Characterization and functional analysis of tandem threonine containing C-type lectin (Thr-Lec) from the ridgetail white prawn *Exopalaemon carinicauda*

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**A B S T R A C T**

As an important pattern-recognition receptor (PRR), C-type lectins (CTLs) play significant roles in recognizing microbes and battle against pathogenic microorganisms in innate immunity. In this study, two tandem threonine containing CTLs (designated as EcThr-LecA and EcThr-LecB) were identified from *Exopalaemon carinicauda*. The full-length cDNA of EcThr-LecA and EcThr-LecB consisted of 1521 and 1518 bp with 1251 and 1242 bp open reading frame encoding a protein with 412 and 413 amino acids, respectively. The genome structure of EcThr-LecA included 10 exons and 9 introns, and the sequences of intron6 and intron7 were variable. The nucleotide sequence of intron2 in EcThr-LecB was specific and different with that of EcThr-LecA. EcThr-LecA and EcThr-LecB proteins were predicted to have a signal peptide, two conserved carbohydrate recognition domain (CRD), and tandem threonine region. The expression levels of EcThr-LecA and EcThr-LecB in the intestine were significantly up-regulated after *Vibrio parahaemolyticus* and white spot syndrome virus (WSSV) challenge. RNA interference (RNAi) was used to explore the effects of EcThr-LecB silencing on the mRNA expression of anti-lipopolysaccharide factor (ALF), crustin (CRU), and lysozyme (LYSO). Knock down of EcThr-LecB could evidently down-regulate the expression of eight different antibacterial peptides (AMPs), including EcALF1, EcALF2, EcCRU1, EcCRU3, EcCRU4, EcLYSO1, EcLYSO2, EcLYSO3, and EcLYSO4, whereas make no effect on the transcription of EcALF1, EcALF3, EcCRU2, and EcLYSO5. The recombinant two CRD domains and tandem threonine region (RLecB) of EcThr-LecB could bind diverse bacteria, lipopolysaccharide, and peptidoglycans in vitro. In addition, RLecB could accelerate the clearance of *V. parahaemolyticus* in vivo. The present data indicated that new-found tandem threonine containing CTLs in *E. carinicauda* may act as PRR to participate in the innate immune defense against pathogens by the recognition of non-self, regulation of AMPs, and clearance of invaders.

**ARTICLE INFO**

**Keywords:**
C-type lectin  
Pathogens  
Antimicrobial peptides  
Innate immunity  
*Exopalaemon carinicauda*

**1. Introduction**

As one of the important economic shrimp species, the ridgetail white prawn *Exopalaemon carinicauda* is distributed mainly in the Yellow sea and the Bohai Sea, which occupies a key position in the field of shrimp culture in eastern China [1]. *E. carinicauda* have many advantages such as fast growth, good fertility, and strong adaptability, which make it good experimental material for basic scientific research [2–4]. With the development of intensive culture and the growing complexity of shrimp farming environment, various diseases caused by bacteria (e.g., *Vibrio parahaemolyticus*) and viruses (e.g., White spot syndrome virus, WSSV) have resulted in serious economic losses to commercial shrimp aquaculture [1]. As invertebrate, *E. carinicauda* lacks the adaptive immunity and mainly relies on the innate immunity to defense against infection by pathogenic microorganisms. Therefore, it’s very important to research emphasis on the innate immune mechanism for disease resistance.

The first step of innate immunity is recognition of non-self, which is accomplished by host germ-line encoded pattern recognition receptors (PRRs) [5]. PRRs can recognize pathogens by binding to conserved pathogen-associated molecular patterns (PAMPs), such as...
lipopolysaccharide (LPS), peptidoglycan (PGN), and lipoteichoic acid (LTA), existing on the surface of the microbes, and then leading to rapid humoral and cellular immune responses [6, 7]. C-type lectins (CTLs), as an important PRR, are a superfamily of protein containing Ca\(^{2+}\)-dependent carbohydrate recognition domain (CRD) of 120–150 amino acid residues. The CRD structure has a characteristic loop-in-a-loop dependent carbohydrate recognition domain (CRD) of 120 amino acids sequence of the primers used in this study. Table 1

The primers used in this study.

| Primer name     | Primer sequences (5′ - 3′)                                                                 |
|-----------------|------------------------------------------------------------------------------------------|
| EcThr-LecA-F    | TGGGAGCTGGTGATAGGTTAATGACAGAGGA                                                          |
| EcThr-LecA-R    | GCTCCTGTCGCTTGGAGGGAGATACAGACGT                                                     |
| EcThr-LecB-F    | TTCTGCGATTAGAAGAGATAGGTTAATGACAGAGGA                                                   |
| EcThr-LecB-R    | AGCTGTGGAACACTACCTGCTGGTGGTGGTGGTTGAGGA                                                 |
| EcThr-LecA-gF1  | GCCCTCTGCCTTCCTACAT                                                                      |
| EcThr-LecA-gR1  | GGCTCCTGTCGCTTGGAGGGAGATACAGACGT                                                     |
| EcThr-LecA-gF2  | AGCTGAGTGGAGTGGTGGTGGAGGGAGATAG                                                       |
| EcThr-LecA-gR2  | GACACTCTCGAATCCTGCTGGTGGTGGTGGTGGATTGAGGA                                                |
| EcThr-LecA-gF3  | CATCTGCTGGTGGTGAGGGAGATAG                                                              |
| EcThr-LecA-gR3  | GGCTCCTGTCGCTTGGAGGGAGATACAGACGT                                                     |
| EcThr-LecB-gF1  | ATCTGAGTGGAGTGGTGGAGGGAGATAG                                                           |
| EcThr-LecB-gR1  | GAGCTGAGTGGAGTGGTGGAGGGAGATAG                                                          |
| EcThr-LecB-gF2  | CTTGTGGAGGAAAGAGATAGGGAGGGAGATAG                                                       |
| EcThr-LecB-gR2  | GACACTCTCGAATCCTGCTGGTGGTGGTGGTGGATTGAGGA                                                |
| EcThr-LecA-qRT-F | ATGAGTGGAGTGGTGGAGGGAGATAG                                                             |
| EcThr-LecA-qRT-R | AGCTGAGTGGAGTGGTGGTGGAGGGAGATAG                                                        |
| EcThr-LecB-qRT-F | ATGAGTGGAGTGGTGGAGGGAGATAG                                                             |
| EcThr-LecB-qRT-R | AGCTGAGTGGAGTGGTGGAGGGAGATAG                                                          |
| EcThr-LecA-gF1  | GCCCTCTGCCTTCCTACAT                                                                      |
| EcThr-LecA-gR1  | GGCTCCTGTCGCTTGGAGGGAGATACAGACGT                                                     |
| EcThr-LecA-gF2  | AGCTGAGTGGAGTGGTGGTGGAGGGAGATAG                                                       |
| EcThr-LecA-gR2  | GACACTCTCGAATCCTGCTGGTGGTGGTGGTGGATTGAGGA                                                |
| 18s RNA-qRT-F   | GGGAGGTAGTGAGGGAGATAGGGAGATAG                                                           |
| 18s RNA-qRT-R   | CAAATGCGGTGTTGAGTGAGGGAGATAG                                                          |
| EcThr-LecB-F    | TCTAGAGTGGAGTGGTGGAGGGAGATAG                                                           |
| EcThr-LecB-R    | GCAATGCGGTGTTGAGTGAGGGAGATAG                                                          |
| EcThr-LecA-F    | ATGGCAGTGAAGTGGGATAC                                                                     |
| EcThr-LecA-R    | GGCATTCATCCGAGACAAGGAGATAG                                                             |
| EcThr-LecB-F    | TCGTGACGAAGTGGGAGATAGGGAGATAG                                                          |
| EcThr-LecB-R    | GTGAGTGGAGTGGTGGAGGGAGATAG                                                             |
| EcThr-LecA-gF1  | ATGACTGTAGCAACGTGACC                                                                    |
| EcThr-LecA-gR1  | AGCTGAGTGGAGTGGTGGAGGGAGATAG                                                          |
| EcThr-LecA-gF2  | GCCACTCTCGAATCCTGCTGGTGGTGGTGGTGGATTGAGGA                                                |
| EcThr-LecA-gR2  | AGCTGAGTGGAGTGGTGGAGGGAGATAG                                                          |
| EcThr-LecA-gF3  | CATCTGCTGGTGGTGAGGGAGATAG                                                              |
| EcThr-LecA-qRT-F | ATGAGTGGAGTGGTGGAGGGAGATAG                                                             |
| EcThr-LecA-qRT-R | AGCTGAGTGGAGTGGTGGAGGGAGATAG                                                          |
| EcThr-LecB-gF1  | ATGAGTGGAGTGGTGGAGGGAGATAG                                                             |
| EcThr-LecB-gR1  | AGCTGAGTGGAGTGGTGGAGGGAGATAG                                                          |
| EcThr-LecB-gF2  | CTTGTGGAGGAAAGAGATAGGGAGGGAGATAG                                                       |
| EcThr-LecB-gR2  | GACACTCTCGAATCCTGCTGGTGGTGGTGGTGGATTGAGGA                                                |
| EcThr-LecL-ex-F | GATGAGTGGAGTGGTGGAGGGAGATAG                                                             |
| EcThr-LecL-ex-R | AGCTGAGTGGAGTGGTGGAGGGAGATAG                                                          |
| EcThr-LecL-ex-R | GATGAGTGGAGTGGTGGAGGGAGATAG                                                             |
| EcThr-LecL-ex-R | GATGAGTGGAGTGGTGGAGGGAGATAG                                                             |
| EcThr-LecL-ex-R | GATGAGTGGAGTGGTGGAGGGAGATAG                                                             |

**Table 1** Sequences of the primers used in this study.

**2. Materials and methods**

**2.1. Animal materials and microorganisms**

Healthy adult *E. carinicauda* with an average body length of 4.2 cm and a body weight of 1.0 ± 0.2 g were purchased from xianlin aquatic market in Nanjing, Jiangsu Province, China. All individuals were acclimatized in the aerated seawater at the temperature of 25 ± 1 °C for 1 week before processing.

**Gram-positive bacteria (Staphylococcus aureus, Bacillus subtilis, B. megaterium, and B. cereus)** and **Gram-negative bacteria (V. parahaemolyticus, Aeromonas hydrophila, and V. alginitolyticus)** were kept in our laboratory. WSSV was kindly provided from Zhejiang University. LPS from *Escherichia coli* 055: B5 and PGN from *Micrococcus*
2.2. Immune challenge and tissue collection

Healthy *E. carinicauda* were challenged with *V. parahaemolyticus* and WSSV. In the *V. parahaemolyticus*-challenged group, each shrimp was injected with 100 μL phosphate buffer saline (PBS: 140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) containing *V. parahaemolyticus* (10⁹ CFU mL⁻¹). The intestine of five random individuals were collected at 0, 2, 6, 12, and 24 h post-bacterial injection. In the WSSV-challenged group, 100 μL of PBS containing WSSV (10⁷ copies mL⁻¹) was injected into the abdominal segment of each shrimp. At 0, 24, 48, and 72 h after virus challenge, the intestines were collected from five random shrimp. The intestine sample from untreated shrimp (0 h) served as the control.

Total RNA was extracted from collected tissues by using an rNAPure High-Purity Total RNA Rapid Extraction Kit (Spin-Column; Biotek, Beijing, China) following the manufacturer’s protocol. The RNA concentration was assessed by Nanodrop 2000 (Thermo Fisher Scientific, USA), and the RNA quality was assessed by electrophoresis on 1% agarose gel. The first-strand cDNA was synthesized from 1 g total RNA with TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (Transgen Biotech, China) in accordance with the manufacturer’s instructions. The cDNA samples were temporarily stored at −20 °C for further use.

2.3. Gene cloning and sequence analysis

SMARTer® RACE 5′/3′ Kit (Clontech, Takara, Japan) was used to generate the 5′- and 3′-RACE-Ready total cDNA samples in accordance with the manual, which were further used as templates for rapid amplification of cDNA ends (RACE) reactions. Based on the partial CTL sequence identified in the hepatopancreas transcriptome of *E. carinicauda*, specific forward (EcThr-LecA-F1/F2/F3 and EcThr-LecB-F) and reverse (EcThr-LecB-R) primers were designed to respectively acquire the 3′ and 5′-end fragments of EcThr-LecA and EcThr-LecB by using Advantage 2 PCR Kit (Clontech, Takara, Japan) under the following conditions: five cycles at 94 °C for 30 s and 72 °C for 3 min; five cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; and 25 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The RACE products were characterized via cloning and sequencing by a commercial company (Springen, Nanjing, China). The full length of *EcThr-LecA* and *EcThr-LecB* were obtained by overlapping EST sequences and 5′ and 3′ fragments. The genomic DNA sequences of *EcThr-LecA* and *EcThr-LecB* were acquired by PCR amplification, screening positive clones, and sequencing. Primers used in this part of the study were listed in Table 1.

The cDNA sequence of EcThr-LecA and EcThr-LecB were analyzed by using the basic local alignment search tool (BLAST) algorithm from the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the deduced protein sequences were obtained by using an Expert Protein Analysis System (https://web.expasy.org/translate/). Domain organization of proteins were predicted in online software Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). Phylogenetic analysis was conducted by the neighbor-joining (NJ) algorithm using the MEGA7 software. The reliability of the tree was tested by bootstrapping using 1000 replications. Multiple sequence alignment of amino acid sequences of EcThr-LecA, EcThr-LecB, and their homologous proteins was performed by using DNAMAN software. The comparison of cDNA sequences of EcThr-LecA and EcThr-LecB were also conducted by DNAMAN software. Theoretical isoelectric point (pI) and MW were determined in ExPaSy (http://web.expasy.org/compute_pi/).

2.4. Quantitative real-time PCR (qPCR)

qPCR was employed to detect the expression profiles of *EcThr-LecA* and *EcThr-LecB* in the intestine after bacterial and viral challenge by using primers (EcThr-LecA-qRT-F, EcThr-LecA-qRT-R, EcThr-LecB-qRT-F, and EcThr-LecB-qRT-R) listed in Table 1. 18S rRNA from *E. carinicauda* was amplified as the internal control. qPCR was conducted by using a TransStart® Top Green qPCR SuperMix Kit (TransGen Biotech, China) in LightCycler® 480 (Roche, USA). The total reaction volume was 10 μL, which contains 5 μL of 2 × TransStart Top Green qPCR SuperMix, 1 μL of 7-fold diluted cDNA, 0.2 μL (10 mM) each of forward and reverse primer, and 3.6 μL of ddH₂O. The qPCR amplification was performed as follows: 95 °C for 30 s; 95 °C for 5 s and 60 °C for 20 s, 40 cycles; a melting curve analysis from 60 °C to 95 °C. Three replicates were set for each sample. The relative expression level of EcThr-LecA and EcThr-LecB were analyzed by using a 2^ΔΔCt threshold cycle (CT) method [21]. Unpaired, two-tailed t-test was conducted for statistical analysis, and p < 0.05 was considered statistically significant.

2.5. Double strand RNA (dsRNA) interference and detection of AMPs

Two pair of primers (*EcThr-LecB*-dsRNA-F/-R and GFP-dsRNA-F/-R, Table 1) with T7 promoter sequences were respectively designed and used to amplify DNA fragments of *EcThr-LecB* and green fluorescent protein (GFP). The obtained DNA fragments were used as templates to synthesize the corresponding dsRNA of EcThr-LecB and GFP using HiScribe™ T7 Quick High Yield RNA Synthesis Kit (BioLabs, USA) following the instruction manual. The concentration of synthesized dsRNAs was assessed by Nanodrop 2000 (Thermo Fisher Scientific, USA) and their qualities were assessed by electrophoresis on 1% agarose gel. Approximately 6 μg of EcThr-LecB-dsRNA or GFP-dsRNA (as control) was injected into the abdominal segment of each shrimp. The intestine from five prawns were randomly collected at 36 h after dsRNA injection. The transcriptional levels of EcThr-LecB and EcThr-LecA in the intestine of dsRNA (*EcThr-LecB*-dsRNA and *GFP*-dsRNA)-silenced shrimp were detected by qPCR. Furthermore, the expression levels of anti-lipopolysaccharide factor (EcALF1, EcALF2, and EcALF3), crustin (EcCRU1, EcCRU2, EcCRU3, and EcCRU4), and lysozyme (EcLYSO1, EcLYSO2, EcLYSO3, EcLYSO4, and EcLYSO5) in the intestine of dsRNA (*EcThr-LecB*-dsRNA and GFP-dsRNA)-silenced shrimp were analyzed by qPCR. Primers (EcALF1/2/3-qRT-F/-R, EcCRU1/2/3/4-qRT-F/-R, and EcLYSO1/2/3/4/5-qRT-F/-R, Table 1) were designed for qPCR analysis.

2.6. Recombinant expression and purification of two CRDs and threonine-rich region of EcThr-LecB

Specific primers (Ec-CRD-Thr-CRD-ex-F and Ec-CRD-Thr-CRD-ex-R, Table 1) containing EcoR I and Xho I sites at their 5′ ends were designed to amplify a cDNA fragment encoding two CRDs and threonine-rich region of EcThr-LecB. The amplified fragment was first digested and then infused into the pGEX-6p-2 vector (Novagen, Germany) digestion with restriction enzymes EcoR I and Xho I (NEB, USA). Recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells (TransGen Biotech, China) for recombinant protein (named RLecB) expression. After that, RLecB was purified by glutathione Sepharose 4B chromatography (Gen-Script, USA) and detected by using 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue R250. The determination of recombinant protein concentration was performed by Bradford protein assay kit (Jiancheng, Nanjing, China).

2.7. Microbial binding assay

The purified RLecB (50 μg) was incubated with four strains of gram-positive bacteria (*S. aureus*, *B. subtilis*, *B. megaterium*, and *B. cereus*) and three strains of gram-negative bacteria (*V. parahaemolyticus*, *A. hydrophila*, and *V. alginolyticus*) (approximately 2 × 10⁸ cells each) in mid-logarithmic phase by gentle rotation for 1 h at 37 °C. Microbes incubated with recombinant GST-tag protein were used as a negative control.
Fig. 1. Sequence feature and genome structure of EcThr-LecA and EcThr-LecB. (A) Nucleotide and deduced amino acid sequences of EcThr-LecA of *E. carinicauda*. The start codon (atg) and the stop codon (tga) were marked in red. The signal peptide and threonine-rich region were underlined and double underlined, respectively. Two CRD domains of EcThr-LecA were marked with pink background. (B) Nucleotide and deduced amino acid sequences of EcThr-LecB. The CRD domains were marked in green. The protein structure of EcThr-LecA and EcThr-LecB were predicted by SMART analysis. (C) The genome organization of EcThr-LecA and EcThr-LecB. Ten exons were represented by blue and green long boxes. Among which, the number of nucleotides that make up the exon7 was different. Full line and dotted line indicated known and possible introns. Unknown sequence of intron was represented by a double-slash (//). Dual linear showed the variant intron6 and intron7. The nucleotide sequence of intron2 in EcThr-LecA and EcThr-LecB was different and specific, which contain 277 and 317 nucleotides, respectively.
Fig. 2. The phylogenetic tree based on the amino acid sequences of EcThr-LecA, EcThr-LecB, and their homologous proteins was constructed by the neighbor-joining distance algorithm. EcThr-LecA and EcThr-LecB were marked with red triangle and pentagram in front of them and highlighted in yellow. Thrs represent multiple threonine. MMR1, MR, and MRC2 is abbreviation of macrophage mannose receptor 1, mannose receptor, and C-type mannose receptor 2, respectively.

Identity = 50.35%

Fig. 3. Multiple sequence alignment of amino acid sequences of EcThr-LecA, EcThr-LecB, and their homologous proteins by using DNAMAN software. The red and pink frame represented the signal peptide and CRDs, respectively. The tandem threonine region was marked in orange broken line with arrows. CoMMR1: macrophage mannose receptor 1 from C. opilio (KAG0712468.1); PtMRC2: C-type mannose receptor 2 from P. trituberculatus (MPC66161.1); PcMR-partial: mannose receptor from P. clarkii (AYD41591.1).
control. The mixtures were centrifuged at 6000 rpm for 5 min for collecting the microorganisms. The harvested cells were washed four times with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) and then detected by 12.5% SDS-PAGE and Western blotting with anti-GST mouse monoclonal antibody (TransGen Biotech, China).

2.8. Carbohydrate binding assay

Enzyme-linked immunosorbent assay (ELISA) was performed to detect the direct binding of RLecB to LPS and PGN. In details, LPS and PGN were initially dissolved in distilled water to a concentration of 80 μg/mL and sonicated thrice for 15 s. Total 4 μg (50 μL) of polysaccharide was added to each well of a microtiter plate. The plate was incubated at 37 °C overnight and heated at 60 °C for 30 min. The wells of the plate were blocked with 200 μL/well of bovine serum albumin (BSA) in TBS (1 mg/mL) at 37 °C for 2 h and then washed four times with TBS (200 μL/well). Afterward, various concentrations of purified RLecB protein (0−15 μg/mL in TBS containing 0.1 mg/mL BSA) was added into the wells (50 μL/well) and incubated at room temperature for 3 h. The wells were washed four times with TBS and then incubated with 100 μL of anti-GST mouse mAb (1:3000 diluted in 0.1 mg/mL BSA) at 37 °C for 2 h. After being washed four times with TBS, each well was incubated with 100 μL of peroxidase-conjugated goat anti-mouse IgG secondary antibodies (1:5000 diluted in PBS containing 0.1 mg/mL BSA) at 37 °C for 1 h. After the last wash with TBS, color was developed by adding 0.01% 3, 3′, 5, 5′-tetramethylbenzidine (Sigma) liquid substrate to each well. The reaction was stopped by adding 2 M H₂SO₄ (2 mol L⁻¹), and the absorbance was read at 450 nm by using a plate reader (BioTek Instruments, USA). The assay was repeated thrice.

2.9. Bacterial clearance assay

Bacterial clearance assay was performed to investigate the involvement of RLecB in V. parahaemolyticus clearance. Total 500 μL of purified RLecB protein in PBS (100 μg/mL) was incubated with 500 μL of PBS containing V. parahaemolyticus (1.0 × 10⁷ cells) at 28 °C for 30 min with gentle rotation. The same number of V. parahaemolyticus was incubated with GST (500 μL, 100 μg/mL), and PBS (500 μL) were used as controls. After incubation, 50 μL of the mixture was injected into shrimp. At 10 and 20 min post-injection, the hemolymph from five random shrimp in each group was collected. After serial dilution with PBS, 50 μL of hemolymph was loaded on LB agar plates and incubated overnight at 37 °C. The number of bacteria clones in the plate was counted. Experiments were repeated thrice.

Fig. 4. Temporal expression patterns of EcThr-LecA and EcThr-LecB in the intestine after bacteria and virus challenges. The mRNA expression levels of EcThr-LecA (A, C) and EcThr-LecB (B, D) in the intestine at different time intervals after V. parahaemolyticus and WSSV challenge treatments were analyzed by qPCR. The reference gene for internal controls is 18S rRNA. Data are shown as mean values ± standard deviations (SD). Significance is compared between the infected and normal group (0 H) at each sampling point. Asterisks indicate significant differences (*p < 0.05, **p < 0.01, and ***p < 0.001).
the control. (Genbank accession number: MZ384247) was 1521 bp, with an open reading frame (ORF) of 1251 bp encoding 412 deduced amino acid (aa) residues. Predicted EcThr-LecA protein contained a signal peptide, two conserved CRD domains, and a low density complex area with rich threonine. The full-length cDNA sequence of EcThr-LecA (Genbank accession number: MZ384248) gene was 1518 bp in length, which contains an ORF of 1242 bp encoding a 413 amino acid protein (Fig. 1B). EcThr-LecB was predicted to have a signal peptide and two conserved CRD (Fig. 3). Furthermore, EcThr-LecA and EcThr-LecB share 78.98% of identity in the cDNA sequences (Fig. S1). The high similarities of these two lectins suggest that they may be produced by gene duplication.

3. Results

3.1. Characterization and sequences analysis of EcThr-LecA and EcThr-LecB

As shown in Fig. 1A, the obtained full cDNA length of EcThr-LecA (Genbank accession number: MZ384247) was 1521 bp, with an open reading frame (ORF) of 1251 bp encoding 412 deduced amino acid (aa) residues. Predicted EcThr-LecA protein contained a signal peptide, two conserved CRD domains, and a low density complex area with rich threonine. The full-length cDNA sequence of EcThr-LecB (Genbank accession number: MZ384248) gene was 1518 bp in length, which contains an ORF of 1242 bp encoding a 413 amino acid protein (Fig. 1B). EcThr-LecB was predicted to have a signal peptide and two conserved CRD domains. It also contained the threonine-rich region.

The genome sequences of EcThr-LecA and EcThr-LecB were obtained by PCR amplification and genome walking. As shown in Fig. 1C, the genome structure of EcThr-LecA included 10 exons and 9 introns. Among which, the number of nucleotides that make up the exon7 was different with that of EcThr-LecB. The sequences of intron6 and intron7 were variable. Intron6 could be made up of 540, 698, 715, or 718 nucleotides. Intron7 could be made up of 867, 1212, or 1837 nucleotides. EcThr-LecB genome contained 10 exons, 1 specific intron, and 8 predicted introns. The nucleotide sequence of intron2 in EcThr-LecA and EcThr-LecB was different and specific, which contained 277 and 317 nucleotides, respectively.

3.2. Phylogenetic analysis and multiple sequence alignment of two Thr-Lecs

Sequence analysis with the BLASTX program revealed that the deduced amino acid sequences of EcThr-LecA and EcThr-LecB exhibited a high identification with macrophage mannose receptor 1 (MMR1), mannose receptor (MR), and C-type mannose receptor 2 (MRC2) from crustaceans. MMR1, MR, and MRC2 are members of CTL superfamily. Based on the amino acid sequences of EcThr-LecA, EcThr-LecB, and their homologs, a Neighbor-Joining phylogenetic tree was constructed to reveal the evolutionary relationships. The information from evolutionary tree showed that MRs fall into two categories. One contained tandem threonine region, the other did not. Moreover, the MRs without tandem threonine region were divided into the types of invertebrate and vertebrate. EcThr-LecA and EcThr-LecB were clustered together and had a closer evolutionary distance with MMR1, MR, and MRC2 from shrimp and crabs that contain tandem threonine region (Fig. 2). The amino acid sequences of EcThr-LecA, EcThr-LecB, and their homologous proteins [CoMMR1 from Chionoecetes opilio (KAG0712468.1), PtMRC2 from Portunus trituberculatus (MPC66161.1), and PeMR-partial from P. clarkii (AYD41591.1)] were compared by using DNAMAN software. The result showed that they have 50.35% of identity in the sequences of amino acids. Moreover, all of them contain the protein domains of signal peptide, tandem threonine region, and CRD (Fig. 3). These results indicated that EcThr-LecA and EcThr-LecB genes in the intestine at 36 h after WSSV injection were detected by qPCR. The samples from E. carinicauda and X. huangi at 36 h after dsRNA (GFP-dsRNA and EcThr-LecB-dsRNA) injection were detected by qPCR. The results showed that the expression of EcThr-LecB in the intestine was significantly increased at 24 and 48 h and decreased at 72 h after WSSV challenge (Fig. 4C), whereas the mRNA expression of EcThr-LecB in the intestine was obviously increased at all tested time points (24, 48, and 72 h) post WSSV injection (Fig. 4D).

3.3. Expression profiles of EcThr-LecA and EcThr-LecB

The time course expressions of EcThr-LecA and EcThr-LecB in the intestine were analyzed after V. parahaemolyticus challenge. As shown in Fig. 4A, the transcriptional level of EcThr-LecA in the intestine was significantly up-regulation at 2, 6, and 24 h post Vibrio injection. The mRNA expression of EcThr-LecB in the intestine was first evidently up-regulated at 2, 6, and 12 h and then down-regulated at 24 h after the challenge by V. parahaemolyticus (Fig. 4B). Moreover, the expression levels of EcThr-LecA and EcThr-LecB in the intestine after the challenge by WSSV were analyzed by qPCR. The results showed that the expression of EcThr-LecA in the intestine was significantly increased at 24 and 48 h and decreased at 72 h after WSSV challenge (Fig. 4C), whereas the mRNA expression of EcThr-LecB in the intestine was obviously increased at all tested time points (24, 48, and 72 h) post WSSV injection (Fig. 4D).

3.4. Effects of EcThr-LecB silencing on the expression of AMPs

As shown in Fig. 5A, the expression level of EcThr-LecB in the intestine at 36 h after EcThr-LecB-dsRNA injection was significantly decreased compared with the GFP-dsRNA injection group (as control). The mRNA expression of EcThr-LecA in the intestine of EcThr-LecB-dsRNA-injected shrimp was also evidently down-regulated (Fig. 5B). These results indicated that EcThr-LecB dsRNA can remarkably inhibit the expression of EcThr-LecB and EcThr-LecA genes in E. carinicauda.

When EcThr-LecB was knocked down, the expression levels of anti-lipopolysaccharide factor (EcALF1, EcALF2, and EcALF3), crustin (EcCCR1U, EcCCR2U, EcCCR3U, and EcCRU4), and lysozyme (EcLYSO1, EcLYSO2, EcLYSO3, EcLYSO4, and EcLYSO5) in the intestine were detected by qPCR. As shown in Fig. 6A, knockdown of EcThr-LecB could down-regulate the expression of EcALF2 and make no effect on the transcription of EcALF1 and EcALF3. In the intestine of EcThr-LecB-silenced shrimp, the expression levels of EcCRU1, EcCRU3, and EcCRU4 were significantly decreased, whereas the mRNA expressions of EcCRU2 were not changed (Fig. 6B). In addition, knockdown of EcThr-LecB obviously decreased the expression levels of EcLYSO1, EcLYSO2, EcLYSO3, and EcLYSO4 (Fig. 6C). The mRNA expression of EcLYSO5 was not influenced by EcThr-LecB silencing.

3.5. Expression and purification of RLecB

A cDNA fragment encoding two CRDs and threonine-rich region of EcThr-LecB was infused into the pGEX-6p-2 vector. After Isopropyl-beta-D-thiogalactopyranoside (IPTG) induction, the RLecB was successfully expressed in E. coli BL21 (DE3). The CRD-Thr-RLecB of EcThr-LecB protein was estimated to have an MW of 33,797.09 Da with PI of 4.41. The apparent molecular mass of the purified RLecB was between 50 and 60 kDa with a GST-tag (approximately 26 kDa) (Fig. 7A). The concentration of RLecB was 500 μg/mL.
Fig. 6. Expression changes of antimicrobial peptides after knockdown of EcThr-LecB. (A) The expression levels of anti-lipopolysaccharide factor (EcALF1, EcALF2, and EcALF3) in the intestine of EcThr-LecB-silenced shrimp. (B) At 36 h after dsRNA (GFP-dsRNA or EcThr-LecB-dsRNA) injection, the expression levels of crustins (EcCRU1, EcCRU2, EcCRU3, and EcCRU4) in the intestine were analyzed by qPCR. (C) After knockdown of EcThr-LecB, the transcriptional levels of lysozymes (EcLYSO1, EcLYSO2, EcLYSO3, EcLYSO4, and EcLYSO5) in the intestine were detected by qPCR. GFP-dsRNA injection group was set as control. Data are shown as mean ± SD. Asterisks indicate significant differences (*p < 0.05 and **p < 0.01).
3.6. Microbial binding and carbohydrate binding activities of RLecB

As one of PRRs, CTL can recognize and bind to pathogens and then initiate multiple immune responses. Microbial binding assay showed that RLecB can bind to all tested gram-positive bacteria (S. aureus, B. subtilis, B. megaterium, and B. cereus) and gram-negative bacteria (V. parahaemolyticus, A. hydrophila, and V. alginolyticus) (Fig. 7B), whereas GST-tag protein (control) can’t bind to these microorganisms. Further study showed that purified RLecB can bind to LPS and PGN in a dose-dependent manner (Fig. 7C). Also, RLecB had a higher binding activity to PGN than that to LPS under the same condition in vitro.

3.7. V. parahaemolyticus clearance assay

Bacterial clearance essay was performed to detect the function of EcThr-Lec in vivo. Purified RLecB or GST-tag (control) protein was first incubated with V. parahaemolyticus and then injected into shrimp. At 10 and 20 min after the injection, the number of the survival bacteria in V. parahaemolyticus only, V. parahaemolyticus + GST, and V. parahaemolyticus + RLecB groups were counted. The result showed that the number of bacteria in RLecB group decreased rapidly compared with that in the control groups (Fig. 7D), suggesting that Thr-Lec in E. carinicauda can facilitate V. parahaemolyticus clearance in shrimp.

4. Discussion

CTLs are a superfamily of proteins that recognize a broad repertoire of ligands and that induce animal immune responses. In insect and crustacean, CTLs are involved in pathogen recognition, opsonization, nodule formation, agglutination, encapsulation, melanization, prophyloloxidase activation, protein trafficking, cell signaling, as well as in maintaining gut microbiome homeostasis [10,22,23]. In this research, we successfully cloned two novel tandem threonine-containing C-type lectins from E. carinicauda (named EcThr-LecA and EcThr-LecB). In the organization of genome, EcThr-LecA and EcThr-LecB had variable two introns (intron6 and intron7) and specific intron2, respectively.
Phylogenetic analysis showed that EcThr-LecA and EcThr-LecB proteins cluster with MMR1, MR, and MRC2 that contain tandem threonine regions of CTL superfamily from shrimp and crabs. All of them contain the conserved and characteristic modules of CRDs, which are essential for binding to multiple ligands. In view of phylogeny relationship and structural features, EcThr-LecA and EcThr-LecB were confirmed to be new members of CTL superfamily in *E. carinicauda*.

Diseases caused by multiple pathogens are huge problems in shrimp farms. The bacteria *V. parahaemolyticus* and the virus WSSV are considered as two major pathogens, which can cause high mortality rates in shrimp and many other crustaceans after infection [24, 25]. To evaluate the possible functions of *EcThr-LecA* and *EcThr-LecB* in shrimp immune defense, their expression patterns were examined by qPCR. The mRNA expression of *EcThr-LecA* and *EcThr-LecB* in the intestine were significantly increased after *V. parahaemolyticus* and WSSV challenges, implicating that *EcThr-LecA* and *EcThr-LecB* were sensitive to the pathogen infection and could be activated after bacterial and viral stimulations. *Mcr CTL* from *Macrobrachium rosenbergi* in hepatopancreas was significantly up-regulated after a challenge with *V. parahaemolyticus* or WSSV [26]. The significant up-regulation of *FmLC3* was manifested in *F. merguiensis* challenged with *V. harveyi* or WSSV [16]. The expression of *LvCTCL3* in gills of *L. vannamei* was up-regulated after *V. parahaemolyticus* and WSSV challenges [11]. These results suggest that bacterial or viral challenge can induce the activation of CTLs.

CTls have been reported to be involved in the regulation of AMPs synthesis in crustacean. For example, a CTL (EsLech) from the Chinese mitten crab that can positively regulate AMP expression via the c-Jun N-terminal kinase (JNK) pathway [27]. The recognition of bacterial glycans by the CRD of MjCC-CL in *M. japonicus* leads to activation of the JAK/STAT pathway via interaction of the coiled-coil domain (CCD) with the surface receptor Domeless, and upregulation of AMP expression [19]. Knockdown of SpCTL-B identified from the hemocytes of mud crab *Scylla paramamosain* could significantly decrease and the transcript levels of AMPs [28]. In the current study, we found that knockdown of *EcThr-LecB* evidently down-regulated the expression of eight different AMPs (EcALF2, EcCRU1, EcCRU3, EcCRU4, EcLYSO1, EcLYSO2, EcLYSO3, and EcLYSO4), whereas make no effect on the transcription of EcALF1, EcALF3, EcCRU2, and EcLYSO5. AMPs are crucial for crustaceans to fight the pathogenic invasion, which have broad spectra of antimicrobial activity, an ability to kill or neutralize gram-negative and gram-positive bacteria, fungi, parasites, and viruses [29, 30]. These results suggest that *EcThr-LecB* may participate in the antimicrobial defenses through selectively positive regulating the mRNA expression of some AMPs. But the complex regulation mechanism of *Thr-Lec* gene on the expression of different AMPs needs further study.

CTls can recognize and noncovalent bind to specific carbohydrate ligands on the cell surface and agglutinate to cells by binding to glycoproteins and glycoconjugates [31]. In this research, the recombinant CRD-Thr-CRD of EcThr-LecB could bind to multiple gram-positive bacteria (*S. aureus, B. subtilis, B. megaterium, and B. cereus*) and gram-negative bacteria (*V. parahaemolyticus, A. hydrophila, and V. alginitolyticus*) in vitro. Many CTls in crustaceans have been reported to bind directly to several kinds of microorganisms selectively, such as *FmLC3* [16], *MnCTL* [32], *FcLec4* [33], *AICTL-3* [34], and so on. It’s known to us that LPS and PGN are the major components exposed on the surface of gram-negative and gram-positive bacteria, respectively. The result from ELISA assay showed that RLeC could directly bind to LPS and PGN (45%), depending on dependent manner. In addition, bacterial clearance assay revealed that RLeC could accelerate the clearance of *V. parahaemolyticus* in shrimp. All the results indicate that two Thr-Lecs are involved in antimicrobial immune defense in *E. carinicauda*.

In conclusion, two tandem threonine containing CTls (named EcThr-LecA and EcThr-LecB) were identified from *E. carinicauda*. The mRNA expression of EcThr-LecA and EcThr-LecB in the intestine were significantly up-regulated after *V. parahaemolyticus* and WSSV challenges. Knockdown of EcThr-LecB significantly inhibited the expression of most tested AMPs. The recombinant two CRDs and threonine-rich region (RLeC) could bind to multiple bacteria and polysaccharides. Furthermore, the RLeC could enhance the clearance of *V. parahaemolyticus* injected in shrimp. All the results indicate that newly discovered Thr-Lec in *E. carinicauda* may act as PRRs to participate in the innate immune defense against pathogens through recognition of non-self, regulation of AMPs, and clearance of invaders.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Acknowledgements**

The current study was supported by the National Science Research of Jiangsu Higher Education Institutions of China (19KJB240002), the National Natural Science Foundation of China (Grant nos. 31902397, 31572647), and the National Science Foundation of Jiangsu Province (BK20190698, BK202171474), and Agricultural Major New Varieties Creation Project of Jiangsu Province (PZCZZ201747).

**Supplementary materials**

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fishimm.2021.100018.

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