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RESEARCH REPORT

Myeloid leukemia factor participates in the antibacterial immune reaction of kuruma shrimp *Marsupenaeus japonicus*

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Abstract

Myeloid leukemia factor (MLF) plays an important role in the development of *Drosophila.* It is reported that MLF could inhibit white spot syndrome virus (WSSV) replication in shrimp. However, the function and mechanism of MLF in shrimp antibacterial immunity are still elusive. In this study, we found *MjMLF* could be upregulated by *Vibro anguillarum* challenge in hemocytes, gills, and intestine of kuruma shrimp. RNAi assay showed that *Mj*MLF could facilitate bacterial clearance in shrimp, and it was beneficial for shrimp survival post *V. anguillarum* infection. Pull down assay showed that r*Mj*MLF could bind to r*Mj*14-3-3 *in vitro*. Some antimicrobial peptide genes could be regulated by *Mj*MLF. The results indicated that *Mj*MLF might participate in the anti-bacterial immune reaction of shrimp.

Key Words: shrimp; *Mj*MLF; *V. anguillarum*; AMPs; *Mj*14-3-3

Introduction

When organisms were challenged by invading pathogens, immune reaction could be initiated. Immune response contains humoral reaction and cellular reaction (Iwanaga *et al*., 2005; Li *et al*., 2013). Humoral reaction includes prophenoloxidase, clotting cascade, and the production of some antimicrobial peptides (AMPs), while cellular reaction consists of apoptosis, encapsulation, nodule formation, and phagocytosis (Iwanaga *et al*., 2005; Li *et al*., 2013).

It is reported production of AMPs is one hallmark in host defense of *Drosophila*, as well as some other holometabolous insects (Lemaitre *et al*., 2007). When microorganism invades, the synthesis and secretion of potent AMPs are induced and accumulated in hemolymph. Microarray studies indicate the gene expression program in *Drosophila* go through intensive change due to the microbial infection. AMP genes and some other immune responsive genes could be regulated by Toll/IMD cascades pathway in *Drosophila* (De Gregorio *et al*., 2002). In *Drosophila*, the infection of some viruses (such as HIV-1 and cytomegalovirus) could also activate Toll/IMD pathway and induce the expression of some immune related genes.

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Myeloid leukemia factor (MLF) was discovered in acute myeloid leukemia (AML) caused by chromosomal rearrangement. MLF protein is a small nucleo-cytoplasmic shuttling protein, and contains a consensus 14-3-3 binding motif (Gobert, *et al*., 2012). Some MLF interaction proteins, such as MLF-interaction protein (MLFIP) and MLF adaptor molecule (MADM), were identified through yeast two hybrid screens (Lim, *et al*., 2002; Hanissian, *et al*., 2004). MLF is able to decrease the occurrence of Trib1-driven acute myeloid leukemia, as tumor suppressor CCAT/enhancer binding protein (C/EBPα) could be stabilized by MLF (Nakamae, *et al*., 2017; Koschmieder *et al*., 2009). MLF plays an important role in regulating mechanosensitive signaling pathway, which is responsible for typical characteristics in neonatal rat cardiomyocytes (NRVCMs). The overexpression of MLF in NRVCMs could inhibit cell proliferation and increase apoptosis, while knockdown of MLF causes opposite results by protecting cell from apoptosis and increasing proliferation. Besides, knockdown of MLF could upregulate D cyclins expression, which indicates MLF functions in regulating cell proliferation (Yoneda-Kato *et al*., 2005; Rangrez *et al*., 2017). In addition, MLF is reported to function as proapoptotic regulator to modulate cell survival by associating with HOP complex molecule (HAX-1 (HS1 associated protein X-1)/Htra2-OMI/PARL (presenilins-associated rhomboid-like)) (Sun *et al*., 2017b). MLF is able to interact with HAX1 and Htra2 physically, and prevent Htra2 from cleavage and activation.

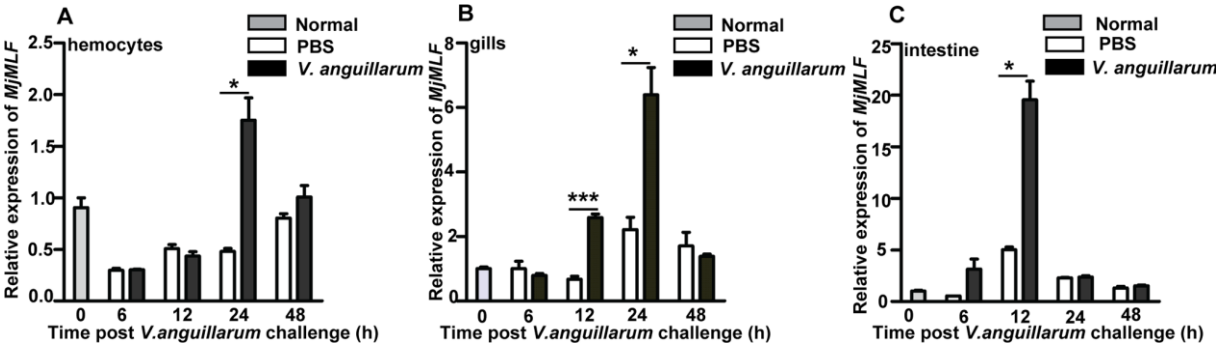

Fig. 1 The temporal course of *MjMLF* in hemocytes, gills, and intestine after *V. anguillarum* injection at 0, 6, 12, 24, 48 h by qRT-PCR at transcriptional level. *β-actin* was used as the control. The significance was detected by *t*-test between PBS group and infected group (**P* < 0.05, ****P* < 0.001)

It is reported that MLF1 could associate with 14-3-3 via a classic RSXSXP sequence in murine and human (Lim *et al*., 2002). MLF1 could be redirected from cytoplasm into nucleus by MLF1-associated nuclear protein (Manp), and also be regulated by 14-3-3 via binding to the 14-3-3 binding motif on MLF1 N terminus (Winteringham *et al*., 2006). And in shrimp, 14-3-3 could inhibit the expression of some AMPs and negatively influence the Akirin-Relish function (Liu *et al*., 2016).

In shrimp, MLF functions importantly by inhibiting WSSV replication and inducing hemocytes apoptosis (Feng *et al*., 2017). In this study, the function of MLF in shrimp innate immunity against bacteria was studied. And results showed that *Mj*MLF could enhance the exogenous *Vibrio anguillarum* clearance, regulate the expression of some AMPs, interact with 14-3-3, and affect shrimp survival. *Mj*MLF participated in the innate immune reaction of kuruma shrimp against *V. anguillarum*.

Materials and methods

Animal and tissue collection

Healthy kuruma shrimp *Marsupenaeus japonicus* (~6-8 g/individual) were purchased from an aquaculture market in Jinan, Shandong Province, China and cultured in artificial aquarium with pumped air (Sun *et al*., 2017a). Three shrimp were randomly selected to extract total RNAs from hemocytes, gills, and intestine with TRIpure Reagent (Bioteke Corporation, Beijing, China) following the manufacture′s instruction.

Expression profiles analysis after V. anguillarum *challenge*

30 μL (1×10^6 CFU) *V. anguillarum* was injected into the last second segment of shrimp. Meanwhile, the same volume of phosphate-buffered saline (PBS) (0.14 M NaCl, 1.8 mM KH_2PO_4, 2.7 mM KCl, and 10 mM Na_2HPO_4) was injected in the other shrimp group, which were used as the control. Tissues including hemocytes, gills, and intestine from *V. anguillarum* injected shrimp and PBS injected shrimp were collected at 6, 12, 24, and 48 h post injection for total RNA extraction. Total RNAs were obtained from at least three shrimp, and reversely transcribed into the first stand cDNAs with primer oligo-anchorR (Feng *et al*., 2017). The obtained cDNAs were diluted 20-fold as the templates for quantitative real time Polymerase Chain Reaction (qRT-PCR). Primer *MjMLF*-RTF1 and *MjMLF*-RTR1 were used as the previous report (Feng *et al*., 2017).

DsRNA preparation

A partial cDNA fragment of *MjMLF* was amplified by PCR with primer *MjMLF*-Fi and *MjMLF*-Ri and used as template for dsRNA synthesis (Feng *et al*., 2017). The control *dsGFP* was produced with the same method (Yang *et al*., 2015). The synthesized dsRNAs were purified by hydroxybenzene and chloroform extraction. And the final dsRNAs were dissolved in RNase-free water, and the concentration was adjusted to 1 μg/μl.

RNA interference (RNAi) was performed as previously reported (Shi *et al*., 2015). At 24 h post the second dsRNA injection, shrimp hemocytes were collected for total RNAs extraction. To test the RNAi efficiency, the first strand cDNAs were reversely transcribed and diluted 20-fold as the qRT-PCR templates with primer *MjMLF*-RTF2 and *MjMLF*-RTR2 (Feng *et al*., 2017).

Bacterial clearance assay

For the bacterial clearance, shrimp was divided into two groups, *dsMjMLF* group and *dsGFP* group. After RNAi, 1×10^6 CFU *V. anguillarum* were injected into the shrimp abdominal segment. At 30 min after *V. anguillarum* injection, hemolymph was extracted from at least three shrimp. The hemolymph was gradiently diluted and spread onto the solid medium plates (1% Tryptone, 0.5% Yeast extraction, 0.1% $FeCl_3$, 3% NaCl, 1.5% Agar) (Zhang *et al*., 2014). After the plates were cultured at 37 °C overnight, the number of bacteria colonies was counted. Results were analyzed by GraphPad Prism 5.0 software with Student′s paired *t* test.

Survival rate assay

For the survival assay, shrimp were divided into three groups, PBS group, *dsGFP* group, and

dsMjMLF group (each group containing 30 shrimp). RNAi assay was performed as the above mentioned. And also, the same volume of PBS was injected into shrimp as the PBS group. After RNAi, 1×10^6 CFU *V. anguillarum* were injected into shrimp of the three groups. The dead shrimp were numbered at 6, 12, 24, 48, 72, 96, 120, 144 h post *V. anguillarum* injection. The *dsGFP*-injected shrimp and PBS-injected shrimp were used as the controls. The *P* value was detected by GraphPad Prism 5.0 software with log–rank (mantel-cox) test.

The expression detection of some AMP genes

V. anguillarum was incubated in liquid medium (1% Tryptone, 0.5% Yeast extraction, 0.1% $FeCl_3$, 3% NaCl) overnight. The bacteria were fully washed by sterile PBS twice. After RNAi, 1×10^6 CFU *V. anguillarum* were injected. The relative expression of some AMP genes including *MjALFE1*, *MjALFE2*, *MjcrustinI-2*, and *MjcrustinI-4* at 6 h post bacteria injection was detected by qRT-PCR in shrimp hemocytes. *β-actin* was the internal control. The primers could be found in the previous report (Bi *et al*., 2015; Yang *et al*., 2017). *P* value was analyzed and accumulated by Student′s *t* test.

Pull-down assay

To detect the interaction of *Mj*MLF with *Mj*14-3-3, GST pull-down assay was performed. 200 μg purified recombinant *Mj*14-3-3 protein (r*Mj*14-3-3, with GST tag) or GST tag protein (as the control) was incubated with the proteinlso GST resin (TransGen Biotech, Beijing, China) respectively for 20 min at room temperature. The resins saturated with r*Mj*14-3-3 or GST protein were sufficiently washed by PBS, till no protein could be detected in the wash solution. 200 μg r*Mj*MLF (with His-tag) were added into the resins separately, and incubated with gentle shaking for 2 h at room temperature. Then the resins was adequately washed by PBS for at least six times to clean up the weak binding protein. The washing solution of the last time was collected as the wash fraction. And finally, the elution buffer (10 mM Glutathione) was added to elute the protein on resin. The eluted fraction and the wash fraction were separated by 15% SDS-PAGE. SDS-PAGE gel was stained by Coomassie brilliant blue G-250 (Sangon Biotech, Shanghai, China). The purified r*Mj*14-3-3, r*Mj*MLF, and GST tag protein were also run on SDS-PAGE as the control.

As the predicted molecular mass of r*Mj*14-3-3 was 53.92 kDa and that of r*Mj*MLF was 52.62 kDa, it was hard to separate the two bands on normal SDS-PAGE. So SDS-PAGE was run at 100 V for about 6 h to separate the two bands on the same lane. GST tag protein was used as the negative control.

Results

MjMLF *was upregulated by* V. anguillarum

To study the function of *MjMLF* in antibacterial immune response, qRT-PCR was carried out to analyze the temporal expression of *MjMLF* in hemocytes, gills, and intestine of shrimp after *V. anguillarum* challenge. The results showed that *MjMLF* could be upregulated in hemocytes, gills, and intestine by *V. anguillarum* challenge (Fig. 1). The expression level of *MjMLF* in hemocytes and gills reached the peak at 24 h after *V. anguillarum* infection (Fig. 1A and 1B), while in intestine it exhibited the highest expression level at 12 h post challenge (Fig. 1C). These results indicated that *MjMLF* might be involved in the immune defense against *V. anguillarum*.

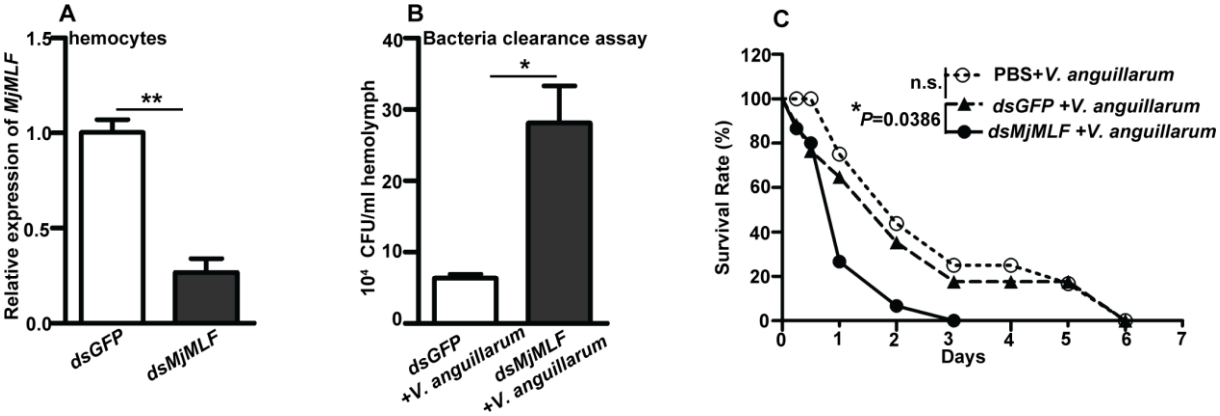

Fig. 2 Bacterial clearance assay and survival assay in shrimp. The *dsGFP* was injected to use as control. (A) The interference efficiency of *MjMLF* in hemocytes was analyzed by qRT-PCR; (B) The bacterial clearance was analyzed by calculating the *V. anguillarum* number per milliliter hemolymph. The significant difference was calculated between *dsMjMLF* group and *dsGFP* group. The significances were calculated by *t*-test ($^*P < 0.05$, $^{**}P < 0.01$). (C) The shrimp survival rate was analyzed by RNAi and *V. anguillarum* challenge. The shrimp mortality was detected for calculation of the shrimp survival rate. The *dsGFP* group and PBS group were used as the control. The survival rate was analyzed with the Kaplan-Meier method. The significant differences were detected by log-rank test ($^*P < 0.05$)

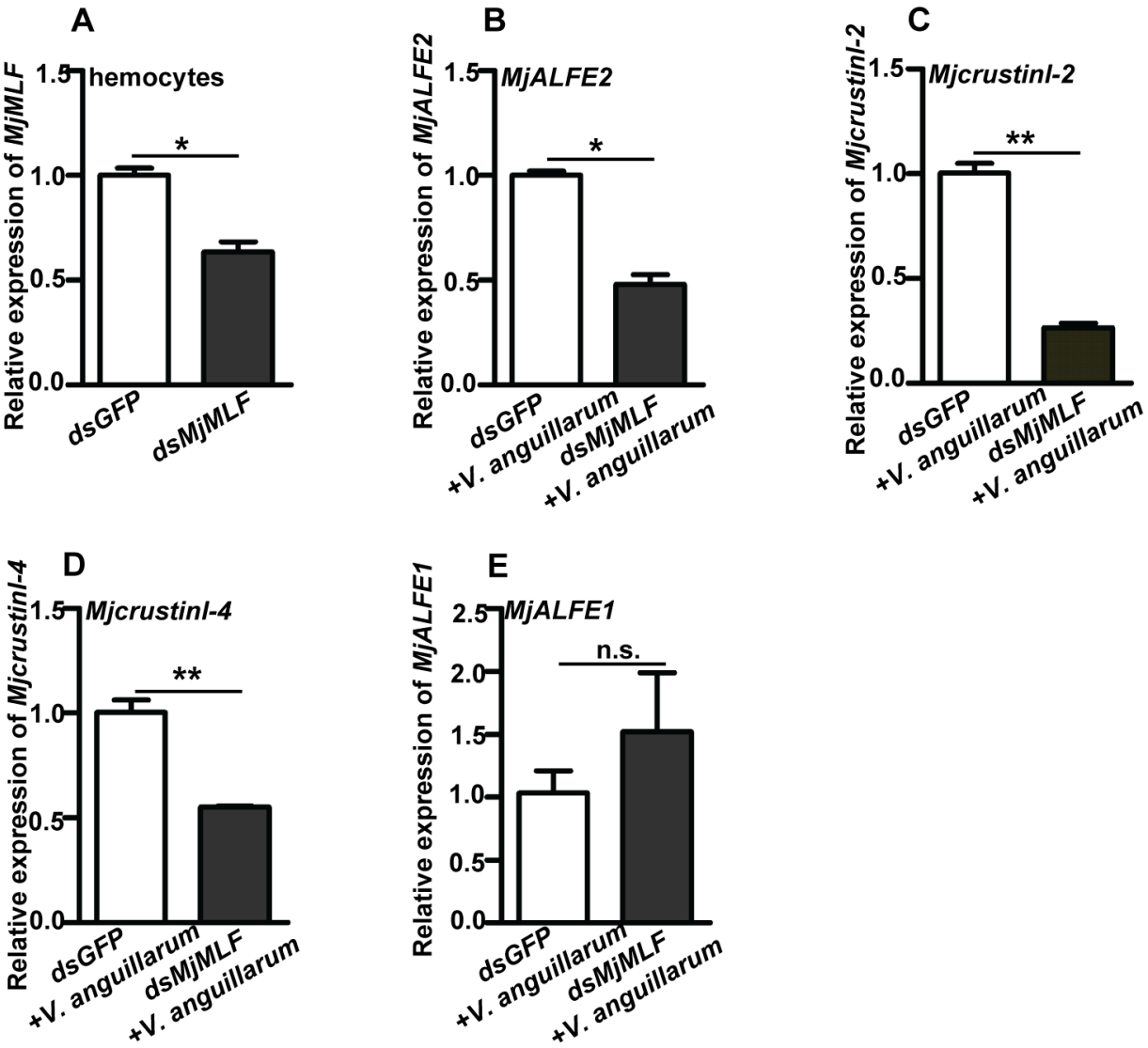

Fig. 3 The expression analysis of AMPs (*MjALFE1*, *MjALFE2*, *MjcrustinI-2*, and *MjcrustinI-4*) in shrimp hemocytes. (A) The knocking down efficiency of *dsMjMLF* in hemocytes was detected after the dsRNA injection. The relative expression of *MjALFE2* (B), *MjcrustinI-2* (C), *MjcrustinI-4* (D), and *MjALFE1* (E) was analyzed by qRT-PCR at 6 h post the *V. anguillarum* challenge. The significant differences were analyzed by *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$)

V. anguillarum *clearance rate decreased in* MjMLF-*silenced shrimp*

To determine the function of *MjMLF* in immune defense against *V. anguillarum*, RNAi and bacterial clearance assay were performed. After knocking down the expression of *MjMLF* (Fig. 2A), *V. anguillarum* were injected into shrimp. Compared to the *dsGFP*-injected shrimp, the *V. anguillarum* number in *dsMjMLF*-injected shrimp was much higher, which indicated the clearance rate in the *dsMjMLF*-injected shrimp was much slower (Fig. 2B). *Mj*MLF could accelerate the bacterial clearance in shrimp. The result implied that *Mj*MLF might have an important function in the immune defense against *V. anguillarum* in shrimp.

The expression of some AMPs was downregulated in dsMjMLF-*injected shrimp*

To test whether some effector molecules could be regulated by *Mj*MLF, the expression of some AMPs (*MjALFE1*, *MjALFE2*, *MjcrustinI-2*, and *MjcrustinI-4*) was analyzed after RNAi by qRT-PCR. After knocking down *MjMLF* (Fig. 3A), the expression of *MjALFE2*, *MjcrustinI-2*, and *MjcrustinI-4* decreased significantly in *dsMjMLF*-injected shrimp compared with the *dsGFP*-injected shrimp (Fig. 3B, C, D), while there was no significant change for the expression of *MjALFE1* (Fig. 3E). The results indicated that *MjMLF* might protect shrimp from *V. anguillarum* infection by regulating some specific AMPs expression.

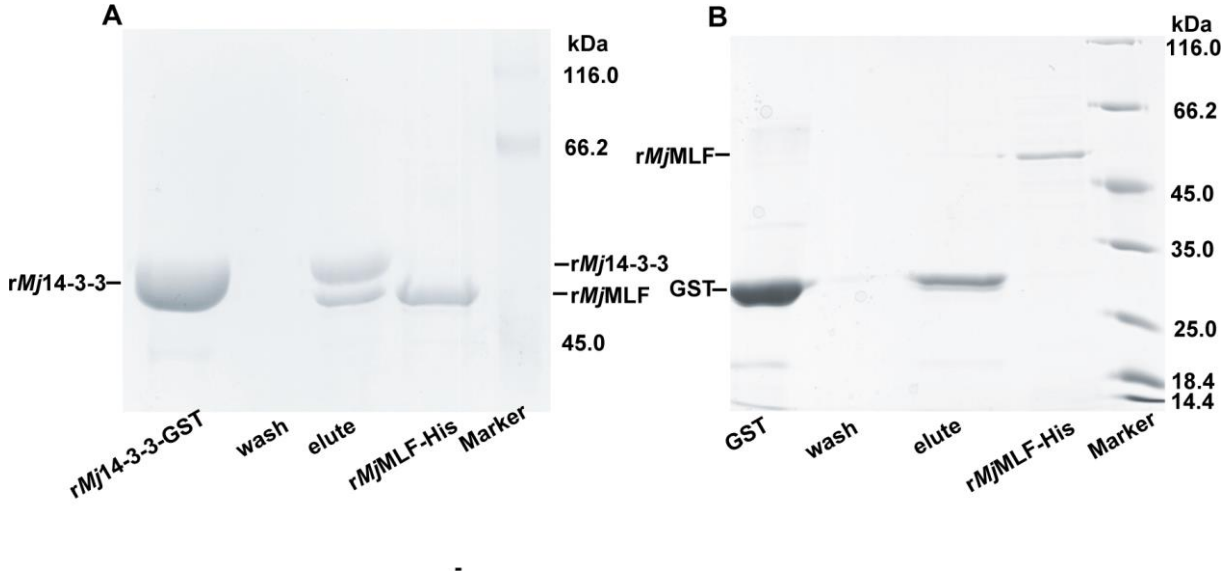

Fig. 4 Pull down assay was used to check the interaction of r*Mj*MLF and r*Mj*14-3-3. (A) Purified recombinant r*Mj*14-3-3 was bound to GST resin, and then r*Mj*MLF was added. After sufficient washing, the eluted fraction was collected. r*Mj*14-3-3, the wash fraction, the elute fraction, and r*Mj*MLF were separated by 15% SDS-PAGE for 6 h at 100 V. (B) Purified GST tag protein was used as the control and separated on 15% SDS-PAGE at 100 V for 2 h. The gels were stained by Coomassie brilliant blue

*r*Mj*MLF interacts with r*Mj*14-3-3 in vitro*

It is reported that MLF could enhance apoptosis, and bind to 14-3-3. And MLF is negatively regulated by 14-3-3 (Sun *et al*., 2015). In shrimp, *Mj*14-3-3 is involved in regulating AMPs expression (Liu *et al*., 2016). To understand the mechanism of *Mj*MLF in antibacterial immunity of shrimp, GST-pulldown assay was performed to investigate the interaction of r*Mj*MLF with r*Mj*14-3-3. The results showed that r*Mj*MLF could interact with r*Mj*14-3-3 *in vitro* (Fig. 4A), while no interaction was detected between r*Mj*MLF and GST protein (Fig. 4B). It demonstrated that r*Mj*MLF had the binding activity to r*Mj*14-3-3, but not the GST tag. And it has been reported His tag protein could not bind to GST tag protein (Xu *et al*., 2017). The results suggested that r*Mj*MLF could bind to r*Mj*14-3-3 directly *in vitro*.

Discussion

In this study, the role of *MjMLF* in antibacterial immune reaction was researched. As far as we know, it is the first report about the function of MLF in antibacterial immunity of kuruma shrimp *M. japonicus*.

As reported previously, the expression of *MjMLF* was upregulated in hemocytes and hepatopancreas by WSSV challenge. And *Mj*MLF inhibits WSSV replication by influencing hemocytes apoptosis. *Mj*MLF could increase the survival rate and play an important function in antivirus immunology (Feng *et al*., 2017). The expression pattern assay showed that expression of *MjMLF* was upregulated in hemocytes, gills, and intestine of shrimp when shrimp were challenged by *V. anguillarum*. The result suggested that the potent role of *Mj*MLF in shrimp antibacterial immunity. To research the function of *Mj*MLF in antibacterial immune reaction, RNAi assay and exogenous bacterial clearance assay were performed. The *V. anguillarum* clearance rate was much slower in *dsMjMLF*-injected shrimp, which indicated the critical role of *MjMLF* in antibacterial immune reaction. Besides, the shrimp survival rate in *dsMjMLF*-injected shrimp was lower than the control groups. *Mj*MLF could be involved in shrimp antibacterial immune reaction.

In *Drosophila*, MLF could promote the expression of JNK (c-Jun N-terninal kinase) (Yanai *et al*., 2014). Further research indicates that JNK pathway is a critical signaling pathway that initiating some relevant effector molecular (Boutros *et al*., 2002; Park *et al*., 2004; Kallio *et al*., 2005; Delaney *et al*., 2006). It is reported that dMLF could associate with DREF (DNA replication-related element-binding factor) and initiate *bsk* promoter. The interaction could further activate the JNK signaling pathway (Yanai *et al*., 2014). In *Drosophila* S2 cells, the JNK signaling pathway plays an essential role in regulating normal AMPs release (Kallio *et al*., 2005). The production of AMPs could regard as local immune response relating to external environment (Lemaitre *et al*., 2007). And in *Drosophila* and other holometabolous insects, the synthesis and secretion of the potent AMPs could be induced and accumulated in hemolymph when invading microorganisms challenge, and it was the critical hallmark of host defense (Lemaitre *et al*., 2007). In *Drosophila*, most of the immune responsive genes are believed to be regulated by immune signaling pathway, such as Toll and IMD pathway (De Gregorio *et al*., 2002).

In our study, we found *Mj*MLF could positively regulate the expression of *MjALFE2*, *MjcrustinI-2*, and *MjcrustinI-4*. Additionally, it is reported that MLF is able to interact with 14-3-3 with phosphorylated motif (Lim *et al*., 2002). In shrimp, we detected their direct interaction *in vitro* by pull down assay. *Mj*14-3-3 was able to regulate the expression of some AMPs (Liu *et al*., 2016). And *Mj*14-3-3 could interact with Akirin, which suppressed the expression of some AMPs. Akirin participates in IMD-Relish pathway via associating with Relish and activate Relish-regulated AMP genes. The interaction of r*Mj*MLF and r*Mj*14-3-3 indicated that *Mj*MLF might suppress the regulation of *Mj*14-3-3 on AMPs expression by binding to *Mj*14-3-3. So *Mj*MLF might participate in shrimp antibacterial innate immunity by regulating the expression of some AMPs. Further research such the sublocation of *Mj*14-3-3 in hemocytes after knocking down *MjMLF* or co-expression of *Mj*14-3-3 and *Mj*MLF in insect cell lines would help us fully understand the mechanism of *Mj*MLF in shrimp antibacterial immunity.

In conclusion, *MjMLF* could protect shrimp from bacterial infection by regulating some AMPs expression, enhancing bacterial clearance, and affecting the shrimp survival rate. *MjMLF* might play a critical function in antibacterial immunity of shrimp.

Acknowledgement

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