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Short communication

A novel C-type lectin with microbiostatic and immune regulatory functions from *Litopenaeus vannamei*

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ABSTRACT

C-type lectins (CTLs) are a group of lectins with at least one carbohydrate recognition domain (CRD), the binding of which to carbohydrates requires the presence of calcium ions. CTLs generally function as pattern recognition receptors (PRRs), essentially participating in innate immunity. In the current study, a novel CTL termed LvCTL5 was identified from Pacific white shrimp *Litopenaeus vannamei*, which shared sequence identities with other crustacean CTLs. LvCTL5 was highly expressed in hepatopancreas and could be activated by infection with bacteria, virus and fungi. The recombinant LvCTL5 protein purified from *E. coli* showed microbiostatic and agglutination activities against bacteria and fungi *in vitro*. Silencing of LvCTL5 *in vivo* could significantly affect expression of a series of immune effector genes and down-regulate the phagocytic activity of hemocytes. Compared with controls, the LvCTL5-silenced shrimp were highly susceptible to *Vibrio parahaemolyticus* and white spot syndrome virus (WSSV) infections. These suggest that LvCTL5 has microbiostatic and immune regulatory activities and is implicated in antiviral and antibacterial responses.

1. Introduction

C-type lectins (CTLs), containing at least one carbohydrate recognition domain (CRD), also called C-type lectin-like domains (CTLDs), are the most diverse family of lectins [1–3]. The CRD of CTLs generally has 110–130 amino acid residues, which construct four Ca^{2+}-binding sites and one characteristic double-loop flanked by conserved disulfide bonds [4–6]. Through the CRD and a Ca^{2+} dependent manner, CTLs bind carbohydrates on the surfaces of pathogens, including lipopolysaccharide, galactose, and mannose, and function as pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) of invading microbial pathogens to initiate subsequent innate immune responses in both vertebrates and invertebrates [7,8].

Pacific white shrimp *Litopenaeus vannamei* (*Penaeus vannamei*) is the most productive aquaculture shrimp in the world [9]. The Pacific white shrimp farming industry is threatened by infectious diseases and adverse water environments nowadays. As a representative species of crustaceans, the immune system of *L. vannamei* has attracted more and more research attention [10–13]. Previous studies have identified several CTL genes from *L. vannamei*, which play important roles in antiviral and antibacterial immunity [14–17]. A recent finding suggests that CTLs could be more essential for survival of shrimp suffering complex microbial stresses in water environments than other immune effector genes, such as antimicrobial peptides (AMPs) [18]. Therefore, further exploring the roles of the CTL family in immunity is of significance for the prevention of diseases in shrimp farming. In the current study, a novel CTL gene termed LvCTL5 was identified from *Litopenaeus vannamei*. We demonstrated that LvCTL5 has inhibitory activity against Gram-positive and -negative bacteria and fungi and plays roles in regulation of both humoral and cellular immune responses, which enrich our knowledge on shrimp CTLs.

2. Materials and methods

2.1. Animals and pathogens

Shrimp (~10 g) from an aquaculture farm in Zhuhai, China were acclimated at ~28 °C for at least 1 week in a recirculating water tank system with air-pumped seawater (2.0% salinity). Before experiments,

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Table 1
Summary of primers used in this study.

Primers	Sequences(5′ to 3′)
For cDNA cloning	
LvCTL5-ORF-F	ATGAAGCTCTCGCTCCTTCTCG
LvCTL5-ORF-R	TCAGTTCTTCTGAAATTTGTACTCGC
RT-PCR analysis	
LvCTL5-F	TGGCTTCTGTCAGGGTTTCC
LvCTL5-R	CGTCCGTCCACACGAACTC
Lv*EF-1α*-F	CCTATGTGCGTGGAGACCTTC
Lv*EF-1α*-R	GCCAGATTGATCCTTCTTGTTGAC
LvSTAT-F	CTTCGCCATCCGTCCTCTAG
LvSTAT-R	GGCTTGATCCTTAGGCACATTC
LvRelish-F	CTGCTTCTCCATACTCAGACCAC
LvRelish-R	CTGTGGCTGCTCCAGTATTTG
LvDorsal-F	TTGCGACCACCAGACAAGAG
LvDorsal-R	GCAAGGTAACGACTAATCTTCTCTG
LvPEN2-F	CCAAGGCGAAGCGTACAG
LvPEN2-R	CAATTGCGAGCATCTGAGAC
LvPEN3-F	CTCCTGCGTCCGCCATG
LvPEN3-R	GTGTAACCGCCCTTGTACAC
LvPEN4-F	GCCCGTTACCCAAACCATC
LvPEN4-R	AACAATCCCCGTATCTGAAGC
LvCTL3-F	GCTCCTGCTGTCCTTCCTG
LvCTL3-R	ACCTCCATCACAAACTCTCTGG
LvCTL4-F	CTTGGACGCTTATGTCACCTAC
LvCTL4-R	CATCCTTGCTCTTGATGTAGTCG
LvLT-F	TGGAGTCGCTACAACTACTTCG
LvLT-R	CAAAGGTCACGAACAAGAGGC
LvLec-F	CTTGGACGCTTATGTCACCTAC
LvLec-R	CATCCTTGCTCTTGATGTAGTCG
LvALF1-F	GGATGTGGTGTCCTGGATGG
LvALF1-R	GCGTCGTCCTCCGTGATG
LvALF2-F	GCGAACAAACTCACTGGACTG
LvALF2-R	ACATGCGACCCTGGAATACAG
LvALF3-F	GACCTGTCCAACCCTGAGC
LvALF3-R	TCGCCTCCTCCTCCGTTATC
LvALF4-F	CCTGGTGGCACTCTTCGC
LvALF4-R	ACGGTGAAGCGGCACTTATG
LvALF-AVK-F	GTTCCTGGTGGCACTCTTCG
LvALF-AVK-R	TCCGTCTCCTCGTTCCTCC
dsRNA production	
dsCTL5-F	GCTCCCAAACCCGAAATAAG
dsCTL5-R	GCAAATGTCATCCCAACCTT
dsCTL5-T7-F	GGATCCTAATACGACTCACTATAGGGCTCCCAAACCCGAAATAAG
dsCTL5-T7-R	GGATCCTAATACGACTCACTATAGGGCAAATGTCATCCCAACCTT
GFP-F	ATGGTGAGCAAGGGCGAGGAG
GFP-R	TTACTTGTACAGCTCGTCCATGCC
T7-GFP-F	GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGAG
T7-GFP-R	GGATCCTAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCATGCC
dsLvSTAT-F	TCAGTATGCCCAGTCCTT
dsLvSTAT-R	CCTAACTCTTTCCGTCTCC
T7-dsLvSTAT-F	GGATCCTAATACGACTCACTATAGGTCAGTATGCCCAGTCCTT
T7-dsLvSTAT-R	GGATCCTAATACGACTCACTATAGGCCTAACTCTTTCCGTCTCC
dsLvRelish-F	AGAGGTGACAGAGGTGGGAT
dsLvRelish-R	CTTGCATGGGTTATCAACTC
T7-dsLvRelish-F	GGATCCTAATACGACTCACTATAGGAGAGGTGACAGAGGTGGGAT
T7-dsLvRelish-R	GGATCCTAATACGACTCACTATAGGCTTGCATGGGTTATCAACTC
dsLvDorsal-F	CTGTTGACCCACCTTACCGAC
dsLvDorsal-R	ATCTTTGACCTCATAGAAACGGAC
T7-dsLvDorsal-F	GGATCCTAATACGACTCACTATAGGCTGTTGACCCACCTTACCGAC
T7-dsLvDorsal-R	GGATCCTAATACGACTCACTATAGGATCTTTGACCTCATAGAAACGGAC
protein expression	
PET32a + -CTL5-BamH I F	CGC **GGATCC** AACAGCGTAGACAGCAAGCAAAG
PET32a + -CTL5-Hind III R	CCC **AAGCTT** ATTA GTTCTTCTGAAATTTGTACTCGCATATCAC

Nucleotides in bold represent the restriction sites introduced for cloning.

shrimp were randomly detected to ensure freeing of white spot syndrome virus (WSSV) and *Vibrio parahaemolyticus* by PCR as previously described [19,20]. The stocks of *V. parahaemolyticus* and WSSV for experiments were prepared as previously described [21,22].

2.2. Gene cloning

An expressed sequence tag (EST) containing a CRD-encoding region was retrieved from a *L. vannamei* transcriptome library. The full length of LvCTL5 ORF was obtained by rapid amplification of cDNA ends (RACE) using a SMARTer RACE cDNA Amplification kit (Clontech, Japan) and sequenced.

2.3. Bioinformatics analysis

The amino acid sequence of LvCTL5 was analyzed using BLAST

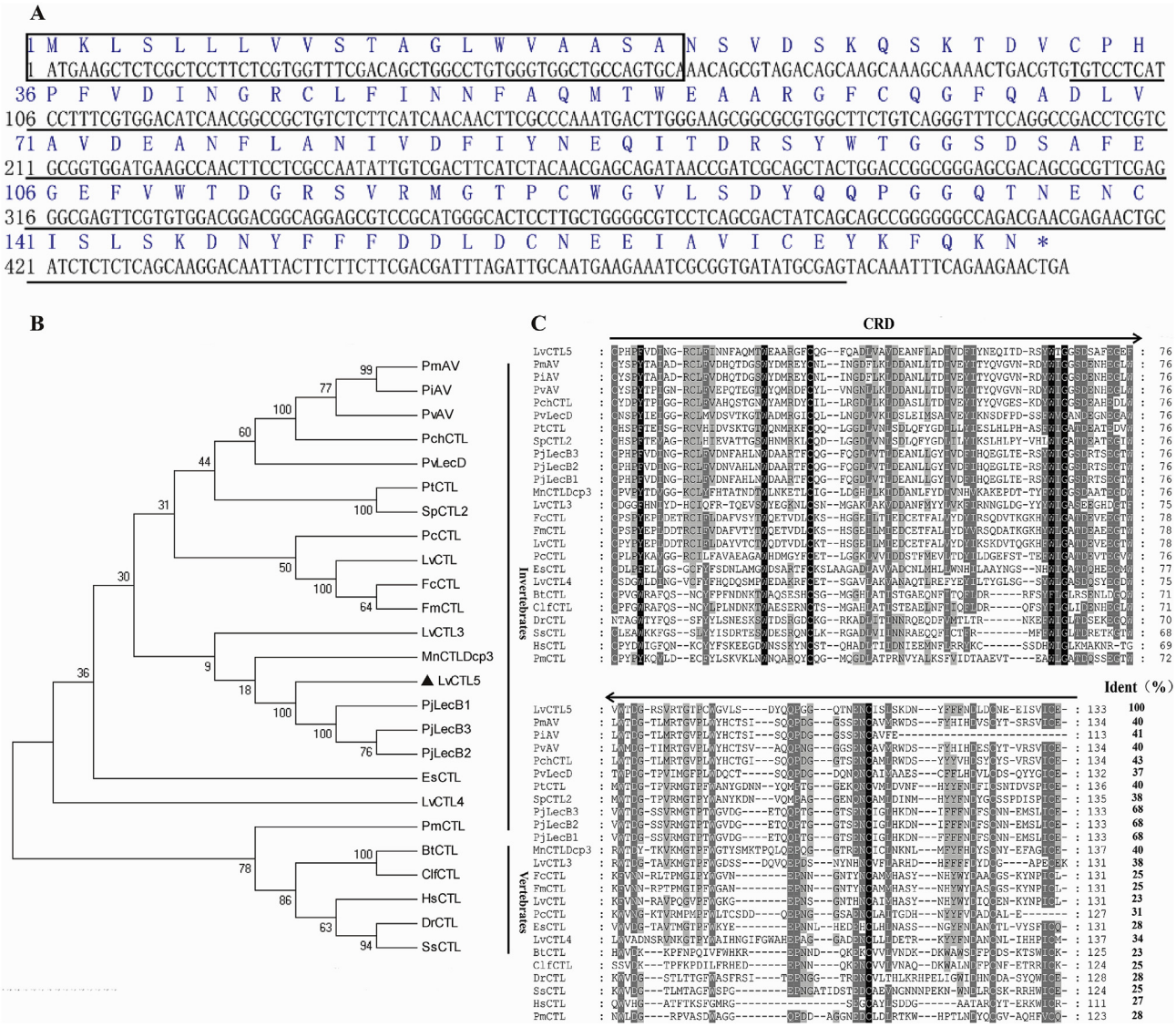

Fig. 1. Sequence analyses of LvCTL5. (A) Nucleotide and deduced amino acid sequences of LvCTL5 ORF. The amino acid sequence was shown above the coding nucleotide sequence and numbered on the left. The signaling peptide and the CRD were boxed and underlined, respectively. (B) Multiple-sequence alignment of the deduced amino acid sequence of LvCTL5 with other crustacean CTLs. The identical amino acid residues were shaded in black and the similar residues in gray. (C) Neighbor joining phylogenetic tree analysis of the protein sequences of CTLs. LvCTL5 was marked with hollow triangle. Proteins analyzed included: PjLecB3, lectin B isoform 3 from *Penaeus japonicas* (GenBank accession No. ADG85660); PjLecB2, lectin B isoform 2 from *P. japonicas* (ADG85661); PjLecB1:lectin B isoform 1 from *P. japonicas* (ADG85668); PchCTL, C-type lectin domain-containing protein from *P. chinensis* (AGL93170.1), PmAV from *P. monodon* (AAQ75589.1); PvAV, antiviral protein from *Penaeus vannamei* (*L. vannamei*) (ROT71312.1); PtCTL, C-type lectin from *Portunus trituberculatus* (AGH68927.1); PvLecD, lectin D from *Penaeus vannamei* (*L. vannamei*) (ROT64978.1); SpCTL2, C-type lectin 2 from *Scylla paramamosain* (AEO92002); PiAV, antiviral-like c type lectin from *Penaeus indicus* (ADV17348.1); LvCTL3, C-type lectin from *L. vannamei* (AGV68681.1); BtCTL, C-type lectin domain family 4 member D from *Bos taurus* (NP_001180046.1); ClfCTL, C-type lectin domain family 4 member D isoform X1 from *Canis lupus familiaris* (XP_005637254.1); DrCTL, C-type lectin domain family 4 member M-like isoform X2 from *Danio rerio* (XP_005172687.1); EsCTL, C-type lectin from *Eriocheir sinensis* (ADK66338.1); FcCTL from *Fenneropenaeus chinensis* (ABA54612.1); FmCTL from *Fenneropenaeus merguiensis* (AEB96259.1); HsCTL from *Homo sapiens* (CAA65480); LvCTL from *L. vannamei* (DQ858900); PcCTL from *Procambarus clarkia* (ADX60057.1); PmCTL from *Penaeus monodon* (AAZ29608.1); SsCTL from *Salmo salar* (ACI68944.1). LvCTL4 from *L. vannamei* (KM387560); MnCTLDcp3, CTLDcp3 from *Macrobrachium nipponense* (ALF45199.1).

software and its homologous genes were retrieved from the National Center for Biotechnology Information (NCBI) Genbank databases. Sequence alignments and Phylogenetic tree were made by Clustal W1.8 and MEGA 5.0 softwares with parameters as previously described [23].

2.4. Real-time PCR

To detect the expression of LvCTL5, tissues were sampled and pooled from 15 healthy *L. vannamei*. For challenge experiments, shrimp were intramuscularly injected with *Aspergillus niger* (10^5 CFU), *Staphylococcus aureus* (10^6 CFU), *V. parahaemolyticus* (10^6 CFU), and WSSV (10^6 copies) in 50 μl PBS buffer. Shrimp injected with PBS buffer containing no pathogens were used as the control group. The hepatopancreas was sampled at 0, 4, 12, 24, 48, 72 and 96 h post injection. RNA extraction, cDNA synthesis and real-time PCR were performed following the methods as previously described [24]. The EF-1α gene (Genbank accession No. GU136229) was used as internal control. Sequences of primers used in this study were listed in Table 1.

2.5. In vitro antimicrobial assays

The ORF of LvCTL5 without the signal peptide coding region was

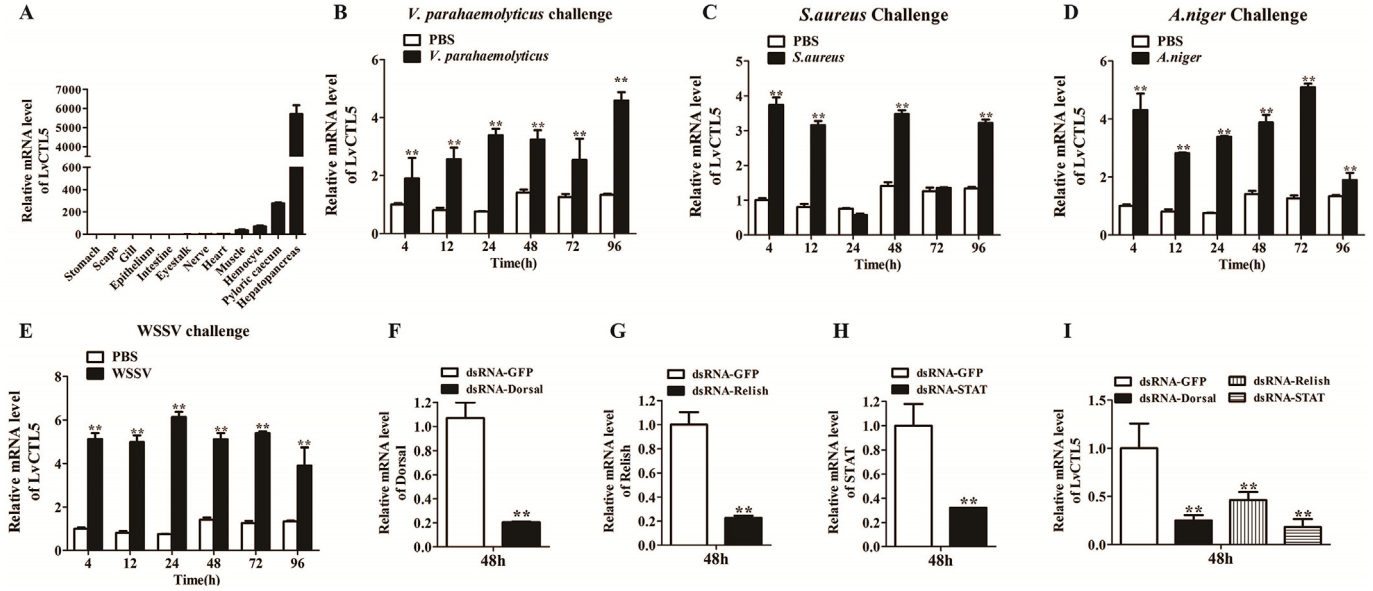

Fig. 2. Expression profiles of LvCTL5. (A) Tissue distribution of LvCTL5 mRNA detected by real-time PCR with EF-1α gene as the internal control. The expression levels were calculated relative to that in intestine (set as 1.0). (B–D) Expression of LvCTL5 in hepatopancreas from *V. parahaemolyticus*, *S. aureus*, *A. niger* and WSSV-challenged shrimps analyzed by Real-time PCR. (F–H) Knockdown efficiencies of Doral, Relish and STAT. (I) The mRNA level of LvCTL5 in Dorsal-, Relish-, and STAT-silenced shrimp. Each bar represents the mean ± SD of the three sample (*$p < 0.05$, **$p < 0.05$).

cloned into the pET-32a (+) vector (Merck Millipore, Germany) and transformed into the *Escherichia coli* strain BL21 (DE3) (Invitrogen, USA) and induced using IPTG. The recombinant 6His-tagged LvCTL5 protein was purified with Ni-NTA agarose (Qiagen, Germany) under native conditions and dialyzed against PBS buffer. The recombinant Trx protein expressed by the empty pET-32a (+) vector was purified as control. The antibacterial activity of LvCTL5 against *V. parahaemolyticus*, *S. aureus,* and *A. niger* was examined following a previously described method [25]. Briefly, microbes were cultured in LB medium to a midlogarithmic phase and diluted to 5×10^4 CFU/ml with LB medium containing gradient concentrations of LvCTL5 or Trx protein. The alcohol of 10% final concentration and TBS buffer were used as positive and negative controls, respectively. After incubation at 37 °C for 16 h, the turbidity of each sample was measured by absorbance at 600 nm (A600) to evaluate the growth of bacteria.

2.6. RNA interference

Double stranded RNAs (dsRNAs) specific to LvCTL5 (dsRNA-CTL5) and green fluorescent protein (dsRNA-GFP) were produced using a T7 RiboMAX™ Express RNAi System (Promega, USA) as previously described [26]. Shrimp were injected with 50 μl PBS containing 5 μg dsRNA-CTL5 or dsRNA-GFP (as control). At 48 h post infection (hpi), the RNA interference (RNAi) efficiency of LvCTL5 and the expression of many immune related genes in hepatopancreas were detected using real-time PCR with primers listed in Table 1. For immune challenge experiments, at 48 h post dsRNA injection, shrimp were further injected with 10^6 CFUs of *V. parahaemolyticus* and 10^6 copies of WSSV ($n = 50$ in each group). Experiments were done in triplicate and the cumulative mortality was recorded.

2.7. Phagocytosis analysis

The phagocytosis of hemocytes was analyzed using flow cytometry following the method as previously described [27]. Briefly, hemocytes extracted from dsRNA-treated shrimp were washed with 2 × Leibovitz's L-15 medium (Gibco, USA) triply and mixed with fluorescein isothiocyanate (FITC)-labled *V. parahaemolyticus* at a 1:100 ratio of cells/bacteria and incubated at 28 °C for 1 h. Hemocytes was then detected using flow cytometry for the signal of FITC and the forward scatter (FSC) values of cells. The FSC threshold was determined by detecting the free FITC-labeled *V. parahaemolyticus* to eliminate cell debris and bacteria, and the fluorescence boundary was set based on detection of the self-fluorescence of untreated hemocytes. A total of 500,000 events were detected for each sample.

2.8. Statistical analysis

The statistical procedures were carried out using SPSS statistical software version 16.0. The mean and standard deviation (SD) from three detections was calculated. Student's t-test was used to compare the two means. The Kaplan-Meier plot (log-rank $\chi 2$ test) was used to analyze the mortalities between different groups.

3. Results

3.1. Cloning and bioinformatics analysis of LvCTL5

The ORF of LvCTL5 gene is bp encoding a protein of amino acids (GenBank Accession No. MK805099). The LvCTL5 protein was predicted to possess a signaling peptide (residues 1–20) and a single CRD (Fig. 1A). The predicted mature LvCTL5 protein without the signal peptide has a theoretical isoelectric point (pI) of 4.18 and a calculated molecular weight of 17.14 kDa. The LvCTL5 protein shared only 23%, 38% and 34% identities with the three previously reported *L. vannamei* LvCTL, LvCTL3 and LvCTL4, respectively, suggesting that LvCTL5 was distinct from other *L. vannamei* CTLs in sequence. In contrast, LvCTL5 shared 68% identity with three CTL isoforms (PjLecB1-3) from *Penaeus japonicus*. Furthermore, there were around 40% of sequence identities between LvCTL5 and CTLs from other shrimp, and 23–28% between LvCTL5 and vertebrate CTLs (Fig. 1B). Phylogenetic tree analysis showed LvCTL5 was clustered into the invertebrate CTL category and was closest to PjLecB1-3 but far from other previously reported *L. vannamei* CTLs.

3.2. Expression profiles of LvCTL5

The distribution of LvCTL5 mRNA in tissues from healthy shrimp

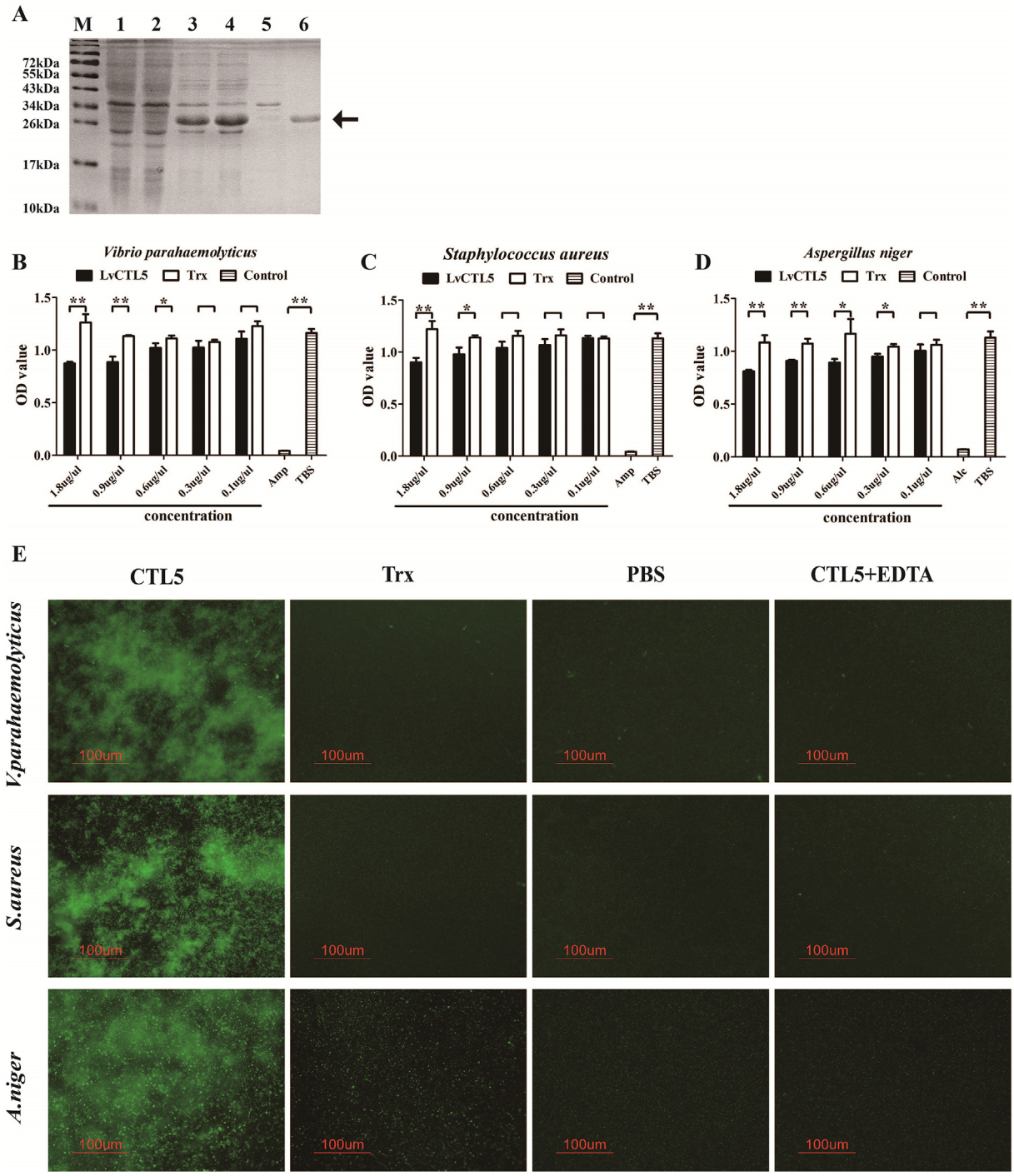

Fig. 3. Microbiostatic and agglutination activities of LvCTL5. (A) SDS-PAGE analysis of the recombinant LvCTL5 protein expressed in *E. coli*. Line 1, untransformed *E. coli* cells; Line 2, uninduced *E. coli* transformed with expressing vectors; Line 3, IPTG-induced recombinant *E. coli*; Line 4, supernatants of lysed recombinant *E. coli*; Line 5, precipitate of ultrasonic lysed recombinant *E. coli*; Line 6: purified recombinant LvCTL5 protein (black arrow). (B–D) Effect of different concentrations of recombinant LvCTL5 and Trx (control) proteins on the growth of *V. parahaemolyticus*, *S. aureus*, and *A. niger*. The TBS buffer and alcohol (Alc) of 10% final concentration were used as negative and positive controls, respectively. The bacterial concentration was determined by absorbance at 600 nm (A600). Each bar represents the mean ± SD of three samples (**$p < 0.01$). (D) Microbe agglutination activity of LvCTL5 against FITC-labeled *V. parahaemolyticus*, *S. aureus*, and *A. niger*.

was analyzed using real-time PCR. As Fig. 2A shown, hepatopancreas expressed the highest level of LvCTL5 mRNA, which was 20.6 times the second highest level found in pyloric caecum. In contrast, the expression of CTL in stomach, scape, gill, epithelium, intestine, eyestalk and nerve was low. The mRNA level of LvCTL5 in hepatopancreas after immune stimulation was further analyzed (Fig. 2B–E). The results demonstrated that expression of LvCTL5 was significantly up-regulated after infection with Gram-negative bacterium *V. parahaemolyticus*, Gram-positive bacterium *S. aureus*, fungus *A. niger* and WSSV, indicating that LvCTL5 could be implicated in a wide spectrum of immune responses. To primarily explore the regulation of LvCTL5 expression, the transcription factors Dorsal, Relish and STAT were knockdown *in vivo* using RNAi strategy (Fig. 2F–H). Compared with the control, with the silencing of these transcription factors, the mRNA

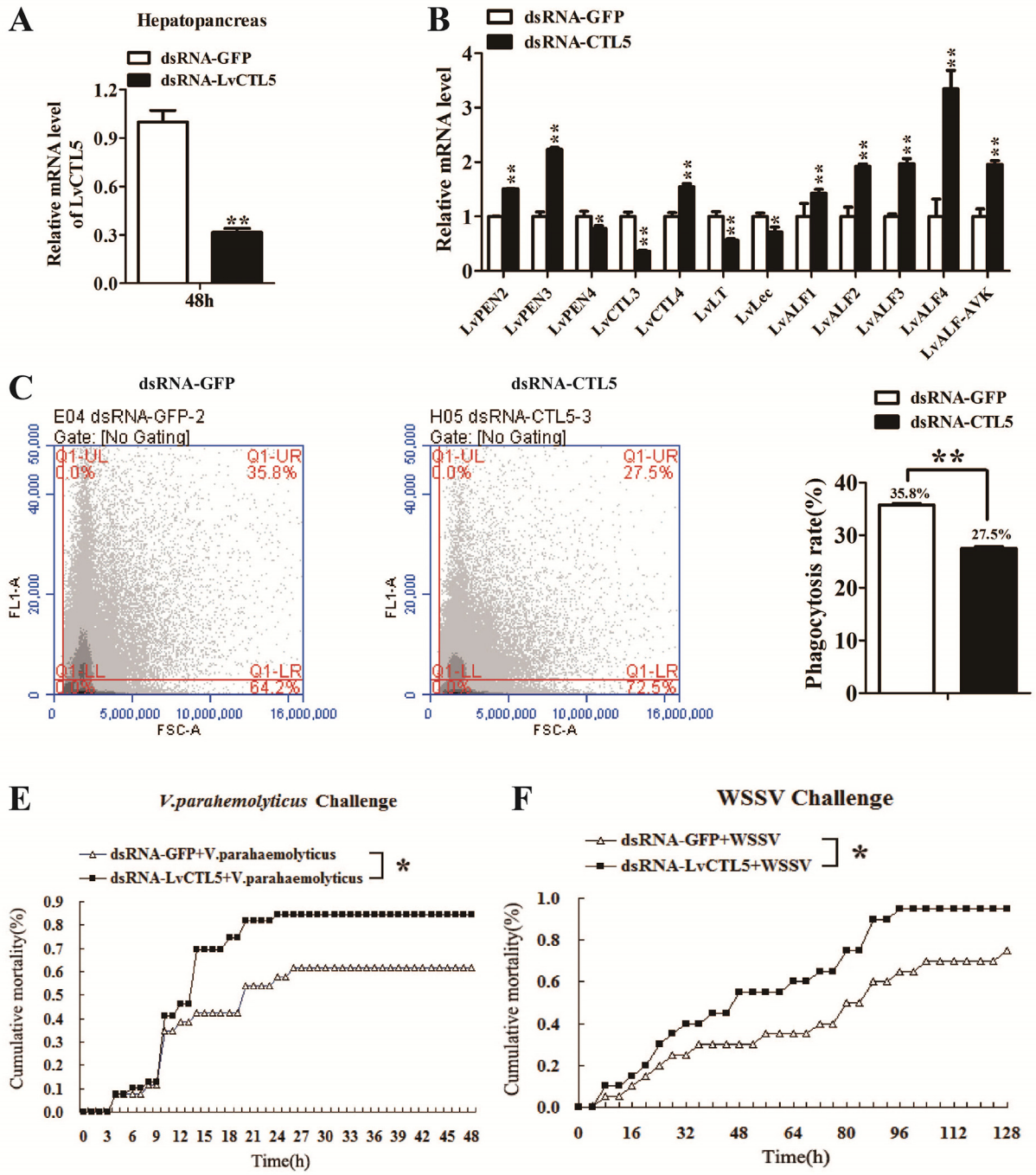

Fig. 4. Immune functions of LvCTL5. (A) Knockdown efficiency of LvCTL5 in hepatopancreas. (B) Expression of many immune effector genes in hepatopancreas of LvCTL5-silencing shrimp analyzed by real-time PCR. $*p < 0.05$, $**p < 0.01$. (C and D) The phagocytic activity of hemocytes from LvCTL5-and GFP-dsRNA treated shrimp against FITC-labeled *V. parahaemolyticus* analyzed by flow cytometry. The result shows a representative of three independently performed experiments. The scatter plots representing one of the three flow cytometric detections were shown in (C) and the data were shown in (D). $**p < 0.01$. Cells were examined by forward scatter (FSC, x-axis) and the phagocytosis of FITC-labeled *V. alginolyticus* was indicated by intracellular green fluorescence (y-axis). (E and F) Mortalities of LvCTL5-and GFP-dsRNA treated shrimp ($n = 50$) infected by *V. parahemolyticus* and WSSV. Differences in cumulative mortality levels between treatments were analyzed by Kaplan-Meier log-rank χ^2 tests. $*p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

level of LvCTL5 also decreased significantly (Fig. 2I).

3.3. Immune function of LvCTL5

The immune function of LvCTL5 was firstly investigated *in vitro*. The recombinant Trx-tagged LvCTL5 protein was purified from *E. coli* and subjected to microbial inhibition assays (Fig. 3A). Compared with the control Trx protein, LvCTL5 significantly inhibited the growth of *V. parahaemolyticus*, *S. aureus*, and *A. niger*, suggesting that LvCTL5 has a broad-spectrum antimicrobial activity (Fig. 3B–D). The agglutination activity of LvCTL5 against these microbes was further investigated (Fig. 3E). The FITC-labeled *V. parahaemolyticus*, *S. aureus*, and *A. niger* were efficiently agglutinated in the presence of Ca^{2+} by recombinant LvCTL5 but not Trx protein. The agglutination activity of LvCTL5 was

eliminated when EDTA was added to chelate Ca^{2+}.

The role of LvCTL5 in shrimp immunity was explored *in vivo* using RNAi strategy (Fig. 4A). Expression of a series of immune effector genes was analyzed in hepatopancreas of LvCTL5-silenced shrimp (Fig. 4B). The results demonstrated that compared with the control, expression of most of the detected antimicrobial peptides (AMPs), including PEN2, PEN3, ALF1-4 and ALF-AVK was significantly up-regulated, while expression of many CTLs, such as LvCTL, LvCTL3 and LvCTL4 was down-regulated, indicating that LvCTL5 could be involved in immune regulation and play different roles in regulating the expression of antimicrobial peptides and other CTLs. The phagocytosis of hemocytes from LvCTL5-silenced and control shrimp against FITC-labeled *V. parahaemolyticus* was further analyzed using flow cytometry (Fig. 4C). The results exhibited that the phagocytic activity of hemocytes was decreased after silencing of LvCTL5, suggesting that LvCTL5 could positively regulate the phagocytosis of hemocytes (Fig. 4D). Furthermore, challenge experiments showed that the mortalities of LvCTL5-silenced shrimp infected with WSSV and *V. parahaemolyticus* were significantly higher than those of the control, suggested that LvCTL5 could be important for antiviral and antibacterial responses in shrimp (Fig. 4E and F).

4. Discussion

At present, dozens of CTLs have been reported in crustaceans, and some of them have been found to possess binding and agglutinating activities against microbes, which is important for their function as PRRs [28,29]. Binding to carbohydrates on the surface of microbes in the presence of calcium is the sine qua non of agglutination activity of CTLs. The binding activity of a few CTLs showed a certain preference for carbohydrates. For instance, the PcLec5 from *Procambarus clarkia*, with a higher affinity to LPS than to peptidoglycan and lipoteichoic acid, has a tendency to bind to Gram-negative bacteria [30], whereas FcLec6 from *Fenneropenaeus chinensis* did not show agglutinating activity against bacteria [28]. In general, most tested crustacean CTLs have a wide-spectrum of agglutinating activity against Gram-negative and -positive bacteria [28]. However, only few studies concern the agglutinating activity of CTLs against fungi, a group of opportunistic infectious pathogens in shrimp. The EsLecB from *Eriocheir sinensis* shows broad-spectrum binding activities to bacteria and fungi, but its agglutinative activity against Gram-negative bacteria and fungi has not been tested [31]. A recent study demonstrated that in addition to agglutinating bacteria, the CRD of a low-density lipoprotein receptor (LDLR) class A domain-containing CTL (LvCTLD) from *L. vannamei* could effectively agglutinate fungi *in vitro* [32]. The current study also showed that LvCTL5 has agglutination activity against both bacteria and fungi. These may indicate that CTLs may play a role in antifungal responses, which is worth of further investigation.

In addition to acting as PRRs, some CTLs also have a direct bacteriostatic function. In vertebrates, several CTLs with a Ca^{2+} dependent carbohydrates binding activity can directly kill bacteria by causing cell wall damage and cytoplasmic leakage [33]. In crustaceans, it has also been reported that some CTLs possess microbicidal activity. The FcLec1 and the Fc-hsL from *F. chinensis* and the EsLecB from *E. sinensis* inhibit the growth of many microorganisms, including bacteria and fungi [31,34]. The current identified LvCTL5 also exhibited *in vitro* inhibitory activity against growth of fungi, Gram-positive and negative bacteria, the underlying mechanism of which needs further studies.

It has also been reported that several crusctacean CTLs with bacterial binding activity did not possess direct antimicrobial activity. Their immune function may be mainly to serve as PRRs to regulate the activation of some immune signaling pathways, thus indirectly playing a bacteriostatic role. For example, although the MjHeCL from *Marsupenaeus japonicus* was shown to be involved in inhibiting microbiota in hemolymph, it did not show direct inhibitory activity against bacteria *in vitro* [35]. The inhibitory effect of MjHeCL on bacterial proliferation *in vivo* is mainly derived from its regulatory effect on antimicrobial peptide gene expression. Similarly, the MjCC-CL from *M. japonicus* also has no direct bacteriostatic or bacteriocidal activity, but it can recognize glycans by its CRD and interact with the Domeless receptor by its coiled-coil domain (CCD) to activate the JAK-STAT pathway, which leads to up-regulation of AMP expression and enhancement of antibacterial immunity [36]. In the current study, knockdown of LvCTL5 *in vivo* also affect expression of a wide range of immune related genes, including AMPs and other CTLs, indicating that LvCTL5 could be involved in immune regulation. Interestingly, LvCTL5 exerted different effects on expression of AMPs and other CTLs, indicating the difference between the mechanisms that regulate expression of AMP and CTLs, which needs further studies. It has been known that shrimp CTLs play important roles in antiviral responses [14–17]. For instance, LvCTL1 exhibited high affinity for WSSV through interacting with several envelope proteins of WSSV and could inhibit WSSV infection *in vivo* [14]. The recombinant LvCTL3 protein was also shown to reduce the mortality of WSSV-infected shrimp [16]. Therefore, the down-regulation of other CTLs in LvCTL5-silenced shrimp could be an important factor leading to increased mortality of shrimp after infection with WSSV. Furthermore, silencing of LvCTL5 signifcantly inhibited the phagocytic activity of hemocytes against bacteria. As opsonization is known as an important function of CTLs [37–39], these suggested an important role of LvCTL5 in regulation of cellular immune responses. Taking together, the current study demonstrated that LvCTL5 has both direct bacteriostatic activity and immunoregulatory function. The higher susceptibility of LvCTL5-silencing shrimp to bacterial infection than the control could be due to both the attenuation of antimicrobial activity and loss of immune regulatory function of LvCTL5 by its specific dsRNA.

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