Fish and Shellfish Immunology 91 (2019) 58–67

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

2-Transmembrane C-type lectin from oriental river prawn *Macrobrachium nipponense* participates in antibacterial immune response

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ARTICLE INFO

Keywords:
Macrobrachium nipponense
Innate immunity
2-Transmembrane C-Type lectin
RNA interference
Antimicrobial activity

ABSTRACT

As a type of pattern-recognition proteins (PRRs), C-type lectins (CTLs) perform important functions in non-self recognition and clearance of pathogens in innate immunity. In this study, a unique 2-transmembrane CTL (designated as Mn-2TM-cLec) with a single carbohydrate recognition domain (CRD) was isolated from *Macrobrachium nipponense*. The full-length cDNA of *Mn*-2TM-*cLec* consisted of 3265 bp with an 837 bp open reading frame encoding a protein with 278 amino acids. *Mn*-2TM-*cLec* was ubiquitously distributed in various tissues of normal prawn, particularly in the hemocytes, hepatopancreas, and gills. The expression of *Mn*-2TM-*cLec* was significantly up-regulated in the gills and hepatopancreas after the prawns were challenged with *Staphylococcus aureus* and *Vibrio parahaemolyticus*. RNA interference knock-down of *Mn*-2TM-*cLec* gene decreased the transcription levels of three antimicrobial peptides (*anti-lipopolysaccharide factor* (*ALF*) *1*, *ALF2*, and *Crustin* (*Crus*) *1*) after *V. parahaemolyticus* infection. The recombinant CRD of Mn-2TM-cLec could bind lipopolysaccharide, peptidoglycans, and diverse bacterial strains and agglutinate *S. aureus* and *V. parahaemolyticus* in a Ca^{2+}-dependent manner. In addition, the rCRD enhanced the clearance of *V. parahaemolyticus* injected in prawns. In summary, Mn-2TM-cLec might act as a PRR to participate in the prawn immune defense against pathogens through its antimicrobial activity.

1. Introduction

As the first line against infections, innate immunity is activated by a wide range of pattern recognition receptors (PRRs) that can recognize conserved pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycans (PGN), and β-1,3-glucans [1,2], thus eventually leading to detoxification, clearance, and lysis of pathogens [3,4]. As an important PRR, lectins are widely distributed in almost all organisms [5] and serve various biological functions, such as pathogen recognition [6], cell–cell interaction [7], protein synthesis and transport [8], and signal transduction [9]. Animal lectins can be classified into the following 13 groups based on their structures and functions: C-type, F-type, I-type, L-type, M-type, P-type, R-type, F-box lectins, calnexin, chitinase-like lectins, ficolins, galectins, and intelectins [10,11]. Among these, C-type lectins (CTLs) are the most abundant and widely studied. The first animal lectin discovered in 1906 was Bovine conglutinin, which belongs to the CTL family [12].

The term CTL was originally used to distinguish a group of Ca^{2+}-dependent (C-type) carbohydrate-binding proteins from other types of lectins [13]. Typical CTLs contain at least one carbohydrate recognition domain (CRD) with 120–130 amino acid residues [13,14]. The CRDs have a characteristic double-loop structure stabilized by two or three disulfide bridges that are formed by four types of Ca^{2+} binding sites [15]. Ca^{2+} binding site 2 is relatively conserved and contains two motifs, EPN (Glu-Pro-Asn) or QPD (Gln-Pro-Asp) and WND (Trp-Asn-Asp) [15,16]. These motifs contribute to the specific binding of CTLs to distinct PAMPs; however, some CTLs do not have such motifs [17]. In vertebrates, CTLs including collectins, selectins, lymphocyte lectins, and proteoglycans are initially divided into two types: type I and type II [18]. Dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN, designated as CD209) and its homolog, liver/lymphnode-specific intercellular adhesion molecule 3 grabbing non-integrin L-SIGN that is also known as DCSIGN-related receptor (DC-SIGNR), are two kinds of type II membrane-associated CTLs that share a

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https://doi.org/10.1016/j.fsi.2019.05.029
Received 29 January 2019; Received in revised form 7 May 2019; Accepted 13 May 2019
Available online 13 May 2019

similar structure and function as cell adhesion and phagocytic PRRs [19]. However, transmembrane CTLs are rarely reported in crustaceans.

To date, a growing number of crustacean CTLs have been identified as PRRs or effectors participating in a series of immune defense response. The PmLec of *Penaeus monodon* has a strong hemagglutinating and bacterial-agglutinating activity and an opsonic effect that enhances hemocyte phagocytosis [20,21]. In *Litopenaeus vannamei*, two CTLs (LvCTL3 and LvCTL4) act as downstream molecules of the NF-κB signaling pathway involved in immunity defense against bacterial and viral infections [22,23]. MrLec from *Macrobrachium rosenbergii* contains a single CRD, exhibits binding activities to PAMPs and bacteria, and regulates antimicrobial peptide (AMP) expression [24]. MjGCTL with a CRD from *Marsupenaeus japonicus* shows a calcium-dependent agglutinating capability against various types of bacteria and may act as an opsonin by binding directly to hemocytes to activate their adhesive state for their encapsulation [25]. FmLC6, a dual-CRD lectin from *Fenneropenaeus merguiensis*, can recognize invading microorganisms and cooperate during pathogenic elimination via its binding, agglutination, and antimicrobial activity [26]. FmLdlr, another *F. merguiensis* CTL containing a low-density lipoprotein receptor (LDLR) domain, may function as a receptor for vitellogenin transportation in hemolymph during shrimp vitellogenesis [27]. Thus, research on the roles of CTLs in crustacean immunity, especially its activity after recognizing and binding with pathogens, will help understand the complicated immune network.

Oriental river prawn *Macrobrachium nipponense* is one of the most important economic crustaceans in China [28]. However, frequent disease outbreaks have led to mass mortalities [29]. As invertebrates, crustaceans rely completely on their innate immune system to defend against invading pathogens [1]. In this study, a novel 2-transmembrane C-type lectin with a single CRD (designated as *Mn*-2TM-*cLec*) was characterized from *M. nipponense*. The tissue distribution and temporal response of *Mn*-2TM-*cLec* mRNA to bacterial challenge was investigated in prawns. Recombinant CRD of Mn-2TM-cLec (rCRD) was synthesized using a prokaryotic expression system to analyze its antibacterial functions *in vitro*. Our results suggested that Mn-2TM-cLec might act as a powerful effector in crustacean *M. nipponense* innate immunity.

2. Materials and methods

2.1. Experimental animals and microorganisms

M. nipponense (mean body weight, 3–4 g) were purchased from a local aquatic market in Nanjing, Jiangsu Province, China and cultured in tanks at 23 °C–25 °C with freshwater and an aeration system for 1 week before processing.

LPS from *Escherichia coli* 055:B5 and PGN from *Micrococcus luteus* were purchased from Sigma (St. Louis, MO, USA). Gram-positive bacteria *Staphylococcus aureus*, *M. luteus*, and *Bacillus subtilis* and Gram-negative bacteria *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, and *E. coli* were kept in our laboratory.

2.2. Immune challenge of prawns and tissues collection

For immune challenge, the prawns were randomly divided into two experiment groups, namely, *S. aureus*- and *V. parahaemolyticus*-challenged groups and a control group. In the control group, 50 μL of PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) was injected into the abdominal segment of prawn using a 1 mL sterile syringe. In each experiment group, the prawns were inoculated with 50 μL of *S. aureus* (3×10^7 cells) or *V. parahaemolyticus* (3×10^7 cells). After the treatment, the prawns were returned into the freshwater tank. Gills and hepatopancreas were randomly collected from five individuals per group at 0, 2, 6, 12, and 24 h post-injection. For the assay on the tissue distribution of *Mn*-2TM-*cLec* mRNA, hemolymph was extracted from health prawns by using a 1 mL syringe with 1/3 volume of anticoagulant (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM EDTA; pH 4.6) and then immediately centrifuged at 2000 rpm for 10 min at 4 °C to isolate the hemocytes. Other tissues including heart, hepatopancreas, gills, stomach, and intestine were also dissected for RNA extraction.

2.3. Total RNA isolation and cDNA synthesis

Total RNA was extracted from the above samples by using an RNA pure high-purity total RNA rapid extraction kit (Spin-column; Bioteke, Beijing, China) according to the manufacturer's protocol. RNA quality was assessed by electrophoresis for 15 min on 1.2% agarose gel, and RNA concentration was measured by the absorbance at 260 nm using NanoDrop 2000 (Thermo Fisher). First strand cDNA for real-time PCR was synthesized with Oligo-d(T) Primer by using the PrimeScript® 1st Strand cDNA Synthesis Kit (Takara, Japan). The first strand cDNA synthesized was produced with 5′-CDS primer A and SMARTer IIA oligo (5′-RACE Ready cDNA) for 5′ fragment cloning and with 3′-CDS primer (3′-RACE Ready cDNA) for 3′ fragment cloning by using the SMARTer™ RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA). The detailed procedures were performed according to the protocol.

2.4. Cloning the full-length cDNA of Mn-2TM-cLec

Partial cDNA sequence of *Mn*-2TM-*cLec* was obtained from our high-throughput transcriptome database from *M. nipponense*. 5′ and 3′ RACE were performed with gene-specific primers (Mn-2TM-cLec-F: 5′-ATGCTACTACTTCGCCCAACACAACGC-3′ and Mn-2TM-cLec-R: 5′-AGGCGTTGTGTTGGGCGAAGTAGTAGC-3′) and adaptor primers (UPM: 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′) using an Advantage® 2 PCR Kit (Clontech, USA) to obtain the full-length cDNA. PCR amplification conditions for 5′ and 3′ RACE were as follows: five cycles at 94 °C for 30 s and 72 °C for 3 min, followed by five cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min, and 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The PCR amplicons were analyzed by 1.0% agarose gel electrophoresis, and those of expected sizes were purified with a DNA gel extraction kit (Shanghai Generay Biotech Co., Ltd.) and inserted into the pEasy-T3 vector (TransGen Biotech, China). Positive clones were sequenced by M13F and M13R primers (Springen, China).

2.5. Sequence and phylogenetic analysis

BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to analyze the nucleotide sequences and search for protein sequences in the databases. Gene translation and prediction of the deduced protein were conducted with ExPASy Translate tool (http://web.expasy.org/translate/). Motif and domain were predicted using the SMART program (http://smart.emblheidelberg.de/). Calculated molecular mass and theoretical isoelectric point were estimated by ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi). Sequence alignments of Mn-2TM-cLec and CTL homologues from other species were analyzed using ClustalX v2.0 program (http://www.ebi.ac.uk/tools/clustalw2) and GENEDOC software. A phylogenetic tree was constructed using MEGA 7.0 software with the neighbor-joining (NJ) method, bootstrapping procedure with a minimum of 1000 bootstraps [30].

2.6. Tissue distribution and expression profiles of Mn-2TM-cLec after bacteria challenge

qRT-PCR was conducted using the TransStart® Top Green qPCR SuperMix Kit (TransGen Biotech, China) on the LightCycler® 96 (Roche, USA). Gene specific primers (Mn-2TM-cLec-RT-F: 5′-CACCGTCCTCGGGTTCCTC-3′ and Mn-2TM-cLec-RT-R: 5′-CTCCGGCCCTGCATCTTTC-3′) were designed to analyze the tissue distribution of *Mn*-2TM-*cLec* and the expression profiles in gills and hepatopancreas upon *S. aureus* and *V. parahaemolyticus* challenge. Samples were run in triplicate and normalized to the control gene β-actin (Mnβ-actin-RT-F: 5′-TATGCACTTCCTCATGCCATC-3′ and Mnβ-actin-RT-R: 5′-AGGAGGCGGCAGTGGTCAT-3′), and *Mn*-2TM-*cLec* expression levels were calculated by the $2^{-\Delta\Delta Ct}$ comparative CT method [31]. PCR conditions were as follows, 95 °C for 30 s; followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and a melting curve analysis from 65 °C to 95 °C. PCR amplifications were performed in triplicates for each sample. Unpaired sample *t*-test was used for statistical analysis, and significant differences was found if $p < 0.05$.

2.7. RNA interference and the expression patterns of AMPs

Gene specific primers (Mn-2TM-cLec-dsRNA-F: 5′-GCGTAATACGACTCACTATAGGGCCCCTTCGACTGGGTATC-3′ and Mn-2TM-cLec-dsRNA-R: 5′-GCGTAATACGACTCACTATAGGCTCTGGGTTGTTCTCGAGG-3′; GFP-dsRNA-F: 5′-GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC-3′ and GFP-dsRNA-R: 5′-GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC-3′) were designed to obtain DNA templates containing a T7 promotor. Double stranded RNA (dsRNA) specifically targeted to *Mn*-2TM-*cLec* and green fluorescent protein (GFP) were synthesized with an *in vitro* transcription T7 kit (Fermentas, USA) by using a previously described method [32]. In the RNAi experiment, 30 μg of dsRNA for *Mn*-2TM-*cLec* or GFP (as negative control) and 50 μL of *V. parahaemolyticus* (3×10^7 cells) were injected into the second abdominal segment of prawns. *V. parahaemolyticus* alone and PBS injection group were also set and served as the control groups. After 36 h, the hepatopancreas and gills of five prawns from each group were sampled for the detection of RNAi efficiency by qRT-PCR.

After RNAi, the expression levels of anti-lipopolysaccharide factor (ALF) 1, ALF2, and Crustin (Crus) 1 were also analyzed. The primers of genes were as follows: MnALF1-RT-F: 5′-GTGGTGCCCAGGATGGACTT-3′ and MnALF1-RT-R: 5′-AGAGGATGGTGGAGGAAATT-3′, MnALF2-RT-F: 5′-AGAACCACCTGAACCCAACG-3′, and MnALF2-RT-R: 5′-TGACAGATTAAGCCAGCCCC-3′, MnCrus1-RT-F: 5′-AGGTTTTACCCCATCATTG-3′, and MnCrus1-RT-R: 5′-TGTGGCGTTATTCTTTCCC-3′.

2.8. Expression and purification of recombinant protein of Mn-2TM-cLec

One pair of primers, namely, Mn-2TM-cLec-CRD-ex-F (5′-GGATCCCCAGGAATTCCCTGCCCCTTCGACTGGGTATCG-3′) and Mn-2TM-cLec-CRD-ex-R (5′-GATGCGGCCGCTCGAGTTACTCACAGATGACGAAGGCCAT-3′) were designed to amplify a cDNA fragment encoding the CRD of Mn-2TM-cLec. The amplified fragment was then infused into the pGEX-6p-2 vector (Novagen, Germany) digestion with restriction enzymes *EcoR* I and *Xho* I (NEB, USA). Recombinant plasmid was then transformed into *E. coli* BL21 (DE3) cells (TransGen Biotech, China) for recombinant protein expression. The recombinant CRD protein was then purified by glutathione Sepharose 4B chromatography (GenScript, USA) in accordance with the protocols of the manufacturer. The purified proteins were detected by using 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue R250.

2.9. Microbial binding assay

The binding activity of recombinant protein rCRD was tested with Gram-positive bacteria (*S. aureus*, *M. luteus*, and *B. subtilis*) and Gram-negative bacteria (*V. parahaemolyticus*, *A. hydrophila*, and *E. coli*). In brief, 500 μL of rCRD (200 μg/mL) was incubated with microbes (2.0×10^8 cells/mL) in mid-logarithmic phase by gentle rotation for 1 h at room temperature. Microbes incubated with rGST were used as a negative control. After centrifugation, the harvested cells were washed three times with TBS. Binding between microbes and recombinant protein was analyzed by 12.5% SDS-PAGE and was detected by Western blot with anti-GST antibody (TransGen, China). The experiments were repeated thrice.

2.10. Microbial agglutination assay

S. aureus and *V. parahaemolyticus* were used for agglutinating test. Bacteria cultured to mid-logarithmic phase in LB medium were harvested through centrifugation at 6000 rpm for 5 min, washed three times in Tris-buffered saline (TBS; 20 mM Tris-HCl, 150 mM NaCl, pH 7.4), and resuspended in TBS at 2×10^8 cells/mL. In the presence or absence of 10 mM $CaCl_2$, 25 μL of bacteria suspension was mixed with 25 μL of rCRD (final concentration of 100 μg/mL) or with 25 μL of GST-tag protein (100 μg/mL), respectively. Agglutination reactions were observed by microscopy (Nikon, Japan). All the assays were performed in triplicate.

2.11. ELISA for binding of rCRD to carbohydrates

The direct binding of rCRD to carbohydrates was determined by ELISA as previously described [33]. In brief, 50 μL/well LPS or PGN (80 μg/mL) was added to the wells of a 96-well microtiter plate incubated at 37 °C overnight and heated at 60 °C for 30 min. The wells were blocked with 200 μL/well 3% BSA in TBS for 2 h at 37 °C and washed four times with TBS (200 μL/well). The purified rCRD protein was then diluted with TBS containing 0.1 mg/mL BSA (50 μL/well, 0.78, 1.56, 3.125, 6.25, 12.5, and 25 μg/mL) added at room temperature for 3 h. The negative control used was GST instead of recombinant protein. The wells were washed as previously described and incubated with 100 μL of rabbit monoclonal anti-GST antibody (1:2000 diluted) at 37 °C for 2 h. After being washed four times with TBS, the wells were incubated with 100 μL of peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000 diluted) at 37 °C for 1 h. The plate was washed and developed with 100 μL of 0.01% 3, 3′, 5, 5′-tetramethylbenzidine (Sigma). The reaction was stopped with 2 M H_2SO_4 (2 mol L^{-1}), and absorbance was read at 450 nm. The assay was repeated thrice.

2.12. Bacterial clearance assay

Clearance assay was performed to analyze whether or not Mn-2TM-cLec can enhance bacterial clearance *in vivo*. In brief, 500 μL of *V. parahaemolyticus* cells (2×10^7 cells) were washed, resuspended with TBS, and incubated with 500 μL of rCRD (50 μg/mL) at 37 °C for 30 min with gentle rotation. *V. parahaemolyticus* was also incubated with GST (500 μL, 50 μg/mL), and TBS (500 μL) were used as controls. After incubation, 50 μL of treated bacterial cells was injected into the prawns. At 10- and 20-min post-injection, hemolymph was collected from three prawns from each group. With serial dilution, the samples (100 μL) were plated on LB agar plates and then incubated at 37 °C for 12 h. The number of bacteria colonies on the plates was counted, and the experiments were repeated thrice.

3. Results

3.1. Characterization and sequence analysis of Mn-2TM-cLec

The obtained *Mn*-2TM-*cLec* full-length cDNA sequence was 3265 bp long with a 1616 bp 5′ untranslated region (UTR), a 3′ UTR of 812 bp, and an 837 bp open reading frame encoding a protein with 278 amino acids (Fig. S1). Conserved domain analysis showed that Mn-2TM-cLec contained two transmembrane regions (TMR, residues 46–68, 202–224) and a putative CRD (residues 76–197). The theoretical pI and molecular weight of Mn-2TM-cLec peptide were 4.77 and 30.96 kDa, respectively.

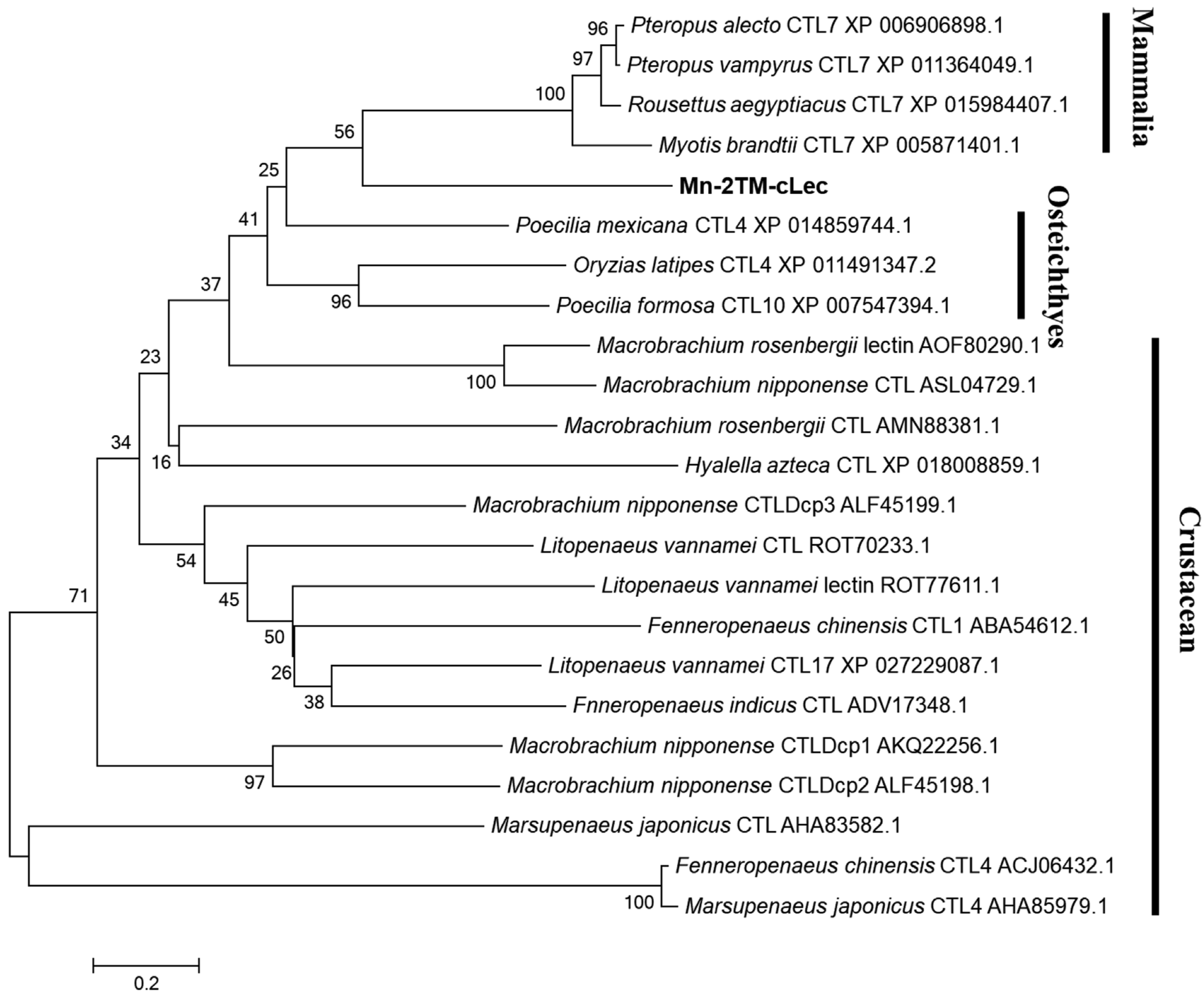

Fig. 1. Phylogenetic analysis of Mn-2TM-cLec and other CTLs from different species. Mn-2TM-cLec from *M. nipponense* was labeled in bold. The phylogenetic tree was produced by MEGA 7.0 software with a bootstrap of 1000.

3.2. Similarity and phylogenetic analyses

Similarity analysis of CRDs in all reported *M. nipponense* CTLs revealed that the amino acid sequence of CRD in Mn-2TM-cLec was highly nonconservative (Fig. S2). BLASTP search analysis showed that Mn-2TM-cLec shared 37% identity with *Poecilia formosa* CTL10 (XP_007547394.1), 33% identity with *Poecilia Mexicana* CTL4 (XP_014859744.1), 32% identity with *Oryzias latipes* CTL4 (XP_011491347.2), and 31% identity with *Pteropus alecto* CTL7 (XP_006906898.1) and *Pteropus vampyrus* CTL7(XP_011364049.1). A NJ phylogenetic tree was constructed based on BLASTP search results and the reported CTLs with one CRD from crustaceans. In this tree, the CTLs were divided into three groups: group I (members from Mammalia), group II (members from Osteichthyes), and group III (members from Crustaceans) (Fig. 1). Mn-2TM-cLec has a close evolutionary relationship with four mammalian CTLs and belongs to group I with a knot value of 56. The domain architectures of 23 CTLs in phylogenetic tree predicted by SMART are shown in Fig. S3. Seven CTLs, namely, PaCTL7, PvCTL7 and RaCTL7 from Mammalia, PmCTL4, OlCTL4, and PfCTL10 from Osteichthyes, and HaCTL from Crustaceans, contain a single TMR. Mn-2TM-cLec with two TMRs may have special physiological function that is different from most of other CTLs.

3.3. Tissue distribution and expression profiles of Mn-2TM-cLec

The transcript levels of *Mn*-2TM-*cLec* in various prawn tissues were characterized by qRT-PCR. *Mn*-2TM-*cLec* was found in all tested tissues, including hemocytes, heart, hepatopancreas, gills, stomach, and intestine, and its highest expression level was detected in hemocytes, followed by in hepatopancreas and gills (Fig. 2). The time course expression of *Mn*-2TM-*cLec* in the gills and hepatopancreas were further analyzed after immune challenge. As shown in Fig. 3, the mRNA expression of *Mn*-2TM-*cLec* in both tissues was up-regulated after the challenge by *S. aureus* or *V. parahaemolyticus*. *Mn*-2TM-*cLec* in the two tissues reached the maximum level at 2 h after the injection of *V. parahaemolyticus*. However, for *S. aureus* group, the highest mRNA level of *Mn*-2TM-*cLec* was detected at 2 h in gills and at 6 h in hepatopancreas after the challenge, respectively, and then declined. Although the expression profiles in the control group injected with PBS fluctuated slightly at different time points, no significant differences were found.

3.4. Interference of Mn-2TM-cLec decreased the expressions of three AMPs

Gene silencing mediated by RNAi was conducted to characterize the potential role of *Mn*-2TM-*cLec* in prawn innate immunity. The expression of *Mn*-2TM-*cLec* was up-regulated by the challenge of *V. parahaemolyticus* in the gills and hepatopancreas at 36 h post-injection and

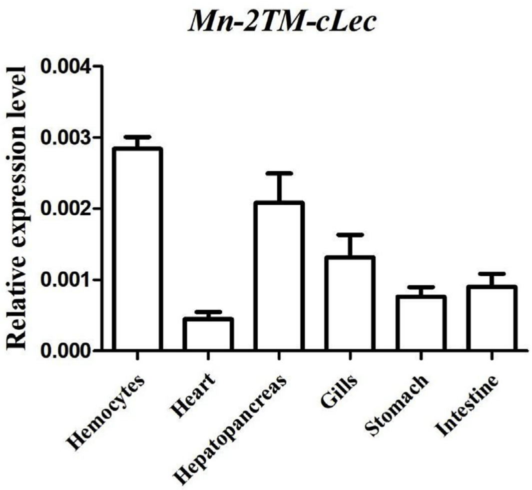

Fig. 2. *Mn*-2TM-*cLec* transcript levels in various tissues of *M. nipponense* as determined by qRT-PCR. β-actin was used as an internal control. Error bars represent the ± S.D. of five biological replicates plus three technical replicates.

was efficiently down-regulated to 44.9% in the gills (Fig. 4A) and 41.6% in hepatopancreas (Fig. 4E) of *Mn*-2TM-*cLec* dsRNA + *V. parahaemolyticus* injected prawns compared with those of the control groups (*V. parahaemolyticus* alone group and GFP dsRNA + *V. parahaemolyticus* group). The transcription levels of three different AMPs (*MnALF1*, *MnALF2*, and *MnCrus1*) were also induced by *V. parahaemolyticus* challenge and were all remarkably declined ($p < 0.05$) by *Mn*-2TM-*cLec* knockdown (Fig. 4). These results revealed that *Mn*-2TM-*cLec* is involved in the anti-*Vibrio* defense of prawns via regulating at least three AMPs.

3.5. Expression and purification of recombinant CRD

Recombinant CRDs were successfully expressed in *E. coli* BL21 (DE3). The predicted molecular mass of CRD was approximately 13.5 kDa. The rCRD contains a GST-tag (approximately 26 kDa) at the N-terminus, and its molecular mass is thus larger than that of CRD. The apparent molecular mass of the purified rCRD was approximately 40 kDa (Fig. 5A), and the concentration of rCRD was 449.3 μg/mL.

3.6. Binding activities of rCRD

Bacterial binding assay was performed to investigate whether or not rCRD can bind microbes. The result showed that the rCRD could tightly bind to all six tested microbes, including the Gram-positive bacteria *S. aureus*, *M. luteus*, and *B. subtilis* and Gram-negative bacteria *V. parahaemolyticus*, *A. hydrophila*, and *E. coli*. In addition, the rCRD has a wide binding spectrum of microbes though with distinct affinities (Fig. 5B). ELISA assay was performed to confirm the carbohydrate-binding capacity of rCRD. The OD 450 values showed that rCRD, but not GST,

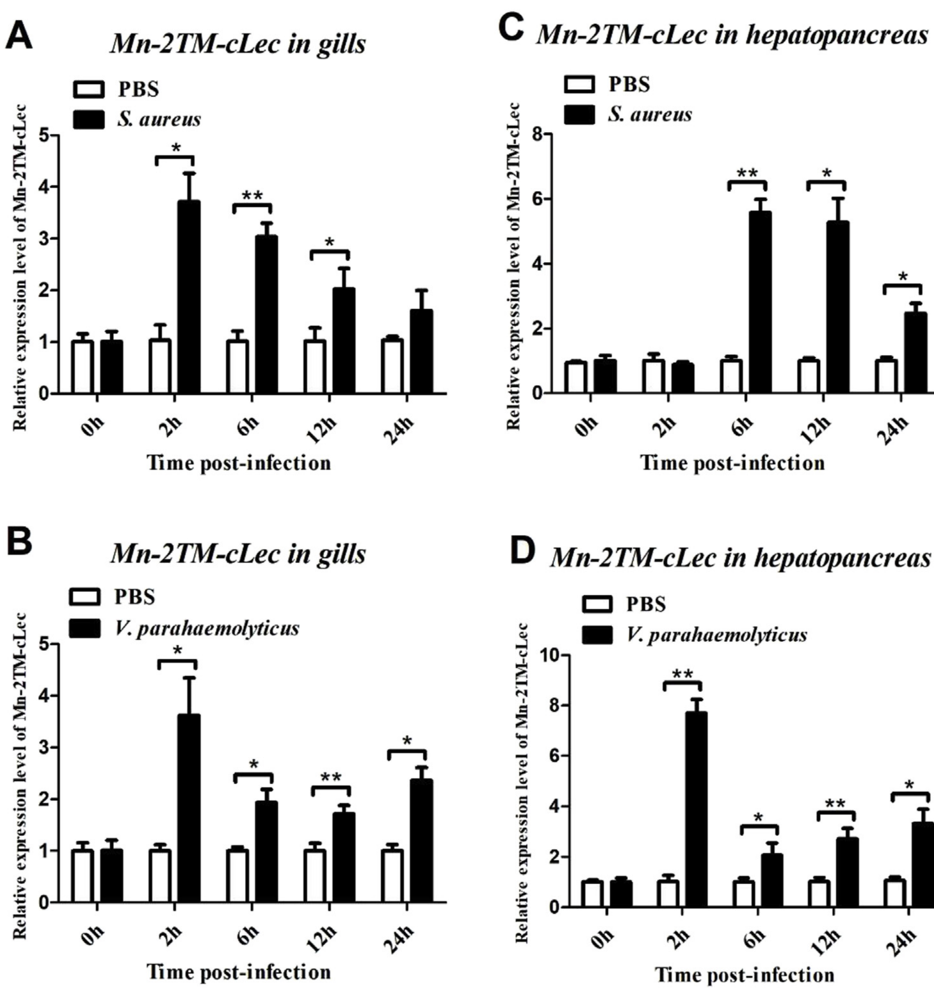

Fig. 3. *Mn*-2TM-*cLec* expression pattern in the gills and hepatopancreas by qRT-PCR at 0, 2, 6, 12, and 24 h after immune challenge by *S. aureus* and *V. parahaemolyticus*. β-actin as the reference gene for internal controls. Error bars represent the ± S.D. of three replicates. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) compared with values of the control.

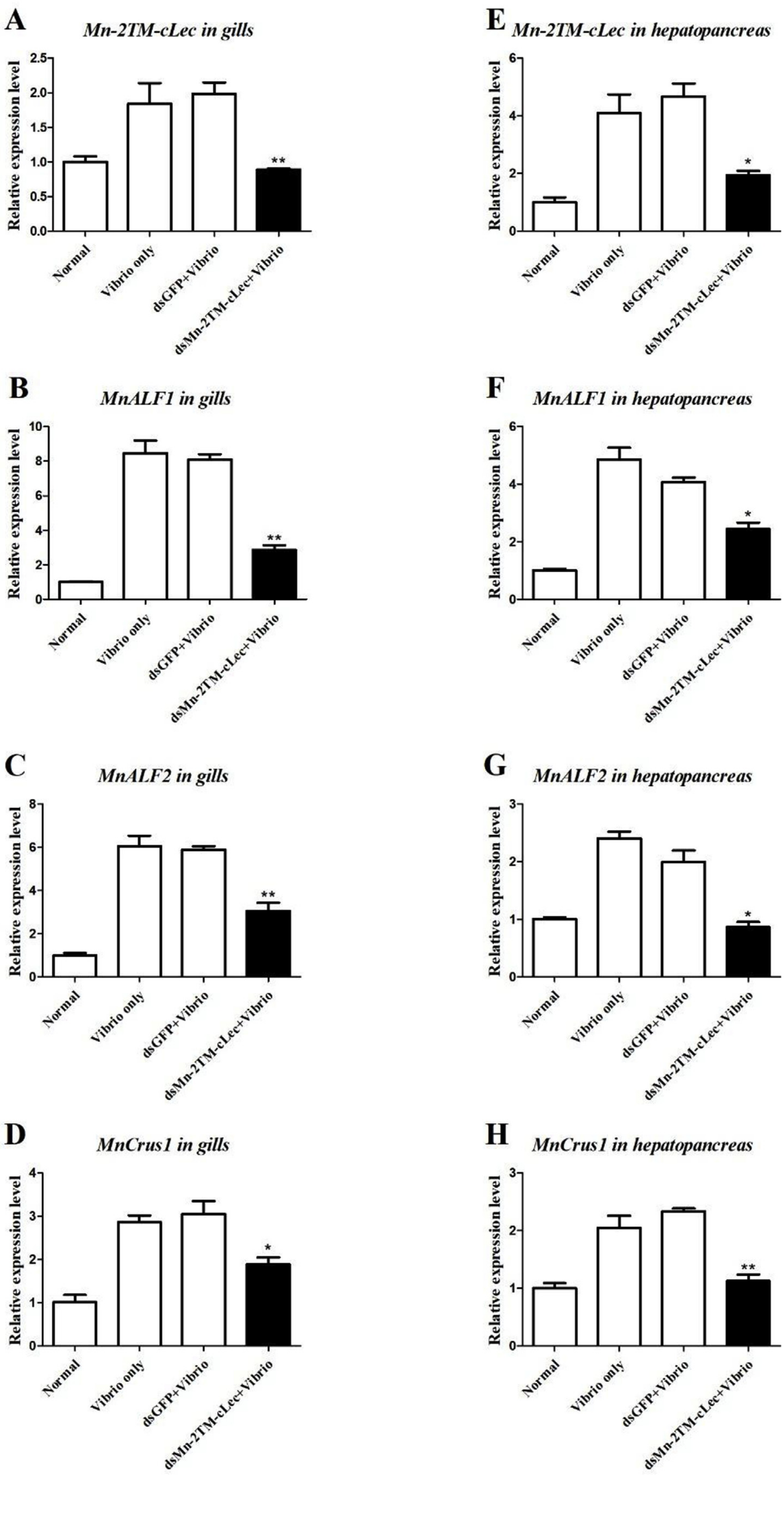

Fig. 4. RNAi of *Mn*-2TM-*cLec* expression and detection of AMPs expression. Transcription level analysis of *Mn*-2TM-*cLec* in the gills (A) and hepatopancreas (E) of *M. nipponense* at 36 h after dsRNA injection. Change of *MnALF1* (B), *MnALF2* (C), and *MnCrus1* (D) at transcription level in gills of prawns at 36 h after dsRNA injection. Change of *MnALF1* (F), *MnALF2* (G), and *MnCrus1* (H) at transcription level in hepatopancreas of prawns at 36 h after dsRNA injection. Normal, *Vibrio* only and dsGFP + *Vibrio* group were used as the control. Normal group represents the sample without any treatment. *Vibrio* only represents the sample after *V. parahaemolyticus* injection. dsGFP + *Vibrio* represents the sample after GFP dsRNA and *V. parahaemolyticus* injection. dsMn-2TM-cLec + *Vibrio* represents the sample after *Mn*-2TM-*cLec* dsRNA and *V. parahaemolyticus* injection. Bars indicate the mean ± S.D. Expression levels were normalized to *M. nipponense* β-actin. Statistical significance was calculated by *t*-test ($^*P < 0.05$, $^{**}P < 0.01$).

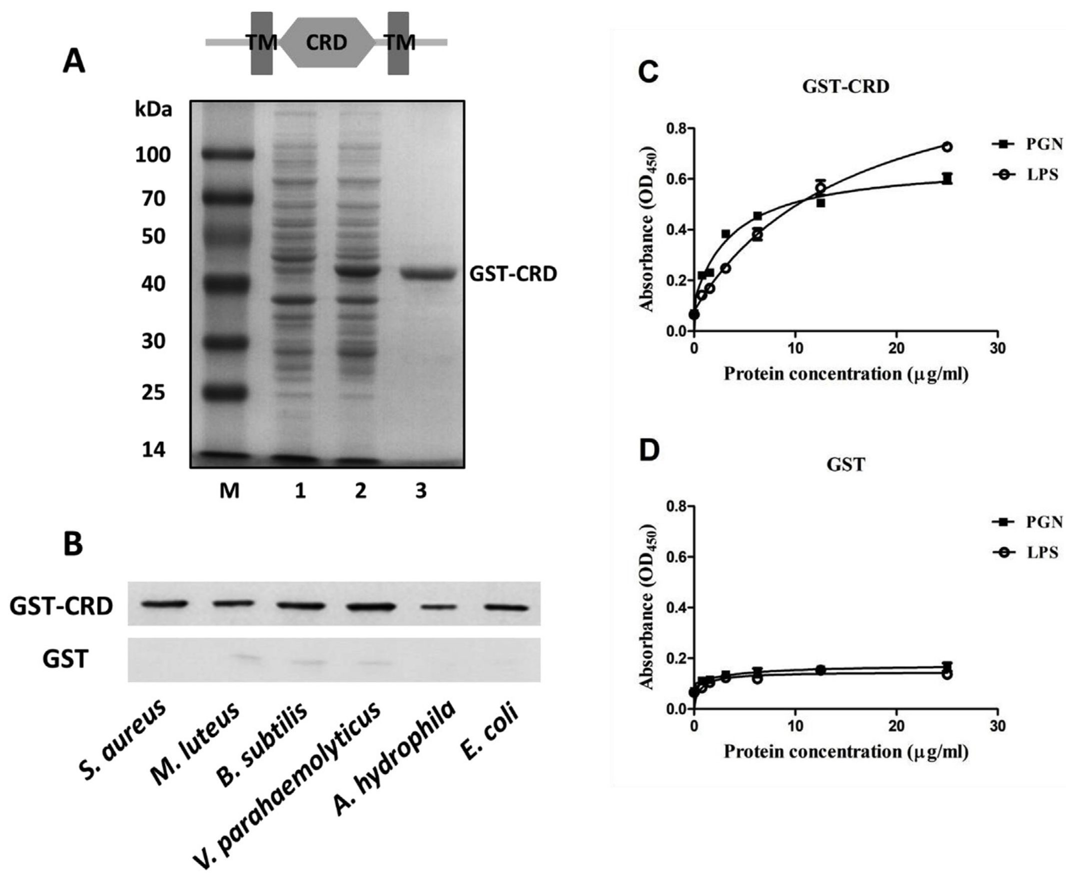

Fig. 5. (A) SDS-PAGE analysis of recombinant CRD. M: low molecular weight marker; lane 1, non-induced *E. coli* with pGEX-6p-2-CRD; lane 2, induced *E. coli* pGEX-6p-2-CRD by IPTG; Lane 3: purified rCRD. Direct binding of rCRD and GST (B) to microbes. Bacteria were incubated with rCRD and then washed three times with TBS. Binding was confirmed by Western blot with anti-GST antibody. Data are shown as the mean ± S.D. derived from three repeats. Binding assay of rCRD (C) and GST (D) to carbohydrates by ELISA *in vitro*. The microtiter plates were coated with 4 μg of LPS or PGN and incubated with various amounts of rCRD. Binding was detected using antiserum against GST-tag containing protein.

could bind to LPS and PGN in a dose-dependent manner (Fig. 5C and D).

3.7. Agglutinating activity of rCRD

In the presence of Ca^{2+} (10 mM), rCRD exhibited strong agglutination activity toward *S. aureus* and *V. parahaemolyticus* (Fig. 6). No microbial agglutination occurred when the microbes were incubated in rCRD buffer without Ca^{2+}, indicating that rCRD is calcium-dependent regardless of the kind of bacteria used. The control group treated with rGST also showed no agglutinating activity.

3.8. rCRD facilitated V. parahaemolyticus clearance

Bacterial clearance essay was performed to detect the *in vivo* function of Mn-2TM-cLec. After incubation of *V. parahaemolyticus* with

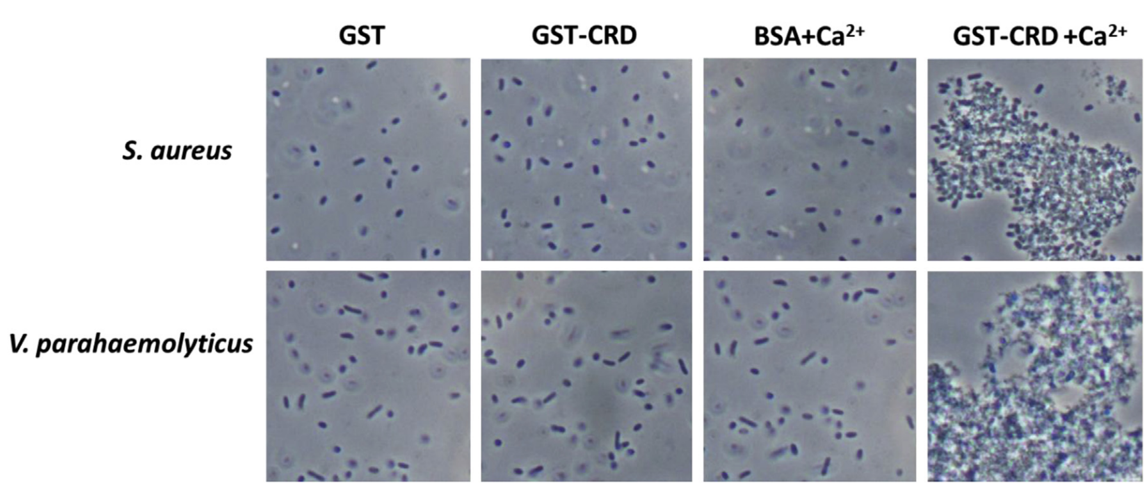

Fig. 6. Agglutination of microorganisms by recombinant CRD with calcium (10 mM). A Gram-positive bacteria *S. aureus* and a Gram-negative bacteria *V. parahaemolyticus* were shown. GST-tag protein was used as a control.

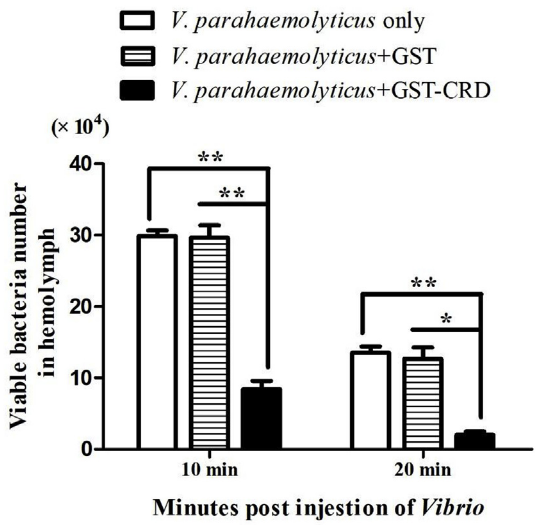

Fig. 7. rCRD enhanced the bacterial clearance *in vivo*. rCRD and GST were firstly incubated with *V. parahaemolyticus* (10^7) at 37 °C for 30 min. The treated bacterial cells were then injected into the prawns. Hemolymph was extracted at 10- and 20-min post-injection, and the bacterial number was counted. Error bars represent ± S.D. of three replicates.

rCRD, the bacteria were injected into prawns, and GST was used as a control. At 10 and 20 min after the injection, the number of the survival bacteria in rCRD group decreased rapidly compared with that in the control group. The results revealed that Mn-2TM-cLec can enhance bacterial clearance in prawns (Fig. 7).

4. Discussion

As important PRRs, CTLs perform functions in non-self recognition and clearance of pathogens in innate immunity [15]. CTLs have been systematically studied in vertebrates but have not been well-characterized in invertebrates [10,34]. In this work, we successfully cloned a novel CTL (Mn-2TM-cLec) based on our previously constructed *M. nipponense* cDNA EST library. The deduced Mn-2TM-cLec protein has a CRD consisting of 122 residues, which are essential for interacting with Ca^{2+} and recognizing carbohydrate ligands [14–16]. Meanwhile, Mn-2TM-cLec possessed two TMRs that are used to immobilize membrane protein on the cell membrane. From higher vertebrates to lower vertebrates, DC-SIGN and L-SIGN amino acid sequences have two domains: a TMR and a CRD [35]. Invertebrates have few CTLs with one TMR, and even fewer CTLs with two TMRs. To date, only one CTL containing two TMRs named Mn-2TM-cLec was found in crustaceans. Although the predicted TMRs of Mn-2TM-cLec have all the features of classical TMRs, including a high degree of hydrophobicity, the prediction of TMRs is not always accurate. Whether or not each TMR can anchor Mn-2TM-cLec to the membrane and the role of these two TMRs in the antibacterial immune response of Mn-2TM-cLec must be further studied. Phylogenetic analysis was conducted to further investigate the evolutionary relationships of Mn-2TM-cLec with CTL proteins from other species. The results indicated that Mn-2TM-cLec originated from crustaceans but were clustered into Mammalia branches, suggesting that Mn-2TM-cLec is a new member of the prawn CTL superfamily.

The hepatopancreas of crustaceans, which are equivalent to the livers of mammals and fat bodies of insects, is a crucial tissue involved in immunity and is the sole biosynthesis site of many CTLs, such as *LvLT* [36], *MjLecC* [37], *FmLC3* [38], *FmLC4* [39], *FmLC6* [26], and *FcLec5* [40]. Different from hepatopancreas-specific CTLs, *Mn*-2TM-*cLec* was expressed in various tissues with the strongest expression found in the hemocytes and hepatopancreas. *LdlrLec1* from *M. japonicus* was mainly distributed in hemocytes, heart, intestines and gill, whereas *LdlrLec2* was abundant in hepatopancreas and heart [41]. *MrLec* was ubiquitously distributed in many tissues of a normal prawn, particularly in its hepatopancreas and gills [24]. *PmCLec* gene was expressed in all tested tissues, and its highest expression level was found in the lymphoid organ, followed by hemocytes and hepatopancreas [21]. The wide distribution of these CTLs may indicate their important role in immunity [11].

Prawn culture worldwide is disrupted by outbreaks caused by devastating bacterial pathogens such as *Vibrio*. The challenge of *M. nipponense* with *V. parahaemolyticus* or *S. aureus* was conducted to investigate the action of CTLs in prawn immune defense. qRT-PCR analysis showed that *Mn*-2TM-*cLec* mRNA in hepatopancreas and gills was dramatically up-regulated after bacteria challenge, implicating that the level of *Mn*-2TM-*cLec* might have been increased in response to microbial infection. *FcLec5* was increased to its peaked expression after the challenge of *F. chinensis* with *Vibrio anguillarum* [40]. *MnCTLDcp2* and *MnCTLDcp3* could be induced effectively by *A. hydrophila* [42]. *FmLC6* was up-regulated after immune challenge by *V. harveyi* and *V. parahaemolyticus* [26]. *MrLec* was up-regulated after a challenge with *V. parahaemolyticus*; in this case, *MrLec* gene was silenced, whereas the expression levels of two AMPs (*ALF1* and *lysozyme 2*) were markedly decreased [24]. *MnCTL* knockdown by RNAi in white spot syndrome virus (WSSV)-challenged prawns significantly decreased *MnALF1* and *MnALF2* transcript levels [43]. Similarity, silencing *Mn*-2TM-*cLec* in gills and hepatopancreas of *V. parahaemolyticus*-challenged prawns may down-regulate *MnALF1*, *MnALF2* and *MnCrus1* transcriptions. AMPs such as ALFs, crustins, and lysozyme are generally small cationic molecules that play important roles in the innate immune defense of vertebrates and invertebrates against invading pathogens [44]. Prawns apparently attempt to clear the infection by up-regulating the expression of *Mn*-2TM-*cLec*, which is involved in regulating the induction of AMPs.

The most important feature of CTLs is the recognition and non-covalent binding of specific carbohydrate ligands on the cell surface and the agglutination of cells by binding to cell surface glycoproteins and glycoconjugates [45,46]. In our study, the rCRD of Mn-2TM-cLec could bind to all tested microbes (three Gram-positive bacteria, *S. aureus*, *M. luteus*, and *B. subtilis* and three Gram-negative bacteria *V. parahaemolyticus*, *A. hydrophila*, and *E. coli*) with similar extent. The binding property of rCRD was similar to those of rFmLC3 [38], rFmLC6 [26], and rMnCRD [43], which also have a wide spectrum of binding activities toward various microbes. By contrast, rFcLec4 selectively binds to several microorganisms [47], and rPmCLec could only bind to two Gram-positive bacteria, *S. aureus* and *S. hemolyticus* [21]. This finding indicated other binding sites on the cell surface. LPS and PGN are the major components on the surface of Gram-negative and -positive bacteria, respectively. The rCRD manifested the direct binding to LPS and PGN in a concentration-dependent manner as analyzed by ELISA. rFcLec5 and its two CRDs all bound to LPS, PGN, lipoteichoic acid (LTA), mannan, and dextran with different binding activities [40]. Purified rFmLC4 could bind to LPS, LTA, and β-1,3-glucan with different Kd values [39]. In the presence of calcium, rCRD agglutinated various bacterial strains (*S. aureus* and *V. parahaemolyticus*), and this result was in agreement with those for rMrLec [24], rFmLC3 [38], rMnCTLDcp2, and rMnCTLDcp3 [42]. Polysaccharide-binding activity triggers microbial agglutination by crosslinking specific polysaccharides (such as LPS and PGN) and forming lattices. Moreover, the *in vivo* clearance of bacterium *V. parahaemolyticus* was conducted to verify the PRR role of rCRD. The result showed that rCRD could mediate the clearance of *V. parahaemolyticus* with the rate faster than prawn themselves as administered by PBS or GST. rFcLec4 from *F. chinensis* promoted the pathogenic bacterium *V. anguillarum* clearance in Chinese white shrimp [46]. rFmLC4 displayed the effective clearance of *V. harveyi* injected in banana prawn *F. merguiensis* [39]. All these results suggest the involvement of Mn-2TM-cLec in prawn immunity against bacterial infection.

In conclusion, a unique lectin with two TMRs (designated as Mn-2TM-cLec) was isolated from *M. nipponense* and classified to the CTL family because its primary structure is composed of a CRD. The expression of *Mn*-2TM-*cLec* was significantly up-regulated in the gills and hepatopancreas after the prawns were challenged with *S. aureus* and *V. parahaemolyticus*. RNAi knock-down of the *Mn*-2TM-*cLec* gene decreased AMP transcription after *V. parahaemolyticus* infection. The recombinant CRD (rCRD) of Mn-2TM-cLec could bind to LPS, PGN, and diverse bacterial strains and agglutinate *S. aureus* and *V. parahaemolyticus* in a Ca^{2+}-dependent manner. The rCRD also enhances the clearance of *V. parahaemolyticus* injected in prawns. In summary, Mn-2TM-cLec might act as PRR to participate in the prawn immune defense against pathogens through its antimicrobial activity.

Author contributions

Y.H. and R.D.Z. carried out the experiments. Y.H. and Q.R. designed the experiments and analyzed the data. T.H.G. H.X. and T.W. contributed reagents/materials. Y.H. and Q.R. wrote the manuscript. All authors gave final approval for publication.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments and funding

The current study was supported by the National Key Research and Development Program of China (2017YFC0405206), the China Postdoctoral Science Foundation (2019M651666), the Natural Science Foundation of Jiangsu Province (BK20180501, BK20171474), the Fundamental Research Funds for the Central Universities (2018B03214), the National Natural Science Foundation of China (Grant No. 31572647), the Natural Science Fund of Colleges and universities in Jiangsu Province (14KJA240002), the Modern Fisheries Industry Technology System Project of Jiangsu Province (Grant No. JFRS-01), and the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.029.

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