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A toll receptor is involved in antibacterial defense in the oriental river prawn, *Macrobrachium nipponense*

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ABSTRACT

Toll-like receptors (TLRs) play an important role in the activation of innate immune response in animals. In this study, we identified a TLR from the oriental river prawn, *Macrobrachium nipponense* (*MnToll1*) and investigated its functions in immunity. The *MnToll1* protein shares similar structural characteristics with other known Toll family proteins. *MnToll1* transcripts are broadly distributed in all of the examined tissues, and its expression level was significantly up-regulated by bacterial challenge. RNAi-mediated knockdown of MnToll1 significantly impaired the survivability of *Vibrio*-challenged prawns. RNAi experiments also revealed that the expression of several antimicrobial peptide genes were regulated by *MnToll1*. Moreover, we found the extracellular region of MnToll1 could directly bind to bacteria and bacterial glycoconjugates. These findings suggest that MnToll1 function as a pattern recognition receptor to recognize invading pathogen and initiate downstream gene expression, to participate in antibacterial defense of *M. nipponense*.

1. Introduction

Lacking of an adaptive immune system, invertebrates mainly rely on their innate immunity to resist invading pathogens [1]. Studies on the innate immune system of invertebrates have revealed much information regarding the underlying mechanisms of host defense and host-pathogen interaction. Pathogen recognition by the innate immune system is mediated by a set of pattern recognition receptors (PRRs), which can recognize the pathogen associated molecular patterns (PAMPs) on the surface of microbes [2]. One of the best characterized PRRs, Toll-like receptors (TLRs), play important roles in innate immunity against pathogen invasion in both invertebrates and vertebrates [3]. *Toll* was firstly identified in fruit fly as a gene essential for embryonic dorsal-ventral development [4], but was later found to be responsible for the antifungal and antibacterial defense in *Drosophila* [5]. In *Drosophila*, fungal or gram-positive bacterial infection is sensed by extracellular recognition factors and leads to the cleavage of a cytokine-like molecule Spätzle, the ligand of the Toll receptor [5,6]. Binding of Spätzle dimer to Toll receptor activates the Toll pathway, with a cassette of proteins consisting of MyD88, Tube, and Pelle recruited, which form a complex and subsequently degrade Cactus; the degradation of Cactus frees Dorsal and Dorsal-related immunity factor (DIF), which enter the nucleus to initiate the transcription of a set of antimicrobial peptide (AMP) genes [7–9]. Toll pathway was also reported to participate in cellular immune response such as phagocytosis and encapsulation [10].

Since the first report of *Drosophila* Toll, the mammalian Toll homologs were identified one after another. Tolls and TLRs from vertebrates and invertebrates are characterized by an extracellular region containing several leucine-rich repeats (LRRs), a transmembrane domain, and a cytoplasmic tail that contains a conserved region named the Toll/IL-1 receptor (TIR) domain [11]. In humans, a total of 10 TLRs were found either in the plasma membrane or endosome of leukocytes. Unlike the *Drosophila* Toll1 which requires the binding of Spätzle, the human TLRs function as PRRs by binding specifically to PAMPs from bacteria, fungi, or viruses [12]. For instance, human TLR4 can recognize lipopolysaccharide (LPS) of Gram-negative bacteria, as well as several viral proteins [13]. In penaeid shrimps, the involvement of TLRs in immunity has been well established. In *Litopenaeus vannamei*, the identified TLRs (LvToll1-3) can respond to both *Vibrio* and white spot syndrome virus (WSSV) infections [14–16]; In *Fenneropenaeus chinensis* and *Marsupenaeus japonicus*, FcToll and MjToll also respond to bacterial

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and viral immune challenges [17,18]; In *Penaeus monodon,* Toll pathway was reported to respond to WSSV infection [19,20]. The regulation of Toll pathway on AMP gene expression has also been reported in above species. A recent study found that shrimp TLRs can recognize invading pathogens in a Spätzle-independent manner: in kuruma shrimp *M. japonicus*, its three Toll-like receptors (Toll1-3) can directly bind to polysaccharides on pathogen surface through their LRR domains, to activate downstream AMP gene expressions [21]. In contrast to the penaeid shrimps, information regarding Toll pathway in palaemonid shrimps are limited. In the giant freshwater prawn *Macrobrachium rosenbergii*, a Toll receptor was reported to participate in host defense against the bacterial pathogen *Aeromonas caviae* [22]; another Toll gene from the same species was found to be involved in anti-WSSV defense [23]. However, the mechanism of prawn Toll pathway activation by these pathogens remain unclear.

The oriental river prawn, *Macrobrachium nipponense*, is an economically and nutritionally important crustacean species in several Asian countries. The cultivation of *M. nipponense* has been impaired with serious diseases linked with its bacterial pathogen *Vibrio spp.* [24]. In this scenario, we are interested in studying the key molecules and immune pathways of *M. nipponense* against *Vibrio* infection, as well as the mechanism underlying the pathogen recognition and elimination. In the present study, we identified a Toll-like receptor from *M. nipponense* (*MnToll1*) and further investigated its role in antibacterial defense of prawns, especially against the pathogen *V. parahaemolyticus*. Here we report on the outcomes of our investigation.

2. Materials and methods

2.1. Experimental animals and bacteria

M. nipponense was reared in a culture pond located in Henan Normal University. Before experiments, prawns with a body weight around 2 g were kept in an aerated water tank with freshwater for a couple days. The bacteria *S. aureus*, *Bacillus megaterium*, *Bacillus subtilis*, *Escherichia coli, Pseudomonas aeruginosa* and *V. parahaemolyticus* were kept in our laboratory.

2.2. Immune challenge and tissues collection

Hemolymph was collected from the ventral sinus of healthy prawns using equal volume of an anticoagulant solution (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6). The collected hemolymph was further centrifuged at 800 × *g* for 10 min at 4 °C to obtain hemocytes. Other tissues, including heart, hepatopancreas, gills, stomach, intestine and muscle were also collected from healthy prawns to extract the total RNA. For immune challenge, the abdominal segment of each prawn was injected with 2×10^7 cells of *V. parahaemolyticus* or *S. aureus*. Gills were dissected and collected at 0, 6, 12, and 24 h post challenge.

2.3. Total RNA isolation and cDNA synthesis

Total RNA was extracted from the collected tissues using the Trizol reagent (Invitrogen, USA), following the manufacturer's protocols. RNA quality was assessed by agarose gel electrophoresis and RNA concentration was determined using NanoDrop 2000 (Thermo Scientific, USA). Then, 2 μg of RNA was used to synthesize the first strand cDNA using the PrimeScript® 1st Strand cDNA Synthesis Kit (Takara, Dalian, China), with an oligo-dT primer.

2.4. Cloning of full-length cDNA of MnToll1

Based on the unigenes obtained from *M. nipponense* transcriptome data, specific primers (MnToll1-F and MnToll1-R, Table S1) was designed to clone the full length of *MnToll1*, using a Clontech SMARTer™ RACE cDNA amplification kit from Takara (Dalian, China), with the following PCR procedures: 1 cycle at 94 °C for 2 min; 30 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min; and 1 cycle at 72 °C for 2 min. The full-length cDNA of *MnToll1* was obtained by overlapping the obtained fragments.

2.5. Sequence analyses of MnToll1

Homology analysis of *MnToll1* with other *TLR* genes was accomplished using the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The signal peptide was predicted using the online SignalP program (http://www.cbs.dtu.dk/services/SignalP/) [25]. Putative domains and motifs were predicted by the online SMART program (http://smart.embl-heidelberg.de/). The phylogenetic tree was constructed with MEGA 4.0 software, using a Neighborhood-joining method [26].

2.6. Real-time PCR

A pair of primers (*MnToll1*-qRT-F and *MnToll1*-qRT-R, Table S1) was designed to examine the tissue distribution and the expression profiles of *MnToll1* by quantitative real-time PCR, while the β-actin gene was used as an internal control, with primers listed in Table S1. The qRT-PCR was performed using StepOnePlus™ Real-Time PCR System (ABI, USA), following the instructions of the manufacturer. A 2 × SYBR real-time PCR premixture (TransGen Biotech, Beijing, China) was used and the cycling conditions were set up as follows: 94 °C for 30 s, 40 cycles of 94 °C for 5 s and 60 °C for 30 s. The experiments were repeated three times, and obtained data were calculated via the $2^{-\Delta\Delta CT}$ method [27].

2.7. RNAi of MnToll1 expression and detection of AMPs expression

Sequence-specific primers (ds*MnToll1*-F and ds*MnToll1*-R, Table S1) linked to the T7 promoter were used to amplify the partial *MnToll1* cDNA as templates for dsRNA synthesis. The dsRNA synthesizing mixture consisted of 8 μg of the template, 2.4 μl of each NTP (100 mM), 60 U of T7 RNA polymerase, 80 U of the RNase inhibitor and 20 μl of 5 × transcription buffer (Takara), which was incubated at 37 °C for 8 h. 8 U of DNase I was then added into the mixture to remove the DNA template. The resulted dsRNA was purified by phenol/chloroform extraction and then ethanol precipitation, which was further dried and dissolved in distilled RNase-free water. Each prawn of experimental group was injected with 30 μg of *MnToll1*-dsRNA, while that of the control group was injected with same amount of *GFP*-dsRNA. At 48 h post injection of dsRNA, the prawns were further challenged *V. parahaemolyticus*. At 12 h post bacterial challenge, the gill samples were collected and total RNA of gills were extracted from each group. The expression of AMP genes, including *anti-lipopolysaccharide factor 1(ALF1), ALF2, crustin 1(Cru1)* and *Cru2,* were detected in *MnToll1*-dsRNA silenced prawns by qRT-PCR. All primers of *AMPs* were listed in Table S1. The experiment was repeated three times.

2.8. Survival experiment

Prawns were assigned into three groups (PBS, dsGFP and dsToll1) and each group contained 50 prawns. Each prawn of the PBS group was injected with PBS; prawns of the dsGFP group was each injected with 30 μg of *GFP*-dsRNA; prawns of the dsToll1 group was each injected with 30 μg of *MnToll1*-dsRNA. At 24 h post dsRNA injection, 1×10^8 cells of *V. parahaemolyticus* was injected into each prawn, and the mortality was recorded for 7 days after infection.

2.9. Expression and purification of the recombinant proteins

The partial cDNA fragment encoding the extracellular region of MnToll1 protein was amplified, using corresponding primers listed in

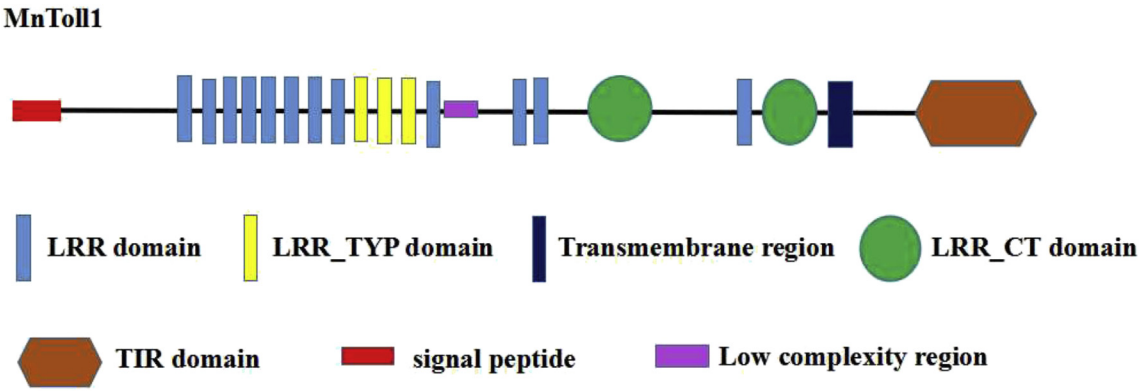

Fig. 1. Schematic representation of domain topology of MnToll1.

Table S1 (MnLRR ExF and MnLRR ExR). The fragments were then ligated into a pET30a (+) plasmid, which were further transformed into *E. coli* BL21 (DE3) cells for protein expression. After inducing with 0.1 mM IPTG, the recombinant protein rMnLRR with a His-tag at N-terminal was expressed in insoluble form. The denaturation, renaturation, and purification of the recombinant protein was performed as described in the literature [28]. Recombinant protein concentration was determined by the Bradford assay.

2.10. Bacterial binding assay

Gram-positive bacteria (*S. aureus*, *B. megaterium* and *B. subtilis*) and Gram-negative bacteria (*E. coli, P. aeruginosa* and *V. parahaemolyticus*) were used to test the binding capacity of rMnLRR. Each bacterium was cultured overnight in 3 ml LB medium (1% Tryptone, 0.5% Yeast Extract, 1% NaCl), and collected by centrifugation. After washing with Tris-HCl buffered saline (TBS, pH 8.0), the bacteria cells were re-suspended in TBS to OD_{600} of 1.0. Purified rMnLRR protein (50 μg) was added into 500 μl of bacterial suspension and incubated at room temperature for 20 min, with gentle rotation. After incubation, bacteria cells were washed four times with TBS and then eluted with 100 μl of 10% SDS. After that, the bacterial cells with elution buffer were subjected to 12.5% SDS-PAGE under reducing conditions. An anti-His-tag monoclonal antibody (Sangon Biotech, China) was used to detect the recombinant protein.

2.11. Glycoconjugates binding assay

An enzyme-linked immunosorbent assay (ELISA) was carried out to detect the binding of rMnLRR to glycoconjugates from bacteria, including LPS (from *E. coli* Serotype 055:B5) and peptidoglycan (from *Staphylococcus staphylolyticus*) (Sigma). The glycoconjugates were prepared in water at a concentration of 80 μg/ml, which were further used to coat the wells of a 96-well amicrotiter plate with 50 μL volume/well. The plate with coating solution was incubated at 37 °C overnight, followed by heating at 60 °C for 30 min. Plate was then blocked with BSA (1 mg/ml, 50 μl) at 37 °C for 2 h and washed with TBS for four times. Purified rMnLRR (0–100 μg/ml) were prepared with gradient dilution and added into the wells. After incubation for 3 h at room temperature, the wells were washed with TBS and the anti-His-tag antibody was added. Another 1 h incubation at 37 °C was performed, following by TBST washing for four times. Each well was then incubated with 100 μl of peroxidase-conjugated goat anti-mouse IgG (1/10000 diluted) for 1 h at 37 °C. For color development, 0.01% 3,3′,5,5′-tetramethylbenzidine (Sigma) was added, and the reaction was stopped by adding 2 M H_2SO_4 to the wells. The absorbance at 450 nm was read and recorded.

2.12. Statistical analyses

To compare difference between experimental groups and control groups, all the gene expression data were analyzed by Student's paired *t*-test, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cloning and sequence analysis of MnToll1

The complete sequence of the *MnToll1* cDNA was 3807 bp in length, containing an open reading frame (ORF) of 3012 bp which encoding a protein of 1003 amino acids. It is predicted that MnToll1 protein has a putative signal peptide of 23 amino acid residues and a mature protein of 980 amino acids (Fig. 1 and Fig. S1). The predicted MnToll1 protein has a molecular mass of 113.0 kDa and an isoelectric point of 5.63. Analysis of its amino acid composition indicated the existence of 12 Leucine-rich repeats (LRR) domains with an amino acid (aa) length of 22–30, two Leucine rich repeat C-terminal (LRR CT) domains, three Leucine-rich repeats typical subfamily (LRR TYP) domains, a transmembrane domain, and an intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domain (Fig. 1). To explore the evolutionary dynamics of TLRs, a phylogenetic tree was constructed using MnToll and other selected TLR sequences. MnToll1 was clustered together with TLRs from *Macrobrachium rosenbergii* and also shared close relationship to TLRs from other crustacean species, distinct from the other cluster of vertebrate TLRs (Fig. S2).

3.2. Stimulation of MnToll1 mRNA expression after bacterial challenge

The mRNA expression of *MnToll1* in different tissues was analyzed by qRT-PCR. Results showed that transcripts of *MnToll1* were distributed in all the tissues examined, with the highest level in gills (Fig. 2).

To better understand the roles of *MnToll1* in antibacterial defense, its expression level after bacterial challenge was examined. After challenged with its pathogen *V. parahaemolyticus*, *MnToll1* in gills was up-regulated at all selected time points (2, 6, 12 and 24 h), with the highest level at 12 h (Fig. 3A). When challenged with gram-positive bacteria *S. aureus*, *MnToll1* in gills showed a continuously upregulated pattern within 24 h (Fig. 3B).

3.3. RNAi of MnToll1 led to increased mortality of vibrio-infected prawns

To further figure out whether *MnToll1* is involved in the immune defense against *Vibrio* infection, a RNA interference experiment followed by a survival experiment was performed. As shown in Fig. 4A, *MnToll1*-dsRNA injection severely reduced accumulations of mRNA encoding MnToll1 in prawns, compared to normal or *GFP*-dsRNA

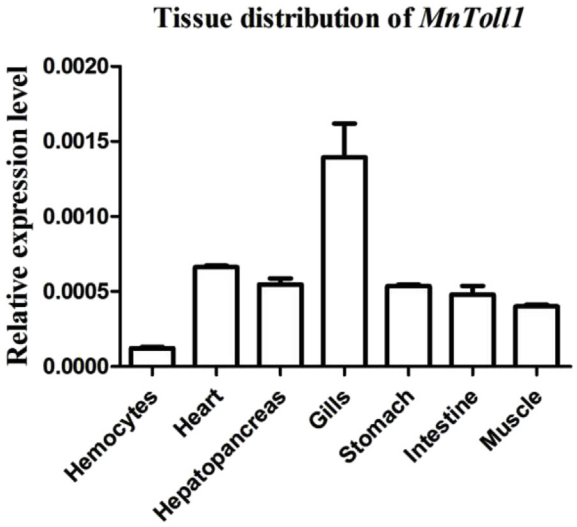

Fig. 2. Distribution of *MnToll1* in different tissues of the oriental river prawn *M. nipponense* analyzed by qRT-PCR. Prawn *β-actin* was amplified as an internal control. Error bars represent the mean value ± S.D. of three independent experiments.

groups, indicating the successful knockdown of *MnToll1*. In the following survival experiment, RNAi-mediated knockdown of MnToll1 led to decreased survival rate of *Vibrio*-infected prawns: by 7 days after *V. parahaemolyticus* infection, the prawns in PBS or dsGFP-treated groups had a survival rate of 40%, whereas the dsMnToll1-treated group had a survival rate lower than 10% (Fig. 4B).

3.4. MnToll1 is involved in regulating AMP gene expression in prawns

An RNAi assay was performed to determine the function of *MnToll1* in regulating AMP gene expression. After *MnToll1*-dsRNA injection into the prawns, *MnToll1* in the gills was significantly knocked down compared to normal or *GFP*-dsRNA controls (Fig. 5A). When challenged with *V. parahaemolyticus*, the expressions of examined AMP genes (*ALF1*, *ALF2, Cru1* and *Cru2*) in *M. nipponense* were increased to higher levels. However, upon *MnToll1* knockdown following *Vibrio* challenge, the transcripts of *ALF2*, *Cru1*, *Cru2* were down-regulated than those without RNAi treatments or dsGFP-treated controls (Fig. 5C–E). The expression of *MnALF1* was not affected by *MnToll1* knockdown (Fig. 5B).

3.5. MnToll1 binds to bacteria and bacterial glycoconjugates

To figure out the activation mechanism of MnToll1 by bacterial pathogens, a Toll1-bacteria binding assay was carried out. The extracellular region of MnToll1 (named MnLRR) was expressed in *E. coli* and purified (Fig. S3). The rMnLRR protein could bind to all the bacteria tested (Fig. 6A). Furthermore, an ELISA assay was performed to detect if rMnLRR could recognize the glycoconjugates on the bacteria surface. As shown in Fig. 6B, rMnLRR could bind to both LPS and PGN with high affinity. The binding curve fits the logarithmic curve, showing the binding between the recombinant protein and glycoconjugates are saturable.

4. Discussion

TLRs function as PRRs to identify various PAMPs, and Toll signaling pathway plays important roles in innate immunity defending against pathogens in both vertebrates and invertebrates. The involvement of TLRs in innate immunity have been confirmed in mammals, insects, as well as in crustaceans. In this study, a Toll-like receptor (designated as *MnToll1*) was identified from the oriental river prawn, *M. nipponense*. MnToll1 is a classical type I transmembrane protein containing 12 LRR domains, units characterized by a conserved pattern of hydrophobic residues with approximately 24 amino acids. MnToll1 has one TIR domain, which is a highly-conserved region of 200 residues and mediates protein-protein interactions between TLRs, as well as TLR and adaptor proteins. There are some unconventional Toll-like receptors having more than one TIRs, for instance, the HcToll2 from *H. cumingii* has two TIRs in tandem [29]. The amino acid sequence of MnToll1 shared similarity with TLR family proteins from other organisms and it was closely matched to crustacean TLRs from *M. rosenbergii* and *M. japonicus*.

MnToll1 mRNA was detected in all tested tissues including hemocytes, hepatopancreas, gills, and intestine. The highest expression level of *MnToll1* was found in the gills, which is consistent with *Toll1-3* in *M.*

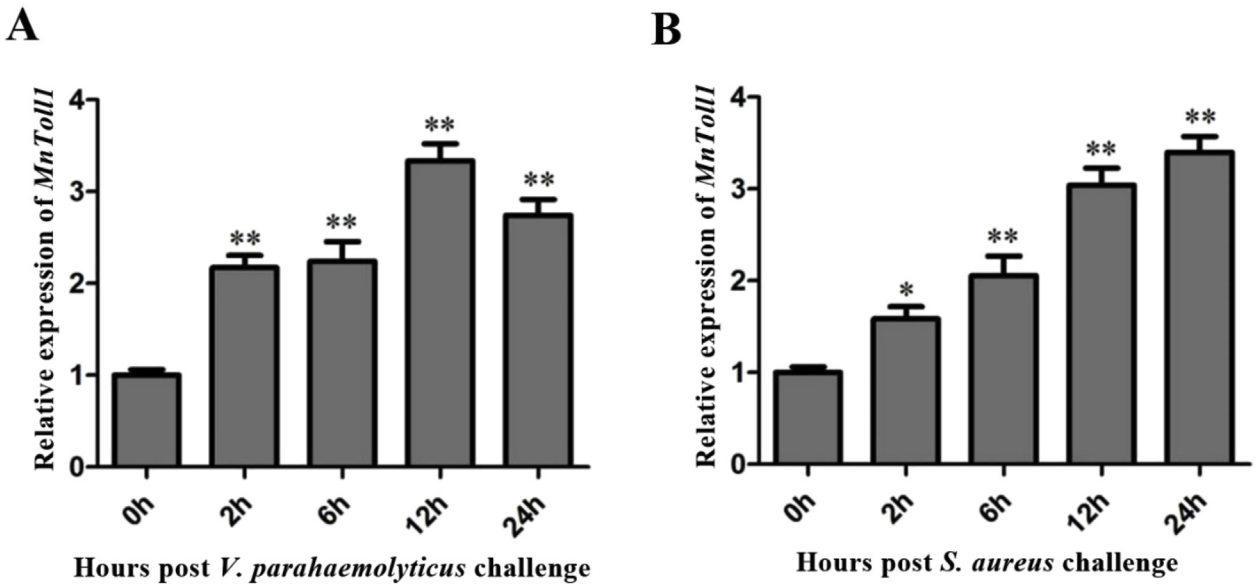

Fig. 3. The expression levels of *MnToll1* in gills of prawns upon *V. parahaemolyticus* (A) or *S. aureus* (B) challenge at 0, 2, 6, 12 and 24 h. Three prawns were chosen to eliminate individual differences at each sampling time point. Error bars represent the mean value ± S.D. of three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) compared with values of unchallenged prawns.

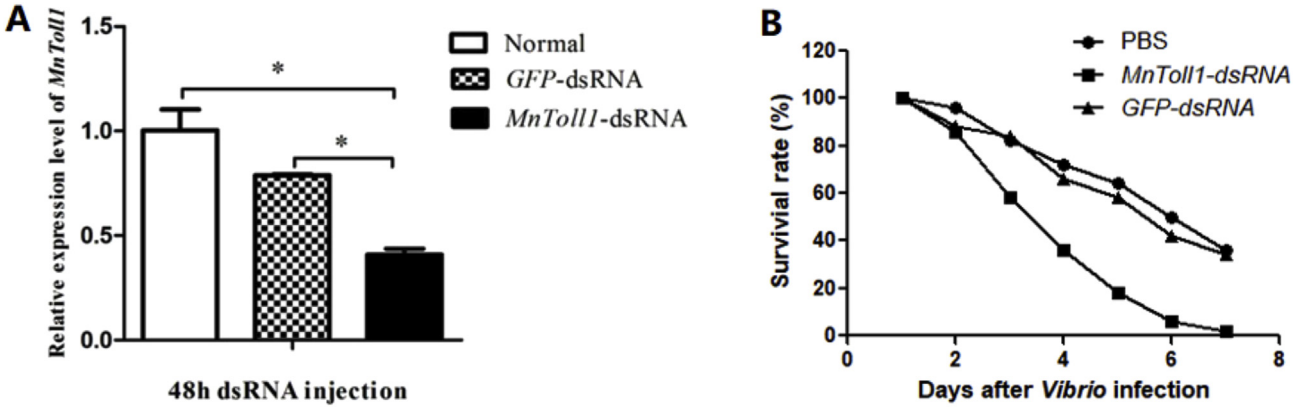

Fig. 4. (A) qRT-PCR analysis of the RNA interference efficiency of *MnToll1* in gills. Samples were collected at 48 h after injection with *GFP*-dsRNA, *MnToll1*-dsRNA. Healthy prawns were sampled as control. Error bars represent the mean value ± S.D. of three independent experiments. Asterisks indicate significant differences (*$P < 0.05$) between experiment group (*MnToll1*-dsRNA group) and control group (*GFP*-dsRNA or normal group). (B) Survival experiment showed that knockdown of MnToll1 led to decreased survivability of *Vibrio*-challenged prawns, compared to control groups.

japonicus, as well as the PcToll from *P. clarkii* [21,30]. The MrToll from *M. rosenbergii* also has a widely tissue distribution pattern but has relatively low expression in gills [22]. It seems most crustacean TLRs have a wide tissue distribution pattern. In the bacterial challenge experiment, we found that expression of *MnToll1* was induced by immune challenge, using Gram-negative bacteria *V. parahaemolyticus* or Gram-positive bacteria *S. aureus*. Similar results have been reported for several other crustacean TLRs, such as the Toll1-3 from *M. japonicus* and PcToll [21,30]. Some shrimp TLRs, including a Toll from *M. rosenbergii*, are also reported to respond to WSSV infection [16,19,23]. It seems that crustacean TLRs have a lower specificity but a broader pathogen-recognition spectrum. This generality of Toll pathway responding to various pathogens is distinct from that in *Drosophila*, in which the Toll pathway only responds to Gram-positive bacteria and fungi infection.

The involvement of MnToll1 in anti-*Vibrio* defense was further confirmed by the survival experiment. dsRNA-mediated knockdown of MnToll1 significantly decreased the survival rate of *Vibrio*-infected prawns, indicating the important role of MnToll1 in anti-*Vibrio* defense. Toll pathway is well known for regulating downstream gene expression, including antimicrobial peptide (AMP) genes. To identify the AMP genes regulated by *MnToll1*, *in vivo* RNAi experiments were conducted. RNAi results showed that *MnToll1* was involved in *V. parahaemolyticus*-

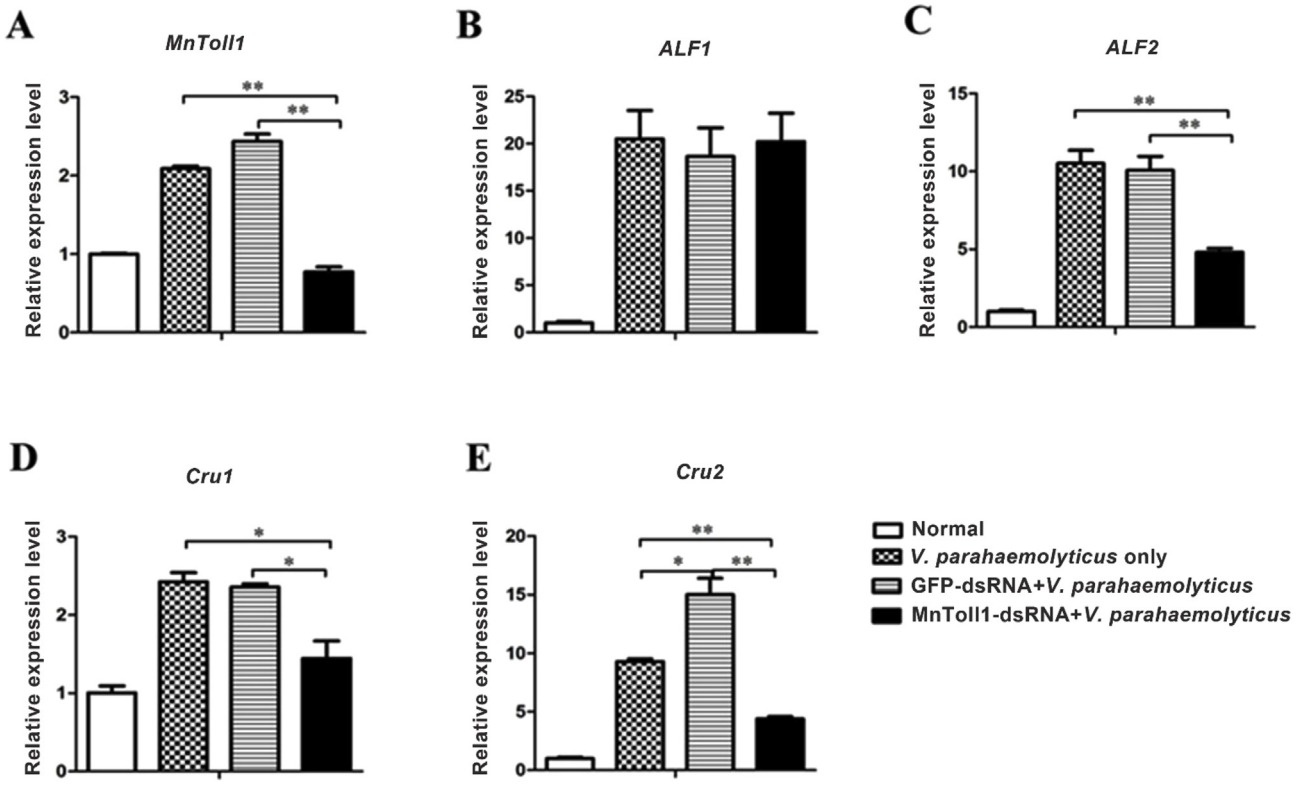

Fig. 5. Expression levels of *AMP* genes (*ALF1*, *ALF2*, *Cru1* and *Cru2*) in gills of prawns with *MnToll1* knockdown. Expression level of *MnToll1* (A) in gills of prawns from control groups (normal, 12 h *V. parahaemolyticus* only challenge, 48 h *GFP*-dsRNA pre-injection plus 12 h *V. parahaemolyticus* challenge) and experiment groups (48 h *MnTol11*-dsRNA pre-injection plus 12 h *V. parahaemolyticus* challenge). B–E: The expression change of *AMP* genes (*ALF1*, *ALF2*, *Cru1*, and *Cru2*) in gills of *MnToll1* knockdown prawns. Error bars represent the mean value ± S.D. of three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) between experiment group and control group.

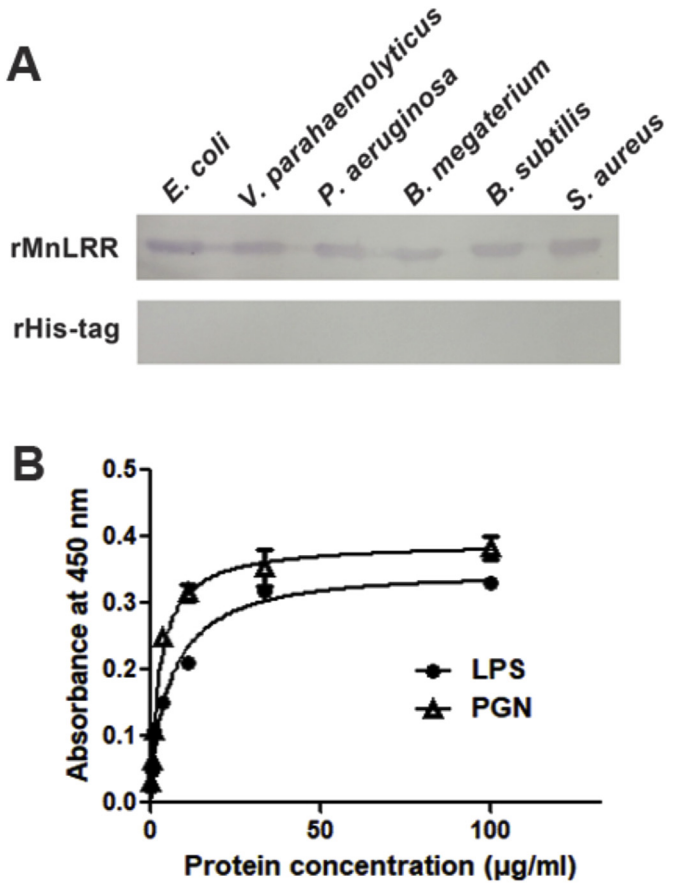

Fig. 6. Bacteria and glycoconjugates binding assays of recombinant MnLRR. (A) Bacterial binding assay showed that rMnLRR could bind to the six bacteria tested, with a rHis-tag protein as negative control. (B) Results of an ELISA assay showed rMnLRR could bind to glycoconjuates originated from bacteria, including peptidoglycan (PGN) and LPS, in a dose-dependent manner.

induced AMP expressions, including AMP genes from the anti-lipopolysaccharide family (*ALF2*) and crustin family (*Cru1* and *Cru2*). AMPs are considered as important effectors of the innate immune system, and induction of AMP genes by MnToll1 can facilitate the clearance of invading pathogens. Based on our results, it could be speculated that *MnToll1* is a functional receptor that can regulate a broad spectrum of AMPs expressions, at least some ALFs and crustins. This is in agreement with other crustacean TLRs, for instance, MrToll from *M. rosenbergii* and PcTolls from *P. clarkii* can regulate the expression of many AMPs including ALFs and crustins [23,30,31]. Although MnToll1 did not regulate the *ALF1* gene we tested, it is possible that its expression is regulated by other TLRs in *M. nipponense*, or other immune signaling pathways such as the JAK-STAT pathway.

In mammals, the Toll signaling pathway is activated by direct recognition of TLRs to PAMPs of pathogens, followed by nucleus entry of NF-κB transcriptional factors and downstream gene expression, resulting in innate and/or adaptive immune responses [32]. However, the activation of *Drosophila* Toll pathway is not via direct recognition of pathogens by TLRs, otherwise by the binding of the active form of a cytokine Spätzle to TLRs [6]. The activation of Spätzle requires other extracellular PRRs to recognize invading pathogens and initiation of a proteolytic cascade [5]. In this study we found that MnToll1 could directly bind to bacterial pathogens and PAMPs on bacteria surface through its extracellular LRR domains and regulate the downstream AMP expression, which is similar to those TLRs in mammals. Similar results have been reported on TLRs from kuruma shrimp *M. japonicus*, as well as two mollusks, *Hyriopsis cumingii* and *Crassostrea gigas* [29,33]. Taken these results together, direct binding of TLRs to pathogens should be one of the activation mechanisms of Toll pathway in shrimps, as well as some other invertebrates. Interestingly, the *Spätzle* gene has been identified in several shrimp species including *F. chinensis*, *L. vannamei*, *M. japonicus* and *P. monodon*, and studies showed that Spätzle is also involved in shrimp immunity and AMP gene expression [34–36]. Our study did not exclude the role of Spätzle in activation of shrimp Toll pathway; on the contrary, we speculate there are two activation mechanisms of Toll pathway in shrimps: one by direct binding of Toll to pathogens, and the other by activation of Spätzle through other PRRs.

In conclusion, a Toll like receptor (designated *MnToll1*) was characterized in the oriental river prawn, *M. nipponense*. The *MnToll1* might function as a PRR to directly recognize the invasion of bacterial pathogens, and regulate the down-stream AMP expression. The identification and functional analyses of *MnToll1* provided new insight to understand anti-*Vibrio* defense of prawns.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.042.

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