shrimp of sur-FKC.

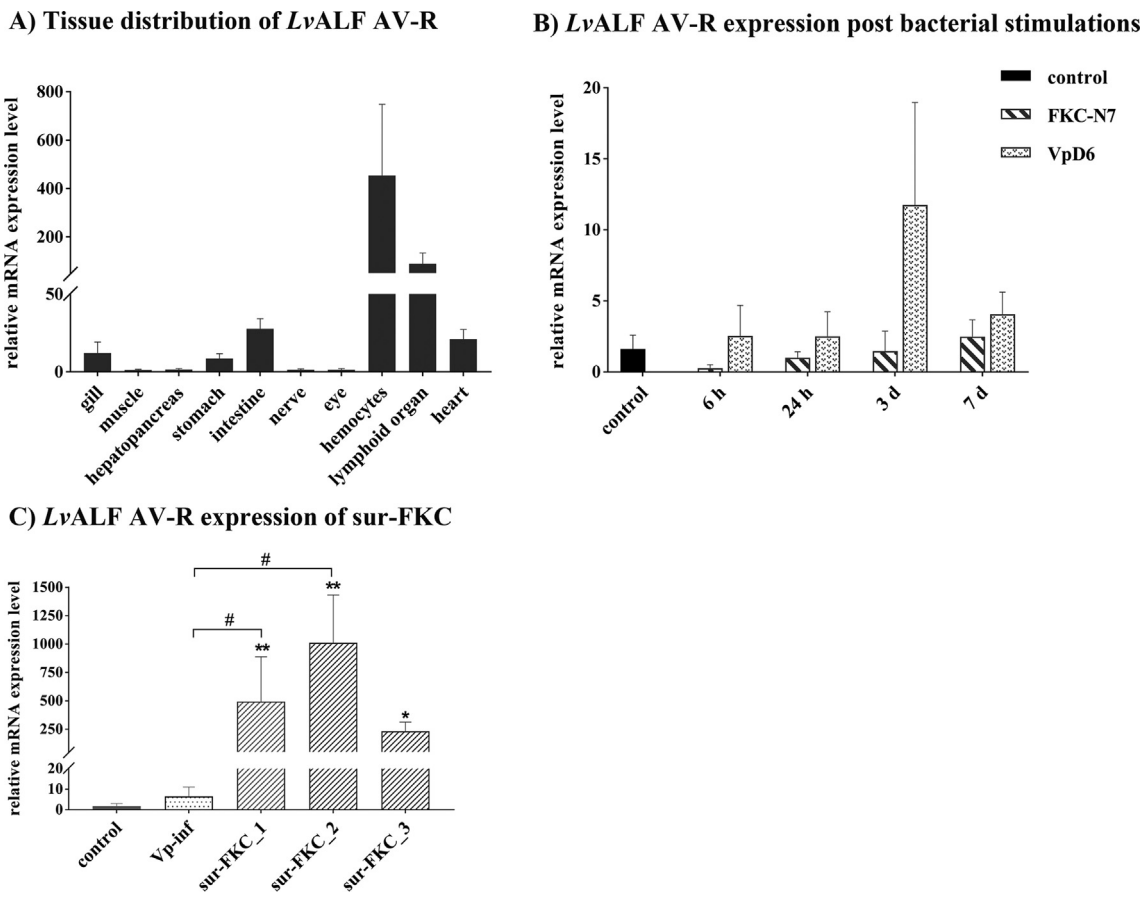

Fig. 3. The mRNA expression of *Lv*ALF AV-R in *L. vannamei* hepatopancreas measured by qPCR. **A)** Expression in different tissues of healthy shrimp (N = 3) normalized to expression in the nerve. **B)** Expression after feeding with FKC-N7 (N = 3) and immersion in VpD6 (N = 3). **C)** Expression in surviving shrimps after feeding with FKC-VpD6 from three trials of sur-FKC-1 (N = 4), sur-FKC-2 (N = 16), and sur-FKC-3 (N = 24), compared with control (* significant difference) and Vp-inf (# significant difference). Data are presented as mean and vertical bars represent ± SEM. Statistical analysis was analyzed by one-way ANOVA with Dunnett's multiple comparison test at P value < 0.05.

3.5. Histological changes in the hepatopancreas

The hepatopancreas of *L. vannamei* after being fed with FKC-VpD6 for 24 h (Fig. 4B), 48 h (Fig. 4C), and 72 h (Fig. 4D) showed signs of AHPND, such as sloughing of tubule epithelial cells, hemocytic infiltration surrounding the hepatopancreas tubules, and enlargement of some nuclei in hepatopancreas tubule epithelial cells. One of the four surviving shrimps examined showed some hemocytic infiltration and enlarged nuclei, but without the sloughing of epithelial tubule cells (Fig. 4E). The hepatopancreases of the other three did not show any signs of AHPND (Fig. 4F–H) and were similar to the hepatopancreas in a non-treated shrimp (Fig. 4A).

3.6. Purified recombinant protein expression

Recombinant r*Lv*ALF AV-R was successfully expressed in insect cell lines. rALF AV-R is composed of 163 amino acid residues with addition of a V5 epitope and HIS tag at the C-terminus. Purified rALF AV-R and rGFP were observed in SDS-PAGE gel at the predicted sizes of 16.4 and 33.7 kDa, respectively (Fig. 5A). A Western blot analysis using an anti-V5 monoclonal antibody detected both recombinant proteins at the predicted size (Fig. 5B).

3.7. Binding activities of LvALF AV-R

LPS and PGN were used as the representatives of cell wall components of Gram-negative and Gram-positive bacteria, respectively. In ELISAs, rALF AV-R bound to LPS and PGN (Fig. 6A) and Gram-negative bacteria (VpD6 and *E. coli*) (Fig. 6B). With respect to Gram-positive bacteria, rALF AV-R bound to *B. amyloliquefaciens*, but not *S. agalactiae*, while rGFP also bound to *B. amyloliquefaciens* but only at the highest concentration (Fig. 6C).

3.8. Interaction of rALF AV-R and Vp_PirAB-like toxin

In ELISAs, rALF AV-R strongly bound to Vp_PirB-like recombinant protein (toxB), but not to native toxin in the VpD6 supernatant or Vp_PirA-like recombinant (toxA) (Fig. 7A). rGFP also bound to Vp_PirB-like recombinant protein (Fig. 7A).

Far-Western blot analysis was performed to confirm the interaction of rALF AV-R and native Vp_PirAB-like toxin. In the left panel of Fig. 7B, rALF AV-R-binding proteins were detected, either in VpD6 or VpN7 at approximately size of 10 kDa in the cell extract and 10–15 kDa in the supernatant. In the right panel of Fig. 7B, rGFP-binding proteins were detected in Vp_PirA-like recombinant (toxA) at size of 15 kDa and in Vp_PirB-like recombinant (toxB) at size of 50 kDa (indicated by arrow).

Western blotting detected the Vp_PirA- and B-like proteins (at their predicted sizes of 15 kDa and 50 kDa, respectively) in the cell extract and supernatant of VpD6, but not in the cell extract or supernatant of VpN7 (Fig. 7C). The recombinant toxA and toxB were used as controls.

3.9. In vivo effect of LvALF AV-R recombinant protein

L. vannamei treated with rALF AV-R, rGFP, and PBS supplemented diets were challenged with VpD6-immersion at final concentrations of 2.5×10^5 and 5×10^5 CFU/ml. The survival rate of shrimp treated

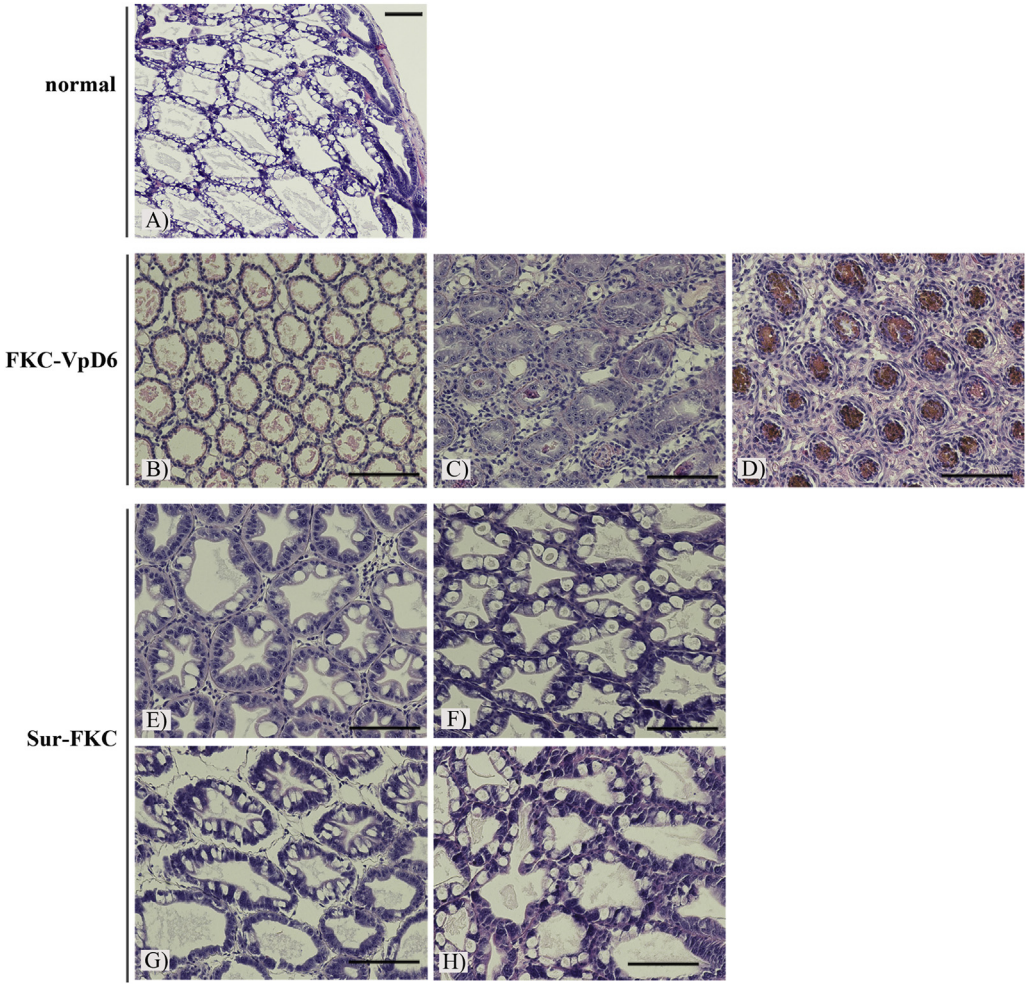

Fig. 4. Histological sections of *L. vannamei* hepatopancreas with H&E staining. **A)** Normal shrimp without treatment. **B-D)** Shrimp after feeding with FKC-VpD6 for 24 h, 48 h, and 72 h, respectively. **E-H)** Four individual surviving shrimps after one week feeding with FKC-VpD6. Scale bars = 100 μm.

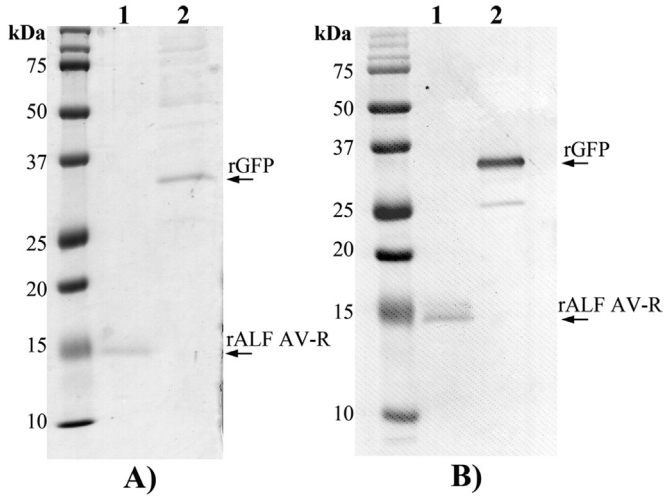

Fig. 5. Expression of purified recombinant proteins rALF AV-R (lane 1) and rGFP (lane 2). **A)** CGP stained gel and **B)** Western blot analysis using anti-V5 monoclonal antibody.

with rALF AV-R was not significantly different from that of shrimp treated with PBS or rGFP groups at 72 h post challenge with either dose (Fig. 8A and B). However, the survival rate of the rALF AV-R group was higher than that of PBS group at most other observation times for both challenges.

4. Discussion and conclusion

Anti-lipopolysaccharide factor (ALF) is a small polypeptide (114–124 amino acid residues) that belongs to an antimicrobial peptide family [27]. ALFs were initially characterized in horseshoe crabs [28–30] and were subsequently identified in other crustaceans, e.g. crab, crayfish, lobster, and shrimp [31–36]. Typically, ALFs contain a hydrophobic N-terminal region and a conserved LPS-binding domain, which is flanked by two conserved cysteine residues that form a disulfide bond [37–39]. The ALFs of penaeid shrimp have a wide-range of functions in shrimp immunity, being involved in anti-bacterial, anti-fungal, and anti-viral activities [40–45].

Our RNA-seq and qPCR results revealed that a gene homologous to an *Lv*ALF AV-R isoform was significantly more strongly expressed in the hepatopancreas of surviving *L. vannamei* after being fed with FKC-VpD6 diet for 1 or 2 weeks. The surviving shrimps (sur-FKC) were hypothesized to be Vp_PirAB-like toxin-resistant. On the other hand, other ALF isoforms such as ALF VV-R, ALF AV-K, and ALF AA-K were not detected in our transcriptomes. It might because of these isoforms are typically expressed in hemocyte [46,47]. To determine whether the high expression of *Lv*ALF AV-R in the hepatopancreas of the resistant shrimp was due to the typical cell components of *V. parahaemolyticus* in the diet, we fed other shrimp with FKC of *V. parahaemolyticus* non-AHPND causing strain. These shrimps did not show a significant increase in the expression of *Lv*ALF AV-R in the hepatopancreas (Fig. 3B). Similarly, a

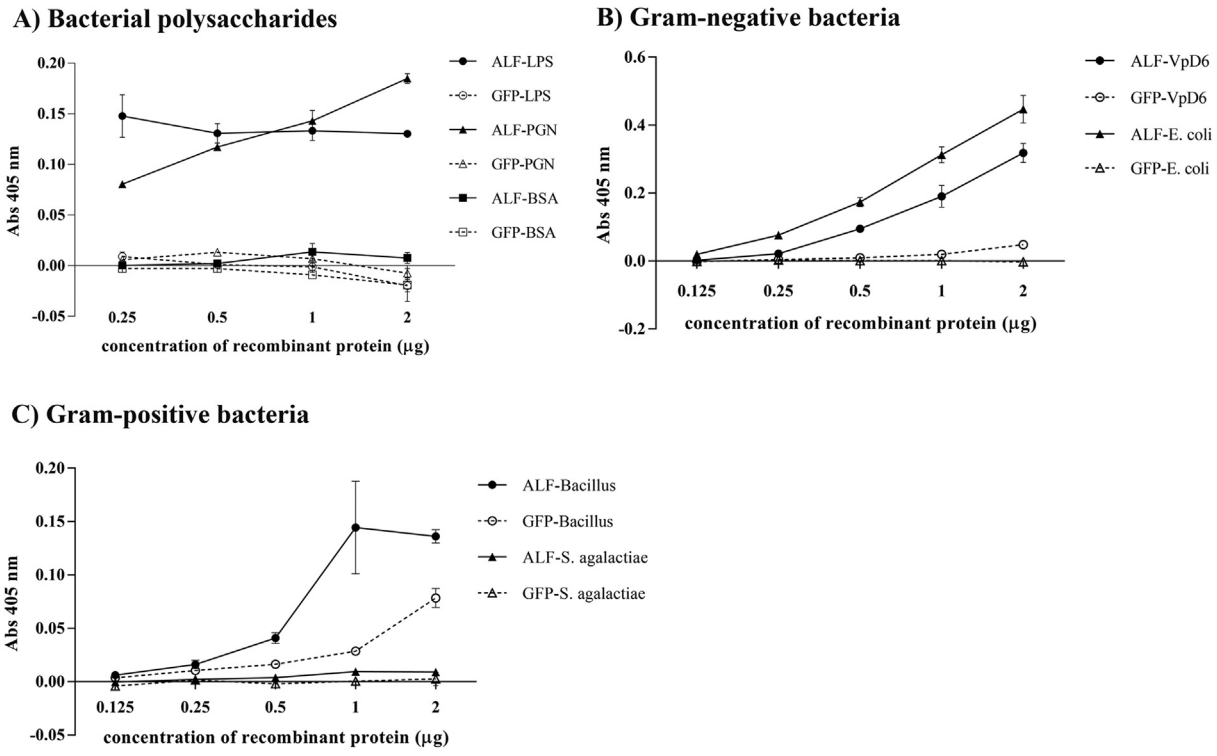

Fig. 6. Binding activity of rALF AV-R to bacterial proteins. **A)** Binding of LPS and PGN polysaccharides. **B)** Gram-negative bacteria binding. **C)** Gram-positive bacteria binding. rGFP was added in all experiments as negative recombinant protein. These data represent the mean ± SEM of at least two independent experiments.

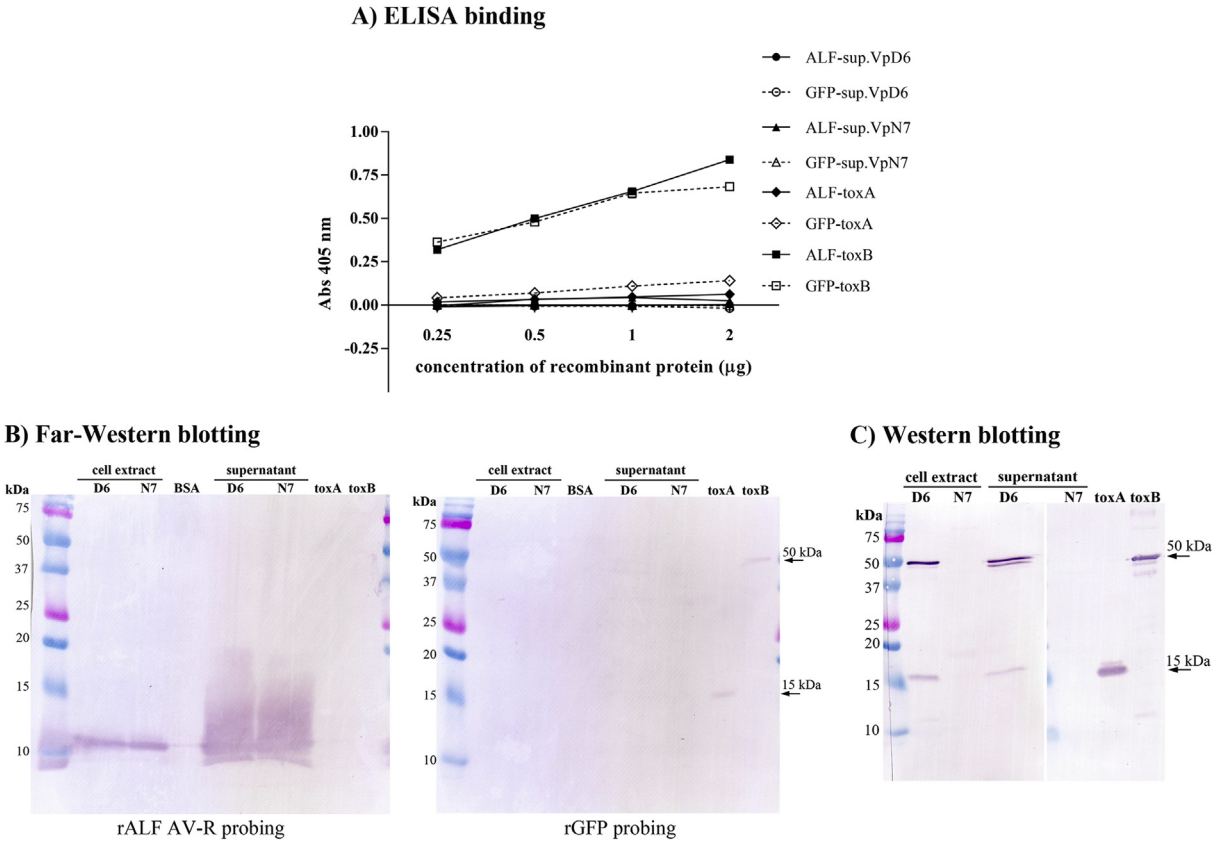

Fig. 7. The interaction of *Lv*ALF AV-R and the Vp_PirAB-like toxin. **A)** ELISA binding assay of native toxin from supernatant of VpD6 (sup.VpD6) and the Vp_PirA- and B-like toxin recombinants (toxA and toxB), whereas the supernatant protein of VpN7 (sup.VpN7) was used as control. These data represent the mean ± SEM of at least two independent experiments. **B)** Far-Western blot analysis of cell extract and supernatant from VpD6 or VpN7, and recombinant Vp_PirA- and −B-like (toxA and toxB), probed with rALF AV-R or rGFP. BSA was loaded as negative control. **C)** Detection of Vp_PirA-like and Vp_PirB-like proteins with specific antibody against Vp_PirA- or B-like toxin in the used sampled of Far-Western blotting.

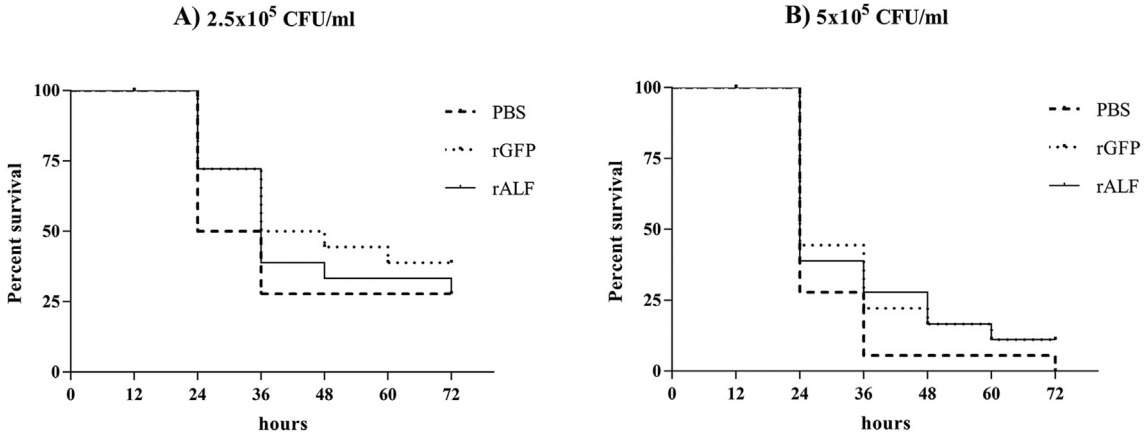

Fig. 8. Survival rate of *L. vannamei* treated with rALF AV-R (rALF) and challenged with two doses of VpD6. **A)** 2.5 × 10^5 CFU/ml and **B)** 5 × 10^5 CFU/ml. rGFP and PBS treated shrimps were used as control groups.

previous report showed that inoculation of heat-killed *V. alginolyticus* did not upregulate *Lv*ALF AV-R in shrimp hemocytes mRNA expression [46]. These results suggest that upregulation of *Lv*ALF AV-R mRNA expression in the shrimp hepatopancreas is a specific response to the Vp_PirAB-like toxin. On the other hand, immersing shrimp in VpD6 for one week (a non-lethal challenge), also did not significantly induce the expression of *Lv*ALF AV-R in the hepatopancreas (Fig. 3B). This was probably because the amount of toxin in the immersion test was lower than that in the FKC-VpD6 feeding experiment.

In healthy shrimp, *Lv*ALF AV-R mRNA was highly expressed in hemocytes but barely expressed in the hepatopancreas (Fig. 3A), in agreement with previous reports [47–49]. Histological sections of FKC-VpD6-fed shrimps showed that epithelial cells were infiltrated with hemocytes surrounding the hepatopancreas tubules (Fig. 4B–D), raising the possibility that these hemocytes may have increased *Lv*ALF AV-R expression. However, this seems to be unlikely in the case of the resistant shrimp, whose hepatopancreases appeared normal, without hemocytic infiltration (Fig. 4G–H).

*Lv*ALF AV-R mature protein has a predicted molecular weight of 11.12 kDa and theoretical pl of 10.17. Based on the theoretical pl of protein, *Lv*ALF AV-R is classified under a highly cationic ALF family and most cationic AMPs bind to bacterial polysaccharides or exhibit antimicrobial activities [50]. Our ELISAs showed that rALF AV-R bound to LPS and PGN (Fig. 6A), which are the major cell wall component of Gram-negative and Gram-positive bacteria, respectively. Moreover, rALF AV-R more strongly bound to Gram-negative bacteria than to Gram-positive bacteria (Fig. 6B and C) as expected because it contains an LPS-binding domain. The unexpected finding that rALF AV-R didn't bind to the Gram-positive *S. agalactiae* (Fig. 6C) might be due to the presence of different cell wall components in this species, compared to *Bacillus* spp. Most Gram-positive bacteria incorporate peptidoglycan, anchored with cell-wall glycopolymers, which have highly variable structures among species [51].

The finding that rALF AV-R bound to recombinant Vp_PirB-like toxin but not to native toxin (Fig. 7A) might be a false-positive result because recombinant Vp_PirB-like also bound to rGFP (Fig. 7A). This was supported by the Far-Western blotting, which showed that rALF AV-R interacted with ~10 kDa V. *parahaemolyticus* proteins but not with the toxin proteins (Fig. 7B). These rALF AV-R-binding proteins may be lipopolysaccharide-related proteins that are mainly found in Gram-negative bacteria. Also, in the Far-Western blotting, unlike the ELISA, rALF AV-R did not bind to Vp_PirB-like recombinant protein, while rGFP bound to both Vp_PirA- and B— like recombinant proteins. These results suggest that the recombinant proteins could differ in structures and functions from the native forms [52]. Moreover, the structures of Vp_PirA- and B-like toxins under non-denaturing (ELISA) and denaturing (Far-Western) conditions were different and may have affected their interactions.

Previous studies have reported that ALF isoform 3 of *Penaeus monodon* (ALF*Pm*3) [53] and a single WAP domain-containing protein of *L. vannamei* (*Lv*SWD) [54], exhibited antimicrobial activities against *V. parahaemolyticus* AHPND-causing strains and promoted survival of *L. vannamei* after infections. For testing the effect of rALF AV-R to Vp_PirAB-like toxin, an immersion challenge with VpD6 was used instead of FKC-VpD6 feeding, to imitate the uptake of toxin in natural condition. In this study, the survival rate of rALF AV-R-fed shrimp after challenge was not significantly different from that of the control groups (Fig. 8). However, the mortality of the rALF AV-R-fed shrimp was not as sudden as it was for the PBS group at both doses of VpD6. Hence, increasing the rALF AV-R concentration in the diet or the feeding time prior to an infection may increase survival. Also, the finding that the survival of rGFP group has higher than that of the PBS group and rALF AV-R group (Fig. 8) might be due to non-self molecule recognition either by innate effector molecules or phagocytosis [55,56] that raised immune defenses against AHPND infection.

On the other hand, the cause of AHPND-resistance might be due to the mutations of some specific genes that affect the action of toxin. The Vp_PirAB-like toxin has structure similar to *B. thuringiensis* Cry toxins, therefore they might also use similar mechanisms to cause cell death in the host [57]. The studies on Cry toxin-resistant insects revealed that the mutations of toxin-receptor genes are the main cause of resistance. Mutated receptor genes alter binding between toxin and receptor, resulting in the reduction of toxicity or resistance [58,59]. To investigate possible occurrence of mutation causing toxin resistance as previously reported, survival shrimp were collected 1 week-post FKC-VpD6 feeding and stocked for 1 week. Shrimp were then re-fed with FKC-VpD6 but result showed that all shrimp died after 3 days (data not shown). Thus, it is highly possible that shrimp survived because of the mechanisms of immune defense, as shown in a study of *L. vannamei* tolerance to AHPND after non-lethal heat shock [60].

In conclusion, our results demonstrate that *Lv*ALF AV-R transcription is upregulated in the hepatopancreas of toxin-resistant *L. vannamei* in response to Vp_PirAB-like toxin. However, *Lv*ALF AV-R may not be the only factor involved in the defense against Vp_PirAB-like toxin virulence.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgments

This research was supported in part by Japan Science and Technology Agency/Japan International Cooperation Agency, Science and Technology Research Partnership for Sustainable Development (JST/JICA, SATREPS), grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Heiwa Nakajima Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.005.

References

[1] F.A.O. Global, Aquaculture Production 1950-2015, (2017) http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en , Accessed date: 30 May 2017.
[2] P.J. Walker, J.R. Winton, Emerging viral diseases of fish and shrimp, Vet. Res. 41 (2010) 51.
[3] S. Thitamadee, A. Prachumwat, J. Srisala, P. Jaroenlak, P.V. Salachan, K. Sritunyalucksana, T.W. Flegel, O. Itsathitphaisarn, Review of current disease threats for cultivated penaeid shrimp in Asia, Aquaculture 452 (2016) 69–87.
[4] FAO, Report of the FAO/MARD Technical Workshop on Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) of Cultured Shrimp (Under TCP/VIE/3304). Hanoi, Viet Nam, on 25–27 June 2013, FAO Fisheries and Aquaculture Report No. 1053. Rome, (2013), p. 54.
[5] T.W. Flegel, Historic emergence, impact and current status of shrimp pathogens in Asia, J. Invertebr. Pathol. 110 (2012) 166–173.
[6] D.V. Lightner, R.M. Redman, C.R. Pantoja, B.L. Noble, L. Tran, Early mortality syndrome affects shrimp in Asia, Global Aquaculture Advocate, 2012, p. 40 January/February.
[7] B. Gomez-Gil, S. Soto-Rodríguez, R. Lozano, M. Betancourt-Lozano, Draft genome sequence of *Vibrio parahaemolyticus* strain m0605, which causes severe mortalities of shrimps in Mexico, Genome Announc. 2 (2014) e00055-14.
[8] I.M. Dabu, J.J. Lim, P.M.T. Arabit, S.J.A.B. Orense, J.A. Tabardillo, V.L. Corre, M.B.B. Maningas, The first record of acute hepatopancreatic necrosis disease in the Philippines, Aquacult. Res. 48 (2017) 792–799.
[9] H. Kondo, S. Tinwongger, P. Proespraiwong, R. Mavichak, S. Unajak, R. Nozaki, I. Hirono, Draft genome sequences of six strains of *Vibrio parahaemolyticus* isolated from early mortality syndrome/acute hepatopancreatic necrosis disease shrimp in Thailand, Genome Announc. 2 (2014) e00221-14.
[10] J.E. Han, K.F. Tang, L.H. Tran, D.V. Lightner, Photorhabdus insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp, Dis. Aquat. Org. 113 (2015) 33.
[11] C.T. Lee, I.T. Chen, Y.T. Yang, T.P. Ko, Y.T. Huang, J.Y. Huang, M.F. Huang, S.J. Lin, C.Y. Chen, S.S. Lin, D.V. Lightner, H.C. Wang, A.H.J. Wang, H.C. Wang, L.I. Hor, C.F. Lo, The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin, Proc. Natl. Acad. Sci. U.S.A. 112 (2015) 10798–10803.
[12] S. Tinwongger, Y. Nochiri, J. Thawonsuwan, R. Nozaki, H. Kondo, S.P. Awasthi, A. Hinenoya, S. Yamasaki, I. Hirono, Virulence of acute hepatopancreatic necrosis disease PirAB-like relies on secreted proteins not on gene copy number, J. Appl. Microbiol. 121 (2016) 1755–1765.
[13] H. Kondo, P.T. Van, L.T. Dang, I. Hirono, Draft genome sequence of non-*Vibrio parahaemolyticus* acute hepatopancreatic necrosis disease strain KC13. 17.5, isolated from diseased shrimp in Vietnam, Genome Announc. 3 (2015) e00978-e001015.
[14] J. Xiao, L. Liu, Y. Ke, X. Li, Y. Liu, Y. Pan, S. Yan, Y. Wang, Shrimp AHPND-causing plasmids encoding the PirAB toxins as mediated by pirAB-Tn903 are prevalent in various Vibrio species, Sci. Rep. 7 (2017).
[15] X. Dong, H. Wang, G. Xie, P. Zou, C. Guo, Y. Liang, J. Huang, An isolate of *Vibrio campbellii* carrying the pirVP gene causes acute hepatopancreatic necrosis disease, Emerg. Microb. Infect. 6 (2017) e2.
[16] J.Y. Roh, J.Y. Choi, M.S. Li, B.R. Jin, Y.H. Je, *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control, J. Microbiol. Biotechnol. 17 (2007) 547.
[17] L. Tran, L. Nunan, R.M. Redman, L.L. Mohney, C.R. Pantoja, K. Fitzsimmons, D.V. Lightner, Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp, Dis. Aquat. Org. 105 (2013) 45–55.
[18] H.C. Lai, T.H. Ng, M. Ando, C.T. Lee, I.T. Chen, J.C. Chuang, R. Mavichak, S.H. Chang, M.D. Yeh, Y.A. Chiang, H. Takeyama, Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp, Fish Shellfish Immunol. 47 (2015) 1006–1014.
[19] M.G. Grabherr, B.J. Haas, M. Yassour, J.Z. Levin, D.A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, Full-length transcriptome assembly from RNA-Seq data without a reference genome, Nat. Biotechnol. 29 (2011) 644–652.
[20] A. Conesa, S. Götz, J.M. García-Gómez, J. Terol, M. Talón, M. Robles, Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics 21 (2005) 3674–3676.
[21] A. Tassanakajon, K. Somboonwiwat, P. Supungul, S. Tang, Discovery of immune molecules and their crucial functions in shrimp immunity, Fish Shellfish Immunol. 34 (2013) 954–967.
[22] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative CT method, Nat. Protoc. 3 (2008) 1101.
[23] S. Tinwongger, P. Proespraiwong, J. Thawonsuwan, P. Sriwanayos, J. Kongkumnerd, T. Chaweepack, I. Hirono, Development of PCR diagnosis for shrimp acute hepatopancreatic necrosis disease (AHPND) strain of *Vibrio parahaemolyticus*, Fish Pathol. 49 (2017) 159–164.
[24] D.V. Lightner, K.W. Hasson, B.L. White, R.M. Redman, Chronic toxicity and histopathological studies with Benlate®, a commercial grade of benomyl, in *Penaeus vannamei* (Crustacea: Decapoda), Aquat. Toxicol. 34 (1996) 105–118.
[25] H. Yasumitsu, Y. Ozeki, S.M. Kawsar, T. Toda, R. Kanaly, CGP stain: an inexpensive, odorless, rapid, sensitive, and in principle in vitro methylation-free Coomassie Brilliant Blue stain, Anal. Biochem. 406 (2010) 86–88.
[26] R. Sirikharin, S. Taengchaiyaphum, P. Sanguanrut, T.D. Chi, R. Mavichak, P. Proespraiwong, B. Nuangsaeng, S. Thitamadee, T.W. Flegel, K. Sritunyalucksana, Characterization and PCR detection of binary, Pir-like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp, PloS One 10 (2015) e0126987.
[27] A. Tassanakajon, K. Somboonwiwat, P. Amparyup, Sequence diversity and evolution of antimicrobial peptides in invertebrates, Dev. Comp. Immunol. 48 (2015) 324–341.
[28] S. Tanaka, T. Nakamura, T. Morita, S. Iwanaga, Limulus anti-LPS factor: an anticoagulant which inhibits the endotoxin-mediated activation of *Limulus coagulation* system, Biochem. Biophys. Res. Commun. 105 (1982) 717–723.
[29] K. Ohashi, M. Niwa, T. Nakamura, T. Morita, S. Iwanaga, Anti-LPS factor in the horseshoe crab, *Tachypleus tridentatus*, FEBS Lett. 176 (1984) 207–210.
[30] T. Morita, S. Ohtsubo, T. Nakamura, S. Tanaka, S. Iwanaga, K. Ohashi, M. Niwa, Isolation and biological activities of limulus anticoagulant (AntiLPS factor) which interacts with lipopolysaccharide (LPS), J. Biochem. 97 (1985) 1611–1620.
[31] Z.G. Hou, Y. Wang, K. Hui, W.H. Fang, S. Zhao, J.X. Zhang, H. Ma, X.C. Li, A novel anti-lipopolysaccharide factor SpALF6 in mud crab *Scylla paramamosain* exhibiting different antimicrobial activity from its single amino acid mutant, Dev. Comp. Immunol. 72 (2017) 44–56.
[32] C. Sun, W.T. Xu, H.W. Zhang, L.P. Dong, T. Zhang, X.F. Zhao, J.X. Wang, An anti-lipopolysaccharide factor from red swamp crayfish, *Procambarus clarkii*, exhibited antimicrobial activities in vitro and in vivo, Fish Shellfish Immunol. 30 (2011) 295–303.
[33] K.M. Beale, D.W. Towle, N. Jayasundara, C.M. Smith, J.D. Shields, H.J. Small, S.J. Greenwood, Anti-lipopolysaccharide factors in the American lobster *Homarus americanus*: molecular characterization and transcriptional response to *Vibrio fluvialis* challenge, Comp. Biochem. Physiol. 3D (2008) 263–269.
[34] K. Somboonwiwat, M. Marcos, A. Tassanakajon, S. Klinbunga, A. Aumelas, B. Romestand, Y. Gueguen, H. Boze, G. Moulin, E. Bachère, Recombinant expression and anti-microbial activity of anti-lipopolysaccharide factor (ALF) from the black tiger shrimp *Penaeus monodon*, Dev. Comp. Immunol. 29 (2005) 841–851.
[35] E. De la Vega, N.A. O'Leary, J.E. Shockey, J. Robalino, C. Payne, C.L. Browdy, G.W. Warr, P.S. Gross, Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection, Mol. Immunol. 45 (2008) 1916–1925.
[36] X. Lv, S. Li, F. Liu, F. Li, J. Xiang, Identification and function analysis of an anti-lipopolysaccharide factor from the ridgetail prawn *Exopalaemon arinicauda*, Dev. Comp. Immunol. 70 (2017) 128–134.
[37] A. Hoses, S. Watson, G.R. Siber, R. Liddington, Crystal structure of an endotoxin-neutralizing protein from the horseshoe crab, Limulus anti-LPS factor, at 1.5 A resolution, EMBO J. 12 (1993) 3351–3356.
[38] J. Aketagawa, T. Miyata, S. Ohtsubo, T. Nakamura, T. Morita, H. Hayashida, S. Iwanaga, T. Takao, Y. Shimonishi, Primary structure of limulus anticoagulant anti-lipopolysaccharide factor, J. Biol. Chem. 261 (1986) 7357–7365.
[39] Y. Yang, H. Boze, P. Chemardin, A. Padilla, G. Moulin, A. Tassanakajon, M. Pugnière, F. Roquet, D. Destoumieux-Garzón, Y. Gueguen, E. Bachère, NMR structure of rALF-Pm3, an anti-lipopolysaccharide factor from shrimp: model of the possible lipid A-binding site, Biopolymers 91 (2009) 207–220.
[40] S. Tharntada, S. Ponpratеep, K. Somboonwiwat, H. Liu, I. Söderhäll, K. Söderhäll, A. Tassanakajon, Role of anti-lipopolysaccharide factor from the black tiger shrimp, *Penaeus monodon*, in protection from white spot syndrome virus infection, J. Gen. Virol. 90 (2009) 1491–1498.
[41] S. Ponpratеep, K. Somboonwiwat, A. Tassanakajon, Recombinant anti-lipopolysaccharide factor isoform 3 and the prevention of vibriosis in the black tiger shrimp, *Penaeus monodon*, Aquaculture 289 (2009) 219–224.
[42] L. Zhu, J.F. Lan, Y.Q. Huang, C. Zhang, J.F. Zhou, W.H. Fang, X.J. Yao, H. Wang, X.C. Li, SpALF4: A newly identified anti-lipopolysaccharide factor from the mud crab *Scylla paramamosain* with broad spectrum antimicrobial activity, Fish Shellfish Immunol. 36 (2014) 172–180.
[43] T. Methatham, P. Boonchuen, P. Jaree, A. Tassanakajon, K. Somboonwiwat, Antiviral action of the antimicrobial peptide ALFPm3 from *Penaeus monodon* against white spot syndrome virus, Dev. Comp. Immunol. 69 (2017) 23–32.
[44] F.Y. Lin, Y. Gao, H. Wang, Q.X. Zhang, C.L. Zeng, H.P. Liu, Identification of an anti-lipopolysacchride factor possessing both antiviral and antibacterial activity from the red claw crayfish *Cherax quadricarinatus*, Fish Shellfish Immunol. 57 (2016) 213–221.
[45] A. Tassanakajon, V. Rimphanitchayakit, S. Visetnan, P. Amparyup, K. Somboonwiwat, W. Charoensapsri, S. Tang, Shrimp humoral responses against pathogens: antimicrobial peptides and melanization, Dev. Comp. Immunol. 80

(2018) 81–93.
[46] F. Jimenez-Vega, F. Vargas-Albores, Isoforms of *Litopenaeus vannamei* anti-lipopolysaccharide and its expression by bacterial challenge, J. Shellfish Res. 26 (2007) 1169–1175.
[47] W. Zhan, L. He, X. Wei, X. Wang, X. Tang, An anti-lipopolysaccharide factor in *Litopenaeus vannamei* participates in the immune defense against WSSV and *Vibrio anguillarum*, J. Crustac Biol. 35 (2015) 670–675.
[48] T. Mekata, R. Sudhakaran, S. Okugawa, T. Kono, M. Sakai, T. Itami, Molecular cloning and transcriptional analysis of a newly identified anti-lipopolysaccharide factor gene in kuruma shrimp, *Marsupenaeus japonicus*, Lett. Appl. Microbiol. 50 (2010) 112–119.
[49] H.S. Jiang, Q. Zhang, Y.R. Zhao, W.M. Jia, X.F. Zhao, J.X. Wang, A new group of anti-lipopolysaccharide factors from *Marsupenaeus japonicus* functions in anti-bacterial response, Dev. Comp. Immunol. 48 (2015) 33–42.
[50] R.D. Rosa, A. Vergnes, J. De Lorgeril, P. Goncalves, L.M. Perazzolo, L. Sauné, B. Romestand, J. Fievet, Y. Gueguen, E. Bachère, D. Destoumieux-Garzón, Functional divergence in shrimp anti-lipopolysaccharide factors (ALFs): from recognition of cell wall components to antimicrobial activity, PloS One 8 (2013) e67937.
[51] C. Weidenmaier, A. Peschel, Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions, Nat. Rev. Microbiol. 6 (2008) 276.
[52] R.B. Zhou, H.M. Lu, J. Liu, J.Y. Shi, J. Zhu, Q.Q. Lu, D.C. Yin, A systematic analysis of the structures of heterologously expressed proteins and those from their native hosts in the RCSB PDB archive, PloS One 11 (2016) e0161254.
[53] P. Supungul, P. Jaree, K. Somboonwiwat, W. Junprung, P. Proespraiwong, R. Mavichak, A. Tassanakajon, A potential application of shrimp antilipopolysaccharide factor in disease control in aquaculture, Aquacult. Res. 48 (2017) 809–821.
[54] S. Visetnan, P. Supungul, A. Tassanakajon, S. Donpudsa, V. Rimphanitchayakit, A single WAP domain-containing protein from *Litopenaeus vannamei* possesses anti-proteinase activity against subtilisin and antimicrobial activity against AHPND-inducing *Vibrio parahaemolyticus*, Fish Shellfish Immunol. 6 (2017) 341–348.
[55] R. Medzhitov, C.A. Janeway, Decoding the patterns of self and nonself by the innate immune system, Science 296 (2002) 298–300.
[56] Buchmann Kurt, Evolution of innate immunity: clues from invertebrates via fish to mammals, Front. Immunol. 5 (2014) 459.
[57] S.J. Lin, K.C. Hsu, H.C. Wang, Structural insights into the cytotoxic mechanism of *Vibrio parahaemolyticus* $PirA^{vp}$ and $PirB^{vp}$ toxins, Mar. Drugs 15 (2017) 373.
[58] L. Pardo-Lopez, M. Soberon, A. Bravo, *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection, FEMS Microbiol. Rev. 37 (2012) 3–22.
[59] B. Peterson, C.C. Bezuidenhout, J. Van den Berg, An overview of mechanisms of Cry toxin resistance in Lepidopteran insects, J. Econ. Entomol. 110 (2017) 362–377.
[60] W. Junprung, P. Supungul, A. Tassanakajon, HSP70 and HSP90 are involved in shrimp *Penaeus vannamei* tolerance to AHPND-causing strain of *Vibrio parahaemolyticus* after non-lethal heat shock, Fish Shellfish Immunol. 60 (2017) 237–246.