



Full length article

Thymosins participate in antibacterial immunity of kuruma shrimp, *Marsupenaeus japonicus*

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ABSTRACT

Thymosins β are actin-binding proteins that play a variety of different functions in inflammatory responses, wound healing, cell migration, angiogenesis, and stem cell recruitment and differentiation. In crayfish, thymosins participate in antiviral immunology. However, the roles of thymosin during bacterial infection in shrimp remain unclear. In the present study, four thymosins were identified from kuruma shrimp, *Marsupenaeus japonicus*, and named as *Mjthymosin2*, *Mjthymosin3*, *Mjthymosin4*, and *Mjthymosin5* according to the number of their thymosin beta actin-binding motifs. *Mjthymosin3* was selected for further study because its expression level was the highest in hemocytes. Expression analysis showed that *Mjthymosin3* was upregulated in hemocytes after challenged by *Vibrio anguillarum* or *Staphylococcus aureus*. The recombinant *Mjthymosin3* protein could inhibit the growth of certain bacteria in an *in vitro* antibacterial test. *Mjthymosins* could facilitate external bacterial clearance in shrimp, and were beneficial to shrimp survival post *V. anguillarum* or *S. aureus* infection. The results suggested that *Mjthymosins* played important roles in the antibacterial immune response of kuruma shrimp.

1. Introduction

Thymosins were originally isolated from calf thymus, and were classified as α , β , and γ thymosin in according to their isoelectric points. Thymosin β proteins, with isoelectric points of pH 5.0–7.0, usually contain 40–44 amino acids, with a molecular mass of about 5 kDa. Thymosin β forms complexes with monomeric actin and prevents G-actin polymerization to filaments, and is thus a member of the actin-binding proteins [1]. Thymosin β has a variety of functions, such as stimulation of angiogenesis; accelerating the healing of skin wounds and corneal scarring; regulation of the immune system; and involved in cancer development; thus having therapeutic potential [1].

Thymosin $\beta 4$ was first isolated and purified from the endocrine thymus, and comprised 43 amino acid residues with an isoelectric point of 5.1 [2]. Thymosin $\beta 4$ plays multiple roles in disease. For example, it promotes significant outgrowth in quiescent adult epicardial explants; functions in restoring pluripotency; and triggers cells differentiation, such as that of fibroblasts and smooth muscle cells [3]. Thymosin $\beta 4$ has a potential role in liver fibrogenesis by affecting the activation of hepatic stellate cells, making it a novel therapeutic target for liver fibrosis [3]. Besides, thymosin $\beta 4$ is able to promote significantly outgrowth in quiescent adult epicardial explants and functions in restoring pluripotency as well as triggering cells differentiation, such as

fibroblasts and smooth muscle cells [4]. It also participates in repair of the cardiovascular system by promoting wound healing and angiogenesis in the heart after myocardial infarction and in the brain after stroke, implying roles in cardioprotection and a therapeutic effect in cardiovascular disease [5,6]. Thymosin $\beta 4$ functions in the preservation and regeneration of the mammalian heart [7], and accelerates dermal healing in some preclinical animal models and patients [8].

In humans, thymosin $\beta 4$ is expressed in ocular surface epithelia, and shows antibacterial activity against common ocular pathogens [9]. Meanwhile, thymosin has been used as a potential target for disease treatment. Thymosin $\beta 4$ has been proven as a novel therapy to treat ocular surface disease [10], and has potential functions in the treatment of nonalcoholic fatty liver disease [11]. Thymosin $\beta 4$ could also be a target for cancer metastasis therapy by accelerating tumor metastasis and angiogenesis [12], and was noted as a potent drug to treat subacute stroke in aged rats [13].

In invertebrates, there are various reports about thymosins. In *Drosophila*, Ciboulot, a novel actin binding protein with three β -thymosin repeats, has important functions in axonal growth during brain metamorphosis by regulating actin assembly [14]. Ciboulot could bind to G-actin via the N-terminal amphipathic helix of the first repeat (D1), like thymosin $\beta 4$, and enhances actin assembly similarly to profilin [15]. In *Caenorhabditis elegans*, tetraThymosin β , consisting of four

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thymosin β repeats, plays a vital role in specific developmental stages [16,17], and the four repeats are all functional in binding multiple actin molecules [16]. Two thymosins are identified in Chinese mitten crab *Eriocheir sinensis*, and their expression levels could be upregulated by *Listonella anguillarum* challenge [18]. Meanwhile, the β thymosins of freshwater crayfish, *Pacifastacus leniusculus*, are involved in regulation of hemocyte homeostasis [19]. Additionally, thymosin β is regarded as a negative effector in ovarian development in the giant tiger shrimp *Penaeus monodon* [20]. In addition, in red swamp crayfish, *Procambarus clarkii*, Pcthy-4 could decrease white spot syndrome virus (WSSV) replication, benefit crayfish survival, and enhance WSSV phagocytosis in hemocytes [21]. Furthermore, thymosin from red claw crayfish, *Cherax quadricarinatus*, could inhibit WSSV replication [22].

Crustaceans are commercially cultured because of their high market value [23]. In 2015, the global shrimp catches were 4.8 million tons, and shrimp culture brought an economic value up to tens of billions US dollars a year [24]. However, shrimp diseases caused by viruses and bacteria occur frequently, leading to huge economic losses worldwide [25]. Shrimp, like other invertebrates, rely only on their innate immune system to protect them from pathogen invasion [26]. Therefore, it is important to study the innate immune system and explore immune-related molecules for shrimp disease prevention and treatment.

In the present study, four thymosins were identified from kuruma shrimp, *Marsupenaeus japonicus*. *Mjthymosin3* was chosen for further research because of its highest expression level in hemocytes. Quantitative real-time polymerase chain reaction (qPCR) was performed to detect the expression patterns of *Mjthymosin3* in shrimp hemocytes post *Vibrio anguillarum* or *Staphylococcus aureus* challenge. Antibacterial tests were performed to determine the antibacterial activity of *Mjthymosins*. RNA interference (RNAi) and bacteria clearance assays were carried out to study the potential antibacterial function of *Mjthymosins in vivo*. A survival rate assay was performed to detect the effect of *Mjthymosins* on shrimp survival. The results indicated that *Mjthymosins* had vital functions in shrimp antibacterial immunity.

2. Materials and methods

2.1. Shrimp and bacterial challenge

Healthy kuruma shrimp (8–10 g each) were obtained from a fish market in Jinan, Shandong province, China. Shrimp were cultured in artificial oxygenated tanks with seawater at 2.4–2.6‰ salinity and 22–25 °C.

For immune challenge, 30 μ L of *V. anguillarum* or *S. aureus* (10^6 colony forming units (CFU)) were injected into shrimp at the last second abdominal segment. Meanwhile, the same volume of phosphate-buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 , pH 7.4) was injected into another group of shrimp as controls [27].

2.2. Gene cloning and sequence analysis

The full-length cDNA sequence of *Mjthymosin3* was obtained from transcriptome sequencing of shrimp hemocytes. Primers ThyF1 and ThyR1 (Table 1) were designed to amplify and verify the cDNA sequence. The PCR amplification program was 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 45 s; and 72 °C for 10 min. The 3'-untranslated region (UTR) was amplified using primers ThyF1 and 3' anchorR (Table 1).

Nucleotide sequence translation was accomplished using the ExPasy Translate tool (<http://web.expasy.org/translate/>), and prediction of the isoelectric point and molecular mass was achieved using ExPasy Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Sequence alignment was performed using MEGA 5.05 and analyzed using GeneDoc software. The thymosin beta actin-binding motif was predicted using the simple modular architecture research tool ([\[smart.embl-heidelberg.de\]\(http://smart.embl-heidelberg.de\)\). A phylogenetic tree of thymosins from kuruma shrimp and other species was constructed using the MEGA 5.05 software \[28\].](http://</p>
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2.3. Tissue distribution and expression profile analysis

To analyze the tissue distribution of *Mjthymosins*, total RNAs from hemocytes, heart, hepatopancreas, gills, stomach, and intestine of at least three healthy kuruma shrimp were extracted using the TRLPure reagent (Biotek Corporation, Beijing, China). Hemolymph was drawn using a 5 mL sterile syringe preloaded with 800 μ L of pre-cooled anticoagulant agent (450 mM NaCl, 10 mM KCl, 10 mM EDTA, and 10 mM HEPES, pH 7.45). Hemocytes were collected by centrifuging the hemolymph at $800 \times g$ and 4 °C for 10 min. First strand cDNAs were reverse transcribed from DNase I digested total RNAs of six tissues, and diluted 10-fold in nuclease-free sterile water as templates for qPCR. Specific primers Thy2RTF with Thy2RTR, Thy3RTF with Thy3RTR, Thy4RTF with Thy3RTR, Thy3RTF and Thy5RTR (Table 1), were designed to analyze the tissue distribution of *Mjthymosin2*, *Mjthymosin3*, *Mjthymosin4*, and *Mjthymosin5* separately.

Hemocytes of the PBS group, the *V. anguillarum* group, and the *S. aureus* group from at least three shrimp were obtained at 0, 6, 12, 24, and 48 h as mentioned above. Total RNAs were extracted and reverse transcribed into the first strand cDNAs, which were diluted 20-fold as templates for qPCR. QPCR was performed in a 10- μ L system (5 μ L of $2 \times$ SYBR Green qPCR Mix (Aidlab, China), 2 μ L of forward primer (1 μ M), 2 μ L of reverse primer (1 μ M), and 1 μ L of cDNA template), and carried out according to previously described methods [29]. Specific primers Thy3RTF and Thy3RTR (Table 1) were designed to analyze the expression pattern of *Mjthymosin3* at the transcriptional level using qPCR. GraphPad Prism software (USA) was used to analyze data.

2.4. RNA interference

Primers Thy3iF and Thy3iR (Table 1) were used to amplify a nucleotide fragment (located from 64 bp to 620 bp in *Mjthymosin3*), as the DNA template for dsRNA synthesis. A 100 μ L reaction system containing 4.8 μ L of ATP (100 mM, ThermoFisher Scientific, USA), 4.8 μ L of CTP (100 mM), 4.8 μ L of UTP (100 mM), 4.8 μ L of GTP (100 mM), 20 μ L of $5 \times$ transcriptional buffer, 8 μ L of T7 RNA Polymerase (160 U, ThermoFisher Scientific, USA), 4 μ L of RNase inhibitor (160 U, Biotek Corporation, Beijing, China), 8 μ L of DNA template, and 40.8 μ L of RNase-free water was incubated at 37 °C for at least 12 h. Then, 10 μ L of DNase I (10 U, ThermoFisher Scientific, USA), 20 μ L of $10 \times$ reaction buffer, and 70 μ L of RNase-free water were added and incubated at 37 °C for 1 h. *GFP* dsRNA was synthesized from a DNA template that was amplified using primers GFPiF and GFPiR (Table 1).

For the RNAi assay, 30 μ g of ds*Mjthymosin3* or ds*GFP* was injected into shrimp, separately; 24 h later, another 30 μ g of dsRNA for *Mjthymosin3* or *GFP* was injected. At 24 h after the second injection, total RNAs were extracted from hemocytes and reverse transcribed into first strand cDNAs as templates for qPCR and semi-quantitative reverse transcription PCR (RT-PCR). Primers Thy3RTF1 and Thy3RTR1 (Table 1) were designed for qPCR to check the knockdown results of *Mjthymosin3* only. To detect the relative expression of other *Mjthymosins* after RNAi, RT-PCR was performed using primers ThyF1 and ThyR1.

2.5. Antibacterial activity test

The full-length cDNA of *Mjthymosin3* was amplified by primer Thy3ExF and Thy3ExR (Table 1), digested and ligated into *EcoRI* and *SalI* restriction enzyme sites site of vector pGEX-4T-1 (GE Healthcare, USA). The resulting recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) competent cells, and induced with 0.5 mM isopropyl β -D-thiogalactopyranoside (IPTG) for recombinant protein

Table 1
Sequences of primers used in this study.

Primers	Sequences (5'–3')	Direction
Gene clone		
ThyF1	ATGAGCGCCGAACTCCCCTCAAG	Forward
ThyR1	TTAGGCCTTCTTCTTCTCTCAAT	Reverse
3'anchorR	GACCACGCGTATCGATGTCGAC	Reverse
qPCR		
Thy2RTF	CCAAACAAGGAGGATATTGAGAAT	Forward
Thy2RTR	TTAGGCCTTCTTCTCTCTCTC	Reverse
Thy3RTF	CCAAACAAGGAGGACGTGGAAGCAGAG	Forward
Thy3RTR	CATTCTCAATATCTTCTTAGCAGGT	Reverse
Thy4RTF	TTTCGCAGCGAAAGACTCAAACGA	Forward
Thy5RTR	GACTTGTCAAATCCTGTTACACC	Reverse
Thy3RTF1	AAGGTCTCCCATGCAGGTA	Forward
Thy3RTR1	AGCACTTTATTGGCTCCC	Reverse
RNAi		
Thy3iF	GCGTAATACGACTCACTATAGGGAGGGATTCTCCGCCGTAA	Forward
Thy3iR	GCGTAATACGACTCACTATAGGCATCGCCTTGCTATTGGT	Reverse
GFPiF	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC	Forward
GFPiR	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC	Reverse
Recombinant expression		
Thy3ExF	TACGAAATTCATGAGCGCCGAACTCCCCTCAAG	Forward
Thy3ExR	TACGTCGACTTAGGCCTTCTTCTTCTCTCAAT	Reverse

expression. After the induced bacteria were collected and disrupted ultrasonically, the recombinant *Mj*thymosin3 protein (r*Mj*thymosin3) was purified from the soluble fraction of bacterial lysate using GST-resin (GenScript, USA). The empty pGEX-4T-1 vector was also transformed into *E. coli* BL21 (DE3) competent cells and induced with 0.5 mM IPTG for expression of the GST tag protein, which was used as the negative control. The GST tag protein was purified in the same way as r*Mj*thymosin3. The purified r*Mj*thymosin3 and GST tag proteins were dialyzed against a solution (0.1 M Tris-HCl, pH 8.0, 5% glycerol) for 16 h twice.

For the antibacterial test, gram-negative bacteria (*E. coli*, *Shewanella colwelliana*, and *V. anguillarum*) and gram-positive bacteria (*S. aureus* and *Micrococcus luteus*) were selected. *V. anguillarum* and *S. colwelliana* were cultured in Luria-Bertani (LB) liquid medium (3% NaCl). *E. coli*, *S. aureus*, and *M. luteus* were cultured in LB liquid medium (1% NaCl). The bacteria were collected, washed, and diluted to 2×10^5 CFU/mL in poor broth medium (PB, 1% Tryptone, 0.5% NaCl, pH 7.5). Then, 190 μ L of bacteria (3.8×10^4 CFU) and 10 μ L of recombinant protein (r*Mj*thymosin3 or GST tag protein) were added and mixed in wells of a 96-well cell culture plate. The plate was then placed at 28 °C for 24 h. The optical density at 600 nm was read using a Universal Microplate Reader (ELX800, BioTek Instruments, USA) [30]. The data were analyzed using GraphPad Prism software.

2.6. Bacteria clearance assay

V. anguillarum was cultured in LB liquid medium (3% NaCl) overnight [31]. After collection and washing using sterilized PBS twice, *V. anguillarum* (10^6 CFU for each shrimp) were injected into ds*Mj*thymosin3-injected shrimp or ds*GFP*-injected shrimp separately. Hemolymph was extracted from at least three shrimp 30 min later. Hemolymph was diluted serially and spread onto plates containing LB solid medium (3% NaCl). The plates were overturned and cultured in a 37 °C incubator overnight. The bacterial colonies of *V. anguillarum* were then counted, and the bacterial colonies per milliliter of hemolymph were calculated. Data analysis and figure construction were accomplished using the Graphpad Prism software.

For the *S. aureus* clearance assay, *S. aureus* was cultured in LB liquid medium (1% NaCl) overnight, and prepared and injected as stated above. Bacterial numbers for *S. aureus* per milliliter hemolymph from ds*Mj*thymosin3-injected shrimp and ds*GFP*-injected shrimp were also analyzed as above.

2.7. Survival assay

For survival assay post *V. anguillarum* challenge, shrimp were divided into two groups, a ds*GFP* with *V. anguillarum*-injected group (30 shrimp) and a ds*Mj*thymosin3 with *V. anguillarum*-injected group (30 shrimp). In the RNAi assay, shrimp were injected with 30 μ g of *GFP* or *Mj*thymosin3 dsRNA separately, twice. At 24 h after the second injection, 1×10^6 CFU of *V. anguillarum* were injected into the shrimp of both groups. Thereafter, the dead shrimp were picked out and counted three times a day.

For survival analysis during *S. aureus* challenge, shrimp were divided into a ds*GFP* with *S. aureus*-injected group (30 shrimp) and a ds*Mj*thymosin3 with *S. aureus*-injected group (30 shrimp). RNAi and *S. aureus* injection were performed stated above.

The survival rate was analyzed using GraphPad Prism 5.0 software, and the *P* value was calculated using a log-rank (Mantel-Cox) test.

3. Results

3.1. Sequence analysis of *Mj*thymosins

To determine the presence of thymosins in shrimp, primers ThyF1 and ThyR1 were designed according to the nucleotide sequence of *Mj*thymosin3 obtained by transcriptome sequencing. Four bands could be detected. After sequencing by BGI (Beijing Genomics Institute, China) and bioinformatic analysis, four *Mj*thymosins were identified, and named as *Mj*thymosin2, *Mj*thymosin3, *Mj*thymosin4, and *Mj*thymosin5, according to their motif numbers (Fig. 1A). *Mj*thymosin2, *Mj*thymosin3, *Mj*thymosin4, and *Mj*thymosin5 contained 273, 387, 501, and 615 base pairs, encoding 90, 128, 166, and 204 amino acids with 2, 3, 4, and 5 thymosin beta actin-binding motifs, respectively (Fig. 1A). As shown in Fig. 1B, the four *Mj*thymosins showed high identities in the sequences of their thymosin beta actin-binding motifs. The isoelectric points of the four *Mj*thymosins were between pH 5.0 and pH 7.0; therefore, they all belonged to the β thymosin family.

To study the evolutionary relationship of thymosins from invertebrates and vertebrates, phylogenetic tree was constructed. In the neighbor-joining phylogenetic tree (Fig. 2), thymosins were separated into two clusters. One cluster contained thymosins from crayfish, crab, mouse, human, and *Drosophila*, while the other cluster consisted of thymosins from shrimp, mosquito, Mediterranean fruit fly, green peach aphid, and termite. *Mj*thymosin2, *Mj*thymosin3, *Mj*thymosin4, and

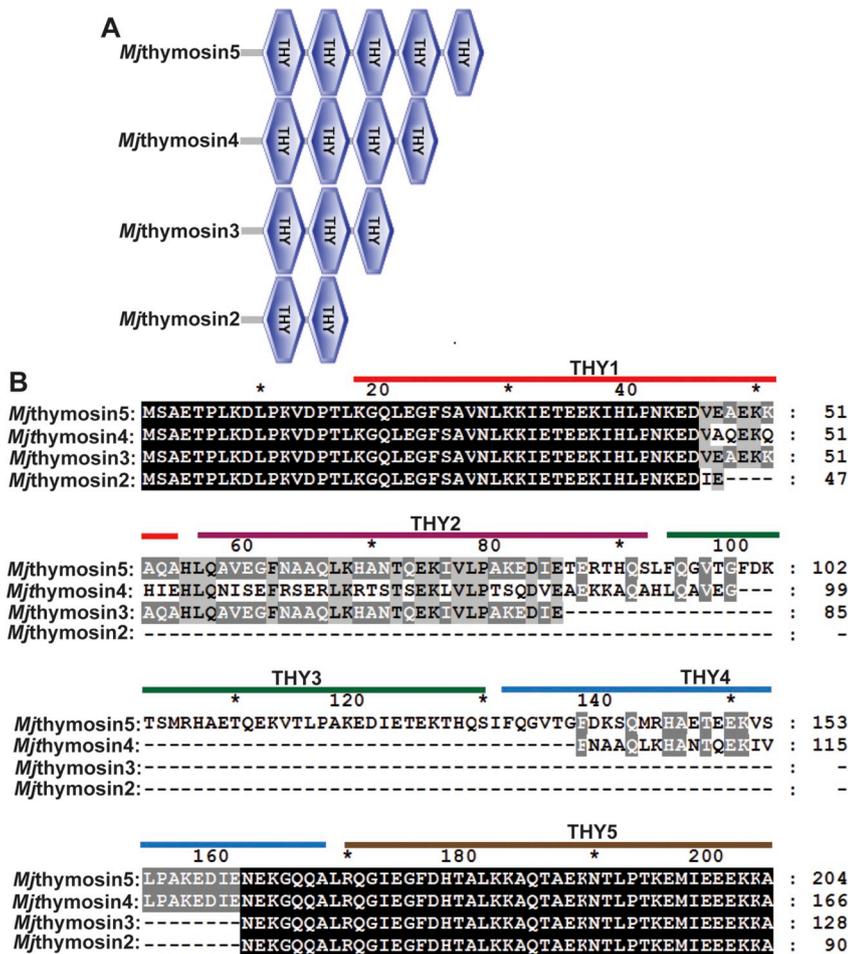


Fig. 1. Sequence analysis of *Mjthymosins*. (A) Schematic graph of the domains in *Mjthymosin*. *Mjthymosin2*, *Mjthymosin3*, *Mjthymosin4*, and *Mjthymosin5* showing that they contained 2, 3, 4, and 5 thymosin beta actin-binding motifs, respectively. (B) Protein sequence alignment of *Mjthymosin2* (GenBank accession no. MH492366), *Mjthymosin3* (GenBank accession no. MH492365), *Mjthymosin4* (GenBank accession no. MH492364) and *Mjthymosin5* (GenBank accession no. MH492363). The thymosin beta actin-binding motifs in *Mjthymosin5* were showed by lines with different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Mjthymosin5 were all located on one sub-branch with the thymosin from black tiger shrimp. The results indicated that *Mjthymosins* were phylogenetically different from the thymosins of other crustacean (crayfish and crab).

To check the expression level of *Mjthymosin2*, *Mjthymosin3*, *Mjthymosin4*, and *Mjthymosin5* in hemocytes, qPCR was performed with specific primers for different *Mjthymosins*. As shown in Fig. 3A, the expression level of *Mjthymosin3* was much higher than the other three *Mjthymosins*. QPCR was performed for the tissue distribution of *Mjthymosins* in hemocytes, heart, hepatopancreas, gills, stomach, and intestine at the transcriptional level. The *Mjthymosins* were ubiquitously expressed in the six tested tissues (Fig. 3B–E). *Mjthymosin2*, *Mjthymosin3*, and *Mjthymosin4* showed the highest expression level in the hemocytes (Fig. 3B–D), while the expression of *Mjthymosin5* in heart was much higher than that in other tissues (Fig. 3E). Hemocytes are critical immune cells in shrimp bacterial immunity [32]; therefore, *Mjthymosin3* was selected for further study.

3.2. *Mjthymosin3* was upregulated by *V. anguillarum* and *S. aureus* infection

To test whether *Mjthymosin3* was involved in bacterial infection, qPCR was carried out to check the temporal expression pattern of *Mjthymosin3* in shrimp hemocytes. The results showed that in hemocytes, *Mjthymosin3* was upregulated by *V. anguillarum* challenge and its expression level reached a peak at 24 h post-infection (Fig. 4A). In addition, after *S. aureus* infection, the expression level of *Mjthymosin3* in hemocytes increased at 6 h (Fig. 4B). Thus, *Mjthymosin3* could respond to *V. anguillarum* and *S. aureus* challenge at the mRNA level. This indicated that *Mjthymosin3* might be involved in the response to bacterial

infection in kuruma shrimp.

3.3. *Mjthymosin3* showed antibacterial activity

To study the function of *Mjthymosins* in shrimp immune responses, an antibacterial activity test was carried out. The *rMjthymosin3* protein was expressed in *E. coli* BL21 (DE3) cells from vector pGEX-4T-1 and induced by IPTG for the antibacterial test *in vitro*. In Fig. 5A, a band corresponding to *rMjthymosin3* was observed around 40 kDa, which was consistent with the predicted molecular mass of 40.9 kDa. The GST tag protein was recombinantly expressed and purified as the control protein.

Gram-negative bacteria *E. coli*, *S. colwelliana* and *V. anguillarum*, and gram-positive bacteria *M. luteus* and *S. aureus* were chosen for the antibacterial assay *in vitro*. To exclude the potential effect of the GST tag in the *rMjthymosin3* protein, the GST tag protein was used as the control. As shown in Fig. 5B, *rMjthymosin3* could inhibit the growth of the five bacteria, which showed that *rMjthymosin3* had a broad-spectrum of antibacterial activity against both gram-negative and gram-positive bacteria. Thus, *Mjthymosin3* might participate in shrimp antibacterial immunity by inhibiting the growth of external bacteria.

3.4. *Mjthymosins* could enhance external bacteria clearance

To investigate the involvement of *Mjthymosin3* in bacteria clearance, RNAi and bacteria clearance assays were performed. dsRNA of *Mjthymosin3* was injected into shrimp. QPCR was used to check the efficiency of *Mjthymosin3* knockdown. Fig. 6A showed that the expression level of *Mjthymosin3* decreased significantly in ds*Mjthymosin3*-injected shrimp compared with that of dsGFP-injected shrimp. This

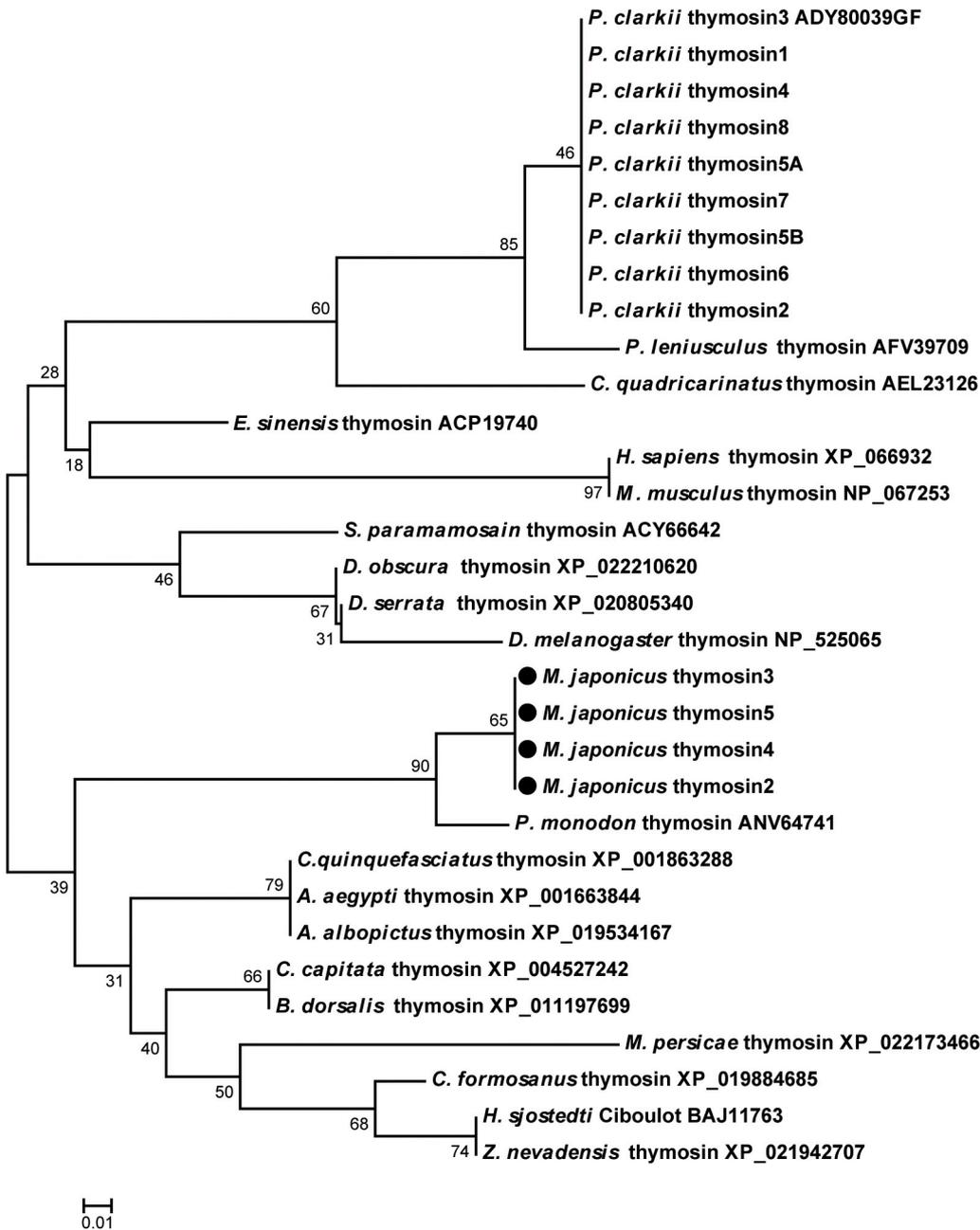


Fig. 2. Phylogenetic tree analysis of thymosins in *M. japonicus* compared with those from other species. All of the thymosin protein sequences collected in GenBank were used to build a neighbor-joining tree using MEGA 5.05 software, with 1000 bootstrap replication. Animals included *Aedes aegypti* (yellow fever mosquito), *A. albopictus* (Asian tiger mosquito), *Bactrocera dorsalis* (oriental fruit fly), *Ceratitidis capitata* (Mediterranean fruit fly), *Cherax quadricarinatus* (red claw crayfish), *Coptotermes formosanus* (Formosan subterranean termite), *Culex quinquefasciatus* (southern house mosquito), *D. melanogaster* (fruit fly), *D. serrata*, *D. obscura*, *Eriocheir sinensis* (Chinese mitten crab), *Hodotermopsis sjostedti*, *Hodotermopsis sjostedti*, *Homo sapiens*, *M. japonicus*, *Mus musculus*, *Myzus persicae* (green peach aphid), *Pacifastacus leniusculus* (freshwater crayfish), *Penaeus monodon* (black tiger shrimp), *Procambarus clarkii* (red swamp crayfish), *Scylla paramamosain* (green mud crab), and *Zootermopsis nevadensis*. *Mjthymosin2*, *Mjthymosin3*, *Mjthymosin4*, and *Mjthymosin5* were marked with solid circles.

showed that *Mjthymosin3* was knocked down successfully by RNAi. As *Mjthymosin2*, *Mjthymosin3*, *Mjthymosin4*, and *Mjthymosin5* shared high sequence identities, RT-PCR was performed to check the expression of the other *Mjthymosins* in *Mjthymosin3*-knockdown shrimp, using the common primers ThyF1 and ThyR1. The results showed that *Mjthymosin2* and *Mjthymosin4* were also knocked down in the ds*Mjthymosin3*-injected shrimp (Fig. 6B).

V. anguillarum or *S. aureus* (10^6 CFU) were injected into the ds*Mjthymosin3*-injected and ds*GFP*-injected shrimp. The bacteria numbers in the hemolymph of the ds*Mjthymosin3*-injected shrimp and ds*GFP*-injected shrimp were calculated. As shown in Fig. 6C, the bacteria number in the *Mjthymosin3*-knockdown shrimp following *V. anguillarum* injection was much higher than that in the control group. A similar result was observed in the *S. aureus*-injected shrimp (Fig. 6D). The results suggested that *Mjthymosins* could accelerate bacterial clearance from shrimp hemolymph. The results implied that *Mjthymosins* functioned in shrimp antibacterial immunity by enhancing bacteria clearance.

3.5. *Mjthymosin3* was beneficial to shrimp survival

To confirm the function of *Mjthymosins* in shrimp antibacterial immunity, survival assay was carried out. Fig. 7A showed that the survival rate of *Mjthymosin3*-knockdown shrimp after *V. anguillarum* injection was much lower than that of shrimp injected with ds*GFP* with *V. anguillarum* injection. Similar results were obtained in *S. aureus*-injected shrimp (Fig. 7B). The results showed that *Mjthymosin3* was beneficial for shrimp survival post-*V. anguillarum* or *S. aureus* infection. Thus, *Mjthymosins* might play significant roles in shrimp antibacterial defense via bacterial inhibition and clearance.

4. Discussion

Four β thymosins were identified and characterized in kuruma shrimp, *M. japonicus*. The function of the *Mjthymosins* in the antibacterial immune reaction was investigated. As far as we know, this study is the first report about participation of thymosins in shrimp

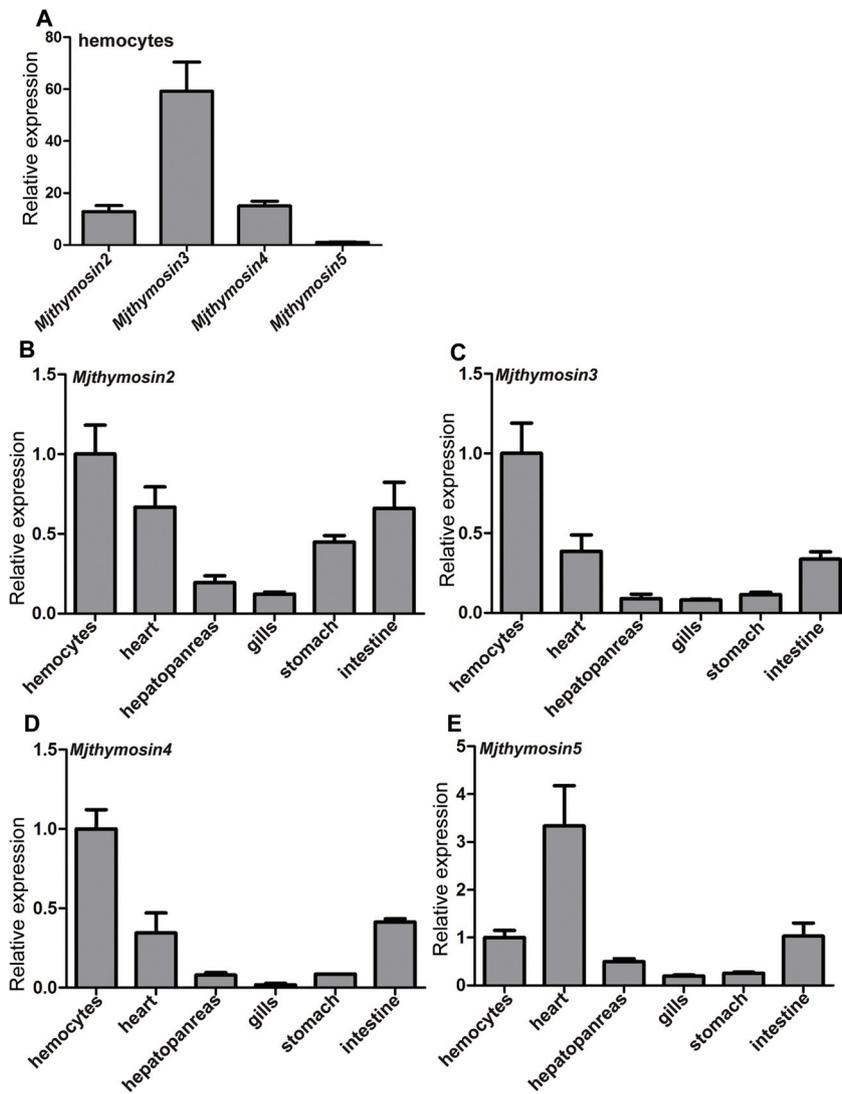


Fig. 3. Tissue distribution analysis of *MjThymosins* of normal shrimp using qPCR. *EF1α* (elongation factor 1α) was used as the internal control. Total RNAs from tissues including hemocytes, heart, hepatopancreas, gills, stomach, and intestines were extracted and reverse transcribed into first strand cDNAs, which were used as the templates for qPCR. (A), the relative expression analysis of *Mjthymosin2*, *Mjthymosin3*, *Mjthymosin4*, and *Mjthymosin5* in hemocytes. (B–E), Tissue distribution analysis of *Mjthymosin2*, *Mjthymosin3*, *Mjthymosin4*, and *Mjthymosin5* in normal shrimp.

antibacterial immunity.

Invertebrates usually have more than one isoform of β-thymosins, which comprise one or more thymosin beta actin-binding motifs. For example, there are nine β-thymosins in red swamp crayfish *P. clarkii*, five different β-thymosins in freshwater crayfish *P. leniusculus*, and two thymosin repeat proteins in Chinese mitten crab *E. sinensis* [18,19,21].

In kuruma shrimp, four thymosins containing 2, 3, 4, and 5 thymosin beta actin-binding motifs, respectively, were identified. They shared high sequence identities and were classified as members of the β-thymosin family.

Tissue distribution assays showed the *Mjthymosins* were present in different tissues with diverse expression levels. This indicated that the

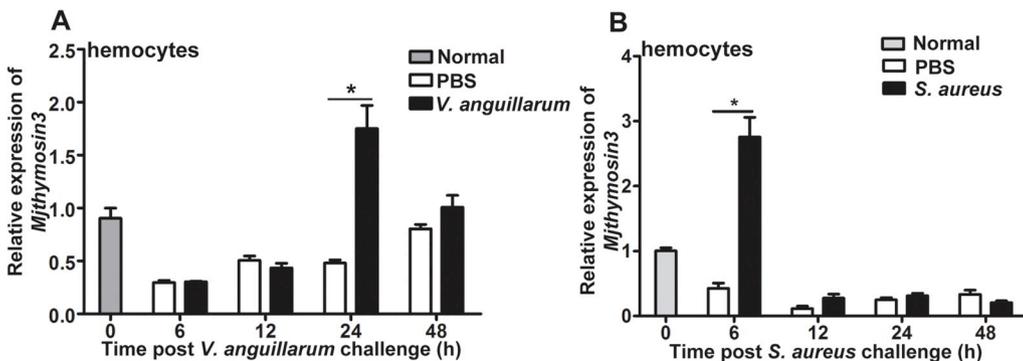


Fig. 4. Expression pattern analysis of *MjThymosin3* at the transcriptional level in hemocytes post challenge of *V. anguillarum* (A) and *S. aureus* (B). Total RNAs were extracted from normal hemocytes and hemocytes at 6, 12, 24, and 48 h after PBS, *V. anguillarum* or *S. aureus* injection. Total RNAs were used as templates to reverse transcribe the first strand cDNAs, which were diluted 20-fold as templates for qPCR analysis. PBS-injected shrimp were used as the control. The expression levels of *EF1α* were used as the internal control. Significance was analyzed between the

V. anguillarum or *S. aureus* challenged group and the PBS-injection group at the same time point using *t*-test analysis (**P* < 0.05).

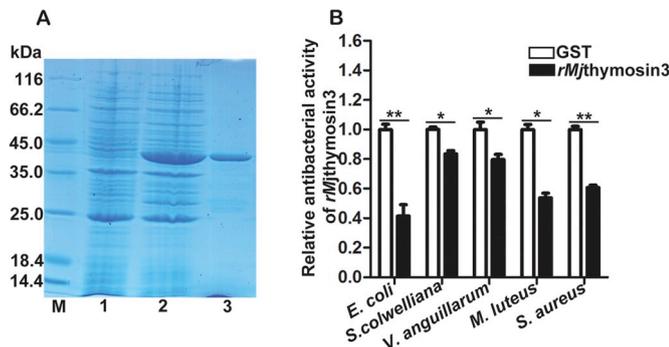


Fig. 5. Antibacterial activity of purified rMjthymosin3 protein *in vitro*. (A) Mjthymosin3 recombinant expression and purification. Lane M, standard protein marker; lane 1, total proteins from *E. coli* with pGEX-4T-1-Mjthymosin3 before IPTG induction; lane 2, total proteins from *E. coli* with pGEX-4T-1-Mjthymosin3 after 0.5 mM IPTG induction; lane 3, purified rMjthymosin3. (B) Antibacterial activity of rMjthymosin3. RMjthymosin3 protein (10 µg) was added into 190 µL bacteria medium, and incubated at 28 °C for 24 h. The OD600 was read using a Universal Microplate Reader. The GST tag protein was used as the control. Significance was calculated using *t*-test analysis (**P* < 0.05, ***P* < 0.01).

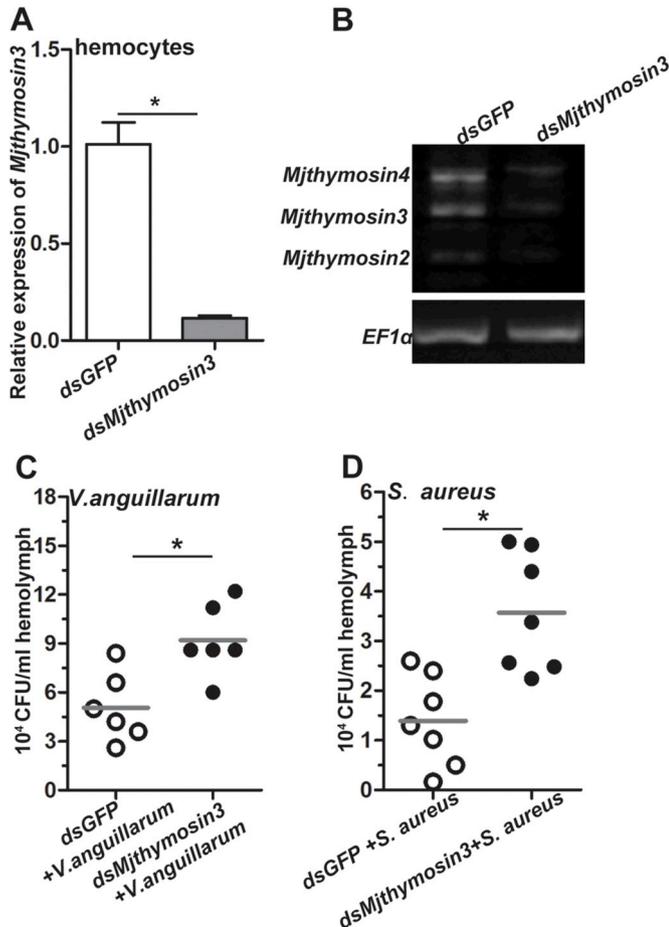


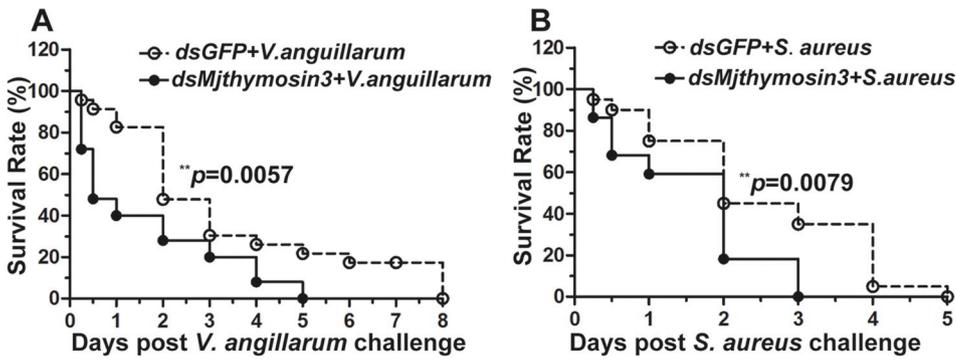
Fig. 6. Mjthymosin3 enhanced bacterial clearance. (A) QPCR was used to analyze the expression level of Mjthymosin3 after RNAi in hemocytes. (B) RT-PCR was used to check the expression level of Mjthymosins after RNAi. EF1α was amplified and used as the internal control. Bacterial clearance assays were performed after Mjthymosin3 knockdown and injection of *V. anguillarum* (C) or *S. aureus* (D). *V. anguillarum* or *S. aureus* were injected into the RNA interfered shrimp and the bacterial count in hemolymph was determined 30 min later. Significance was calculated by *t*-test analysis (**P* < 0.05).

Mjthymosins might function differently in various shrimp tissues. Among the tested tissues, the expression of Mjthymosin3 was relatively high compared to that of the other Mjthymosins, and Mjthymosin3 showed the highest expression in hemocytes. Hemocytes play vital roles in the immune-related processes of shrimp [33]. This suggested a vital function of Mjthymosin3 in shrimp immunity. Therefore, Mjthymosin3 was selected for further study. In the neighbor-joining phylogenetic tree, thymosins from kuruma shrimp were clustered together with thymosin from giant tiger shrimp in the same sub-branch, but not with thymosins from freshwater crayfish, red swamp crayfish, and red claw crayfish. These results indicated that there were some differences between the thymosins from shrimp and those of crayfish, indicating that the thymosins from shrimp and crayfish might have separate origins or functions.

The relative expression level of Mjthymosin3 in hemocytes was up-regulated after *V. anguillarum* and *S. aureus* challenge, implying that Mjthymosin3 might be involved in resistance to bacterial infection of shrimp. Thymosin β has various functions in host defense, inflammation regulation, and antimicrobial activity [34]. Previous studies have reported that thymosin β exhibited immune activities in both vertebrates and invertebrates. In addition, thymosins may be involved in the antiviral immune reactions of insects and crayfish. In *Helicoverpa armigera* testis cell lines, knockdown of thymosin increased AcMNPV (*Autographa californica* multiple nucleocapsid nucleopolyhedrovirus) replication [35]. In red swamp crayfish and red claw crayfish, thymosin could inhibit WSSV replication [21,22]. Thymosin β was also reported to show antibacterial activities. In human ocular surface epithelia, thymosinβ4 could inhibit the growth of *Pseudomonas aeruginosa*, *S. aureus*, and *Staphylococcus epidermidis* [9]. In the teleost fish Golden pompano (*Trachinotus ovatus*), thymosin beta 4 was upregulated by bacterial infection, and showed antibacterial properties, such as enhancing the clearance of bacterial colonization and inhibiting bacteria growth [34]. In the sea urchin, *Paracentrotus lividus*, some fragments of thymosin functioned as potential antimicrobial peptides by controlling biofilms of staphylococcal strains [36]. β-thymosin from Pacific oyster (*Crassostrea gigas*) exhibited antibacterial properties by inhibiting the growth of the gram-negative bacteria *E. coli*, the gram-positive bacteria *Bacillus subtilis*, and the yeast *Candida albicans* [37].

To test whether Mjthymosins had antibacterial activities, *in vitro* bacterial growth inhibition assays were performed. We found that Mjthymosin3 could inhibit the growth of gram-negative bacteria and gram-positive bacteria. The result indicated that Mjthymosins participated in the antibacterial immunity of shrimp by inhibiting the bacterial growth. To further investigate the possible mechanism of Mjthymosins' antibacterial activity in the shrimp immune reaction, bacteria clearance tests were carried out. Mjthymosins could facilitate the clearance of external *V. anguillarum* and *S. aureus* in shrimp hemolymph. Thus, Mjthymosins were beneficial to shrimp survival and might be involved in shrimp antibacterial immune defense via their antibacterial activity and bacteria clearance ability. In addition, several antimicrobial peptides (AMPs) have been reported to exhibit antimicrobial activity in kuruma shrimp, such as penaeidins, crustins, and anti-lipopolysaccharide factors [38–40]. Mjthymosins might be novel members of the protein family with antibacterial activity and may function together to protect shrimp from bacterial infection.

In conclusion, four Mjthymosins were identified in kuruma shrimp (*M. japonicus*), and among them, Mjthymosin3 was chosen for further study. Mjthymosin3 was upregulated in hemocytes by *V. anguillarum* and *S. aureus* challenge. In addition, rMjthymosin3 exhibited antibacterial activity to certain gram-negative and gram-positive bacteria. Mjthymosins enhanced bacterial clearance and were beneficial to shrimp survival. Thus, Mjthymosins play important roles in the antibacterial immune response of kuruma shrimp.



GraphPad Prism 5.0 software (** $P < 0.01$).

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Fig. 7. Survival rate of shrimp after *Mjthymosin3* knockdown. (A) *V. anguillarum* were injected into the *Mjthymosin3*-silenced shrimp to record the shrimp survival rate for eight days. (B) *S. aureus* were injected into the *Mjthymosin3*-silenced shrimp to record the shrimp survival rate for five days. Shrimp were divided into two groups: The dsGFP group and *dsthymosin3* group (30 shrimp in each group). At 24 h after the second dsRNA injection, *V. anguillarum* or *S. aureus* were injected. The dead shrimp were monitored every day after bacterial infection. DsGFP-injected shrimp were used as the control. The significance between two groups was calculated using a log-rank (Mantel-Cox) test in