Fish and Shellfish Immunology Reports 4 (2023) 100094

Contents lists available at ScienceDirect

Fish and Shellfish Immunology Reports

journal homepage: www.sciencedirect.com/journal/fish-and-shellfish-immunology-reports

Identification and characterization of a Reeler domain containing protein in *Procambarus clarkii* provides new insights into antibacterial immunity in crustacean

Ming-Lu Zhang [a,1], Kai-Min Zhou [a,1], Xian-Wei Wang [a,b,*]

[a] Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Sciences, Shandong University, Qingdao, China
[b] State Key Laboratory of Microbial Technology, Shandong University, Qingdao, China

ARTICLE INFO

Keywords:
Procambarus clarkii
Reeler
Biofilm
Antibacterial immunity
Homeostasis

ABSTRACT

Crayfish, as an invertebrate, relies only on the innate immune system to resist external pathogens. In this study, a molecule containing a single Reeler domain was identified from red swamp crayfish *Procambarus clarkii* (named as *Pc*Reeler). Tissue distribution analysis showed that *Pc*Reeler was highly expressed in gills and its expression was induced by bacterial stimulation. Inhibiting the expression of *Pc*Reeler by RNA interference led to a significant increase in the bacterial abundance in the gills of crayfish, and a significant increase in the crayfish mortality. Silencing of *Pc*Reeler influenced the stability of the microbiota in the gills revealed by 16S rDNA high-throughput sequencing. Recombinant *Pc*Reeler showed the ability to bind microbial polysaccharide and bacteria and to inhibit the formation of bacterial biofilms. These results provided direct evidence for the involvement of *Pc*Reeler in the antibacterial immune mechanism of *P. clarkii*.

1. Introduction

Invertebrates rely mostly on innate immunity to defend themselves against microbial infection due to the lack of adaptive immunity [1]. In general, invertebrates innate immune system is made up of humoral immunity and cellular immunity [2]. Humoral immune responses include synthesis and release of certain immune proteins, such as antimicrobial peptides, whereas cellular immune reactions involve phagocytosis, encapsulation and nodulation [3]. These immune responses together generate the defense mechanism against bacterial, fungal, and viral infections in insects, crustaceans and other invertebrates [4,5].

Reeler domain was initially identified in the mouse extracellular matrix protein Reelin. Reelin is a large glycoprotein containing 3461 amino acid residues [6]. Reelin contains 8 Reeler domains that bind to lipoprotein receptors on neurons [7]. Studies in insects suggest that Reeler domain-containing molecules may play a role in immune responses. The first characterized protein with a Reeler domain in insects was a 19 kDa plasma protein from *Locusta migratoria* [8]. This protein is abundant in the hemolymph of adult locusts, and its synthesis is positively regulated by larval hormones, but the specific function of this protein has not been determined. A novel immune protein containing a Reeler domain, named Noduler due to its involvement in nodule formation [9], was identified from the Indian saturniid silkmoth *Antheraea mylitta*, and its homologs have been cloned and identified from other insects, such as *Hyphantria cunea* [10], *Manduca sexta* [11], *Samia cynthia ricini* [12], *Lonomia obliqua* [13] and *Bombyx mori* [14]. The homolog in silkworm genome, Reeler 1, is strongly induced in the hemolymph, fat body and midgut by bacteria. Knockdown of Reeler 1 led to decreased melanization in the hemolymph. When the silkworm was infected with pathogens, the number of nodules formed in Reeler 1-knockdown larvae decreased, and this effect could be rescued by recombinant Reeler 1. These results suggest that *Bombyx mori* Reeler 1 may be involved in nodule formation and is an essential component of the melanization cascade. Although Reeler domain containing proteins have been cloned and reported in insects, their function in crustaceans is largely unknown.

In the present study, we identified a protein containing a Reeler domain from *P. clarkii*, and designed it as *Pc*Reeler. The expression profiles of *Pc*Reeler upon bacterial challenge suggested its involvement in host antibacterial response. The role of *Pc*Reeler was determined by using RNAi and recombinant proteins. The findings that *Pc*Reeler maintains the microbiota in gills and inhibits biofilm formation

* Corresponding author at: School of Life Sciences, Shandong University, Qingdao 266237, China.
E-mail address: wangxianwei@sdu.edu.cn (X.-W. Wang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fsirep.2023.100094
Received 15 November 2022; Received in revised form 16 April 2023; Accepted 17 April 2023
Available online 18 April 2023

suggested that *Pc*Reeler is an important antibacterial effector in *P. clarkii*.

2. Materials and methods

2.1. Animals and microorganisms

Healthy red swamp crayfish (*P. clarkii*, ~10 g) were collected from an aquaculture farm in Huai'an, Jiangsu, China. The animals were cultured in aerated water at 25°C in the laboratory for at least 7 days before the experiments, and fed commercial diets daily. All animals were randomly selected for study. The animal-related experiments were approved by the Animal Ethical Committee of Shandong University School of Life Sciences (SYDWLL-2021–98).

Aeromonas hydrophila, Staphylococcus aureus, Escherichia coli, and *Micrococcus luteus* were cultured overnight at 37°C with rotation in Luria broth medium (1% NaCl, 1% tryptone, 0.5% yeast extract), collected, and resuspended in sterile PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 [pH 7.4]). The suspensions were serially diluted and plated onto agar plates to determine the bacterial counts. Other bacteria used in this study were isolated from the gills and hemolymph of healthy crayfish.

2.2. Immersion infection and sample collection

P. clarkii were maintained in water containing bacteria (10^6–10^7 colony forming units/ml, CFU/ml), and the crayfish gills were completely submerged in a bacterium-containing environment. For hemocytes collection, at specific time points after immersion infection (0 h, 6 h, 12 h, 24 h, 48 h), crayfish hemolymph was extracted using a 5 ml sterile syringe preloaded with pre-cooled anticoagulant (140 mM NaCl, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) at a ratio of 1:1. The hemocytes pellet was isolated by centrifugation at 800 × *g* for 10 min at 4°C. Other tissues such as the heart, hepatopancreas, gills, stomach and intestine were collected simultaneously. Each sample was from at least 4 crayfish. Total RNA was extracted using TRIzol (Invitrogen, USA), and used to synthesize the first strand cDNA using a ReverTra Ace® Kit (Acebio, China) according to the manufacturer's instructions. The protein samples were prepared by homogenizing the tissue in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 0.5 mM PMSF, 0.1% SDS, 0.5% Nonidet P-40, pH 7.5) and centrifuging the homogenate at 13,000 × *g* for 10 min at 4°C to collect the supernatant.

2.3. Expression profile analysis

Semiquantitative RT-PCR was performed to study the tissue distribution of *Pc*Reeler mRNA using specific primers (*Pc*ReelerRTF/R) listed in Table 1, following a conventional procedure: 94°C for 3 min; 30 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 45 s; and a final 72°C for 10 min, using the EasyTaq PCR SuperMix (TransGen Biotech, China). PCR products were analyzed using 1.5% agarose gel electrophoresis. β-actin mRNA was amplified using its primers (β-actinRTF/R) as the internal reference. Quantitative real-time RT-PCR (qRT-PCR) was performed to determine the expression profiles of *Pc*Reeler mRNA after infection. PCR was performed by using an iQ SYBR Green Supermix (Bio-Rad, USA) using the CFX96 Real-time System (Bio-Rad, USA). The cycling conditions were 95°C for 5 min; 40 cycles of 95°C for 10 s and 60°C for 45 s; and melting from 65 to 95°C. The results were calculated using the $2^{-\Delta\Delta Ct}$ method, and the expression level was normalized to that of β-actin.

2.4. Bioinformatics analysis

The sequence of *Pc*Reeler was obtained by analyzing a transcriptome sequencing dataset of the gill after bacterial infection. The signal peptide was predicted using the SignalP 5.0 program (https://services.healthtech.dtu.dk/service.php?SignalP-5.0). The domain architecture was predicted using SMART (http://smart.embl-heidelberg.de/). The Reeler proteins from other species were obtained from the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) by the blast tool for phylogenetic analysis. MEGA 6.0 was used to produce the phylogenetic tree. The multiple sequence alignment was performed with MEGA 6.0 and GENDOC software.

2.5. Expression of recombinant PcReeler and antiserum preparation

The sequence encoding the *Pc*Reeler mature peptide was amplified using the specific primers listed in Table 1 and ligated into the pET30a (+) plasmid. The recombinant vector was transformed into *Escherichia coli* Rosetta (DE3) strain for expression under induction with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h at 37°C. The proteins were expressed as soluble proteins, and were purified by affinity chromatography using Ni-NTA His-Binding Resin (Merck, Germany).

Table 1
Primers used in this study.

Primers	Sequence (5′–3′)
(q)RT-PCR	
*Pc*ReelerRTF	TTGTTGACGGTGATTGTGGT
*Pc*ReelerRTR	CTACCGACCCTTCAGGCA
β-actinRTF	AAACTTTCAACACTCCCGCTATG
β-actinRTR	CGAACGATTTCTCGCTCTGC
16S rDNARTF	ACTCCTACGGGAGGCAGCAGT
16S rDNARTR	TATTACCGCGGCTGCTGGC
RNAi	
*Pc*ReelerSi1F	GCGTAATACGACTCACTATAGGTCTCCGCAGCCAAAGTACCATTTAA
*Pc*ReelerSi1R	TTAAATGGTACTTTGGCTGCGGAGACCTATAGTGAGTCGTATTACGC
*Pc*ReelerSi2F	GCGTAATACGACTCACTATAGGCACACTAACTCAACCGCCAAGTTCA
*Pc*ReelerSi2R	TGAACTTGGCGGTTGAGTTAGTGTGCCTATAGTGAGTCGTATTACGC
CtrlSi1F	GATCACTAATACGACTCACTATAGGGGGAGTTGTCCCAATTCTTGTT
CtrlSi1R	AACAAGAATTGGGACAACTCCCCCTATAGTGAGTCGTATTAGTGATC
CtrlSi2F	AAGGAGTTGTCCCAATTCTTGCCCTATAGTGAGTCGTATTAGTGATC
CtrlSi2R	GATCACTAATACGACTCACTATAGGGCAAGAATTGGGACAACTCCTT
16S rDNA primer	
27F	AGTTTGATCATGGCTCAG
1492R	ACCTTGTTACGACTT
Recombinant expression	
*Pc*ReelerExF	TACCCCGGAGGCGCGCCATT
*Pc*ReelerExR	AAAATGCCTACACTCCTTAA

Purified proteins were dialyzed in PBS thoroughly and stored at −80°C before use. A tag (termed rTag) expressed by the empty vector was prepared and processed simultaneously. The purified protein (1 mg/ml) was thoroughly mixed with an equal volume (1.5 ml) of complete Fred's adjuvant (Sigma-Aldrich, USA) to immunize the New Zealand white rabbit. This process was repeated after 25 days with incomplete adjuvant rather than complete adjuvant. After the second immunization, the titer and specificity of the antiserum were tested, and then rabbit was sacrificed for bleeding to obtain the antiserum.

2.6. RNA interference

The oligonucleotides containing the T7 promoter and the small interfering RNA (siRNA) sequence (Table 1) were commercially synthesized and used as templates to synthesize siRNA, using the *in vitro* T7 Transcription Kit (Vazyme, China). The siRNA specific for GFP sequence was synthesized as a control. Specific siRNAs (30 μg) were injected into the crayfish hemocoel at the abdominal segment, and the control group was injected with an equal amount of control siRNA. The RNA interference (RNAi) efficiency was determined using qRT-PCR at 24 h, 48 h and 72 h after siRNA injection.

2.7. Quantitation of bacteria using qPCR for the 16S rDNA

The gills from crayfish were homogenized in PBS. The genomic DNA from the homogenate was extracted following the instruction of the Genomic DNA Extraction Kit (Toyobo, Japan). The bacterial abundance in crayfish gills were determined by qPCR with universal primers for bacterial 16S rDNA listed in Table 1. The β-actin was used as an internal reference for calibration. Data were analyzed using the $2^{-\Delta Ct}$ method, expressed as the ratio of 16 s rDNA to β-actin. Each sample was from 1 crayfish, and each group consisted of at least 10 samples.

2.8. Survival analysis

Thirty healthy crayfish were randomly selected to inject with an equal dose of *siPcReeler* or ctrl siRNA, and raised in water containing *A. hydrophila*. Crayfish death was recorded every day for a period of 6 days. In addition, to generate an overexpression-like effect, 30 μg of recombinant protein (rReeler) was injected into crayfish, and the control group was injected with the same dose of rTag.

2.9. Carbohydrate-binding assay

The binding activity of r*Pc*Reeler to LPS, PGN or zymosan was analyzed using enzyme linked immunosorbent assay (ELISA). Briefly, the 96-well plates were coated with LPS, PGN or zymosan, with 4 μg of carbohydrates per well. The plates were incubated at 37°C overnight, followed by incubation at 60°C for 30 min and then blocked with 5% BSA in TBS at 37°C for 2 h. Next, r*Pc*Reeler or the rTag was added into the wells with serial doses, and the mixture was maintained at room temperature for 3 h. After TBS washing, bound proteins were detected by adding mouse anti-His-tag Abs (1:1000 dilution; Zhongshan Golden Bridge Biotechnology, China) at 37°C for 2 h. After washing three times with TBS, alkaline phosphatase conjugated horse anti-mouse secondary Ab (1:10,000 dilution; Zhongshan Golden Bridge Biotechnology, China) added into the wells and incubated at 37°C for 1 h. Finally, 50 μl of p-nitrophenyl phosphate (1 mg/ml in 10 mM of diethanolamine with 0.5 mM of $MgCl_2$) was added to each well for visualization for 30 min at 25°C. The absorbance at 405 nm was detected using a BioTek plate reader (BioTek, USA). The results were presented as the mean ± SD derived from three independent repeats.

2.10. Bacterial binding assay

Bacteria (10^8 CFU/ml) were incubated with 20 μg of r*Pc*Reeler and 0.5 mM $CaCl_2$ for 2 h with gentle shaking at 25°C. After centrifugation at 12,000 × *g* for 10 min, the bacteria were collected and washed three times with TBS. Finally, the bacterial pellet was collected and analyzed by Western blot using anti-His-tag Abs.

2.11. 16S rDNA sequencing

Total microbial DNA was extracted from crayfish gills using a PowerFecal DNA Isolation Kit (Qiagen, USA) according to the manufacturer's instructions. The quality of DNA was verified by agarose gel electrophoresis. Commercial sequencing and analysis were performed by OE Biotech (Shanghai, China).

2.12. Isolation and identification of microbiota components

The gills were homogenized in sterile PBS. The hemolymph was collected with a sterile syringe. The homogenate or hemolymph were spread on agar plates and incubated overnight at 30°C. The single colony on the plate was picked, and inoculated to LB medium for overnight culture. The 16S rDNA of each bacteria was amplified by PCR using bacterial universal primers (27F and 1429R, listed in Table 1), and sequenced. The sequences were analyzed by BLAST to determine the species.

2.13. Biofilm detection

The bacteria (10^6 CFU/ml, 20 μl) were inoculated into sterile 96-well plates containing 160 μl of LB medium before adding 20 μg of r*Pc*Reeler or rTag. The mixture was maintained at 37°C for 24–48 h. The medium was discarded, and the 96-well plate was washed with sterile PBS to remove unabsorbed bacteria. Methanol (200 μl) was added to fix the biofilm for 15 min. After discarding the methanol, 200 μl of 0.1% crystal violet solution (Sangon Biotech, China) was added to the wells for staining for 30 min. Unbound crystal violet was removed by washing, and 150 μl of 30% glacial acetic acid was added to the well to fully dissolve the crystal violet for 30 min. The absorbance at 620 nm was then determined by using the BioTek microplate reader.

2.14. Confocal laser scanning microscopy

The bacteria (10^6 CFU/ml, 20 μl) were added into the 6-well plate containing 2 ml LB medium. r*Pc*Reeler or rTag were added to the final concentration was 10 mM, 5 mM, 2.5 mM. The mixture was incubated at 30°C for 24–48 h. The medium was discarded, then washed with PBS. Acridine orange (2 mg/ml, Sangon Biotech, China) was used to stain the attached bacteria for 15 min, followed by wash with distilled water. The morphology of the biofilm was observed using a spinning disk confocal microscope (SDCM) (Spin SR10, Olympus, Japan).

3. Results

3.1. Identification and expression profiles of PcReeler

The open reading frame (ORF) of *Pc*Reeler was 456 bp and encoded a polypeptide of 151 amino acids (Fig. 1A). The domain analysis showed that *Pc*Reeler contained a single Reeler domain and signal peptide (Fig. 1B). The molecular weight and isoelectric point of the mature *Pc*Reeler were 14.1 kDa and 6.10, respectively. Phylogenetic analysis of *Pc*Reeler and other Reeler proteins suggested that *Pc*Reeler was more closely related to its homologs from *Pollicipes pollicipes* and *Dermacentor silvarum* (Fig. 1C). Multiple sequence alignments showed that some residues were conserved among the Reeler family, suggesting the possible importance of them for Reeler proteins (Fig. 1D).

The expression profiles of *Pc*Reeler were studied to check whether it was involved in the innate immune response of crayfish. As shown in Fig. 2A, *Pc*Reeler transcripts were mostly localized in gills, an important

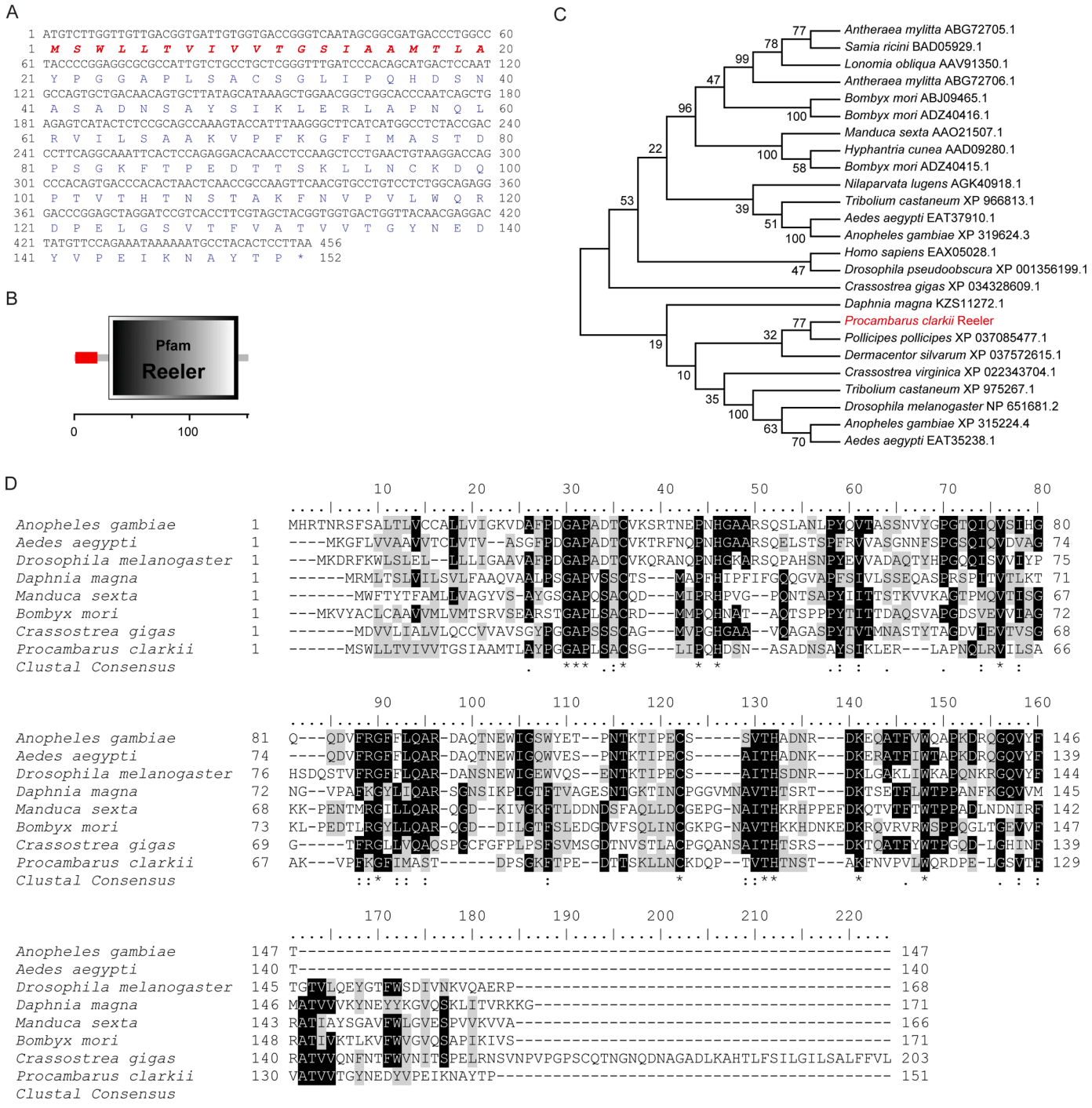

Fig. 1. Sequence analysis of *Pc*Reeler. (A) Sequence of the open reading frame of *Pc*Reeler. The number of nucleotides and amino acids were indicated at left. The signal peptide was indicated in red font. Mature peptides were shown in blue font. (B) The domain architecture of *Pc*Reeler. (C) Phylogenetic analysis of Reeler proteins. The neighbor-joining tree was built using MEGA 6.0 with 1000 bootstraps. (D) Multiple sequence alignment of representative Reeler proteins. The alignment was performed with MEGA 6.0 and GENDOC software. The accession numbers of these sequences were shown in (C) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

immune organ of crayfish. Lower expression of *Pc*Reeler was detected in hemocytes, heart, hepatopancreas, stomach, and intestine. To determine whether *Pc*Reeler expression was influenced by bacterial challenge, its expression after *S. aureus* and *A. hydrophila* stimulation in gills was detected. The results showed that the expression level of *Pc*Reeler mRNA was significantly induced after bacterial immersion infection, and the induction was sustainable for at least 48 h (Fig. 2B). We also examined the expression profiles of *Pc*Reeler protein. As shown in Fig. 2C, the expression of *Pc*Reeler protein was relatively high in the gills and stomach of crayfish. The *Pc*Reeler protein level in gills was also significantly induced after immersion infection with *A. hydrophila* (Fig. 2D). Based on the above data, we suppose that *Pc*Reeler may play an important role in the antibacterial immunity of crayfish.

3.2. *PcReeler maintains the gill microbiota homeostasis*

In order to study the function of *Pc*Reeler, RNAi was performed to knock down its expression. As shown in Fig. 3A, the expression of the

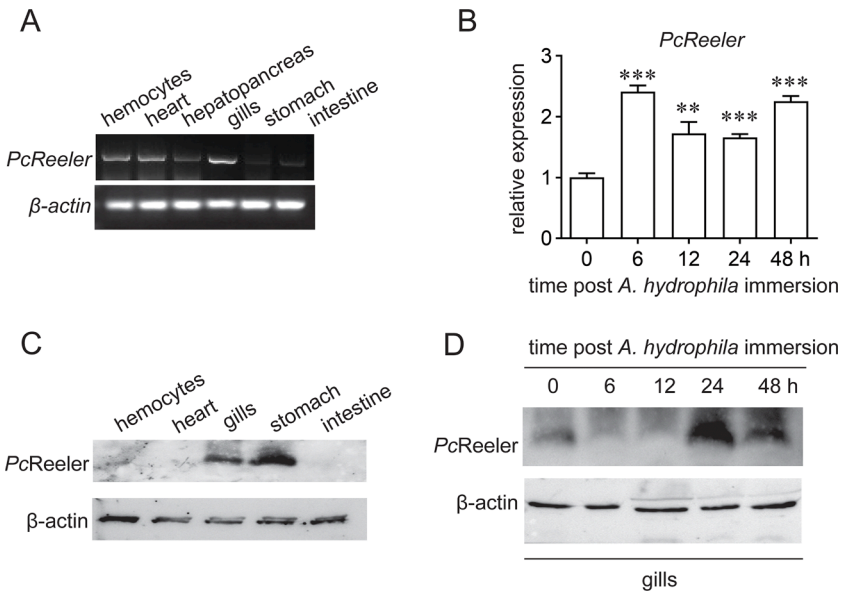

Fig. 2. The expression profiles of *Pc*Reeler. (A) Tissue distribution of *Pc*Reeler mRNA. qRT-PCR was performed with β-actin as the internal reference gene. Each sample was from at least four crayfish. The data were representative from two independent repeats. (B) The expression profiles of *Pc*Reeler mRNA after immersion infection with *A. hydrophila*. The *Pc*Reeler expression was normalized to that of β-actin. The relative expression was further calibrated to that at 0 h after infection. Each sample was collected from at least four crayfish. The results were shown as mean ± SD from three repeats. Student's t-test, ***, $p < 0.001$. **, $p < 0.01$. (C) Tissue distribution of *Pc*Reeler protein. The expression was examined by Western blotting. β-actin was used as the internal control. The experiment was repeated twice independently. (D) The expression profiles of *Pc*Reeler protein in gills after immersion infection by *A. hydrophila*. Data shown were the representative from three independent repeats.

*Pc*Reeler gene was significantly suppressed by *siPcReeler* injection, and this silencing effect lasted for at least 72 h. We also investigated the efficiency of RNAi at the protein level. The results showed that the *Pc*Reeler protein level was also inhibited after *siPcReeler* injection (Fig. 3B).

Because *Pc*Reeler is abundantly expressed in crayfish gills, we detected whether there was any change in bacterial abundance in gills after RNAi. The results showed that in the absence of exogenous bacterial infection, the abundance of total bacteria in the gills was significantly increased after *Pc*Reeler knockdown (Fig. 3C). We further determined the effects of *Pc*Reeler knockdown on gill microbiota composition by sequencing bacterial 16 s rDNA. As shown in Fig. 3D, silencing the expression of *Pc*Reeler significantly increased the α-diversity of the microbiota. At the phylum level, the proportion of *Bacteroidia* in gills decreased. However, the proportion of proteobacteria increased, and the abundance of other bacteria did not change significantly compared with the control group (Fig. 3E). At the genus level, the proportions of *Hydrogenophaga* and *Vibrio* increased, while the abundance of other bacteria decreased (Fig. 3F). This indicated that in the absence of exogenous bacterial stimulation, inhibition the expression of the *Pc*Reeler disrupted the homeostasis of the bacterial community structure in crayfish gills.

3.3. *PcReeler plays a protective role against external infection*

In order to reveal whether knockdown of *Pc*Reeler affects host defense against exogenous bacteria, we explored the changes in the gills bacteria load after *Pc*Reeler knockdown and immersion infection. As shown in Fig. 4A, the abundance of total bacteria in the crayfish gills significantly increased when external *A. hydrophila* was introduced into the living environment after *Pc*Reeler knockdown. Correspondingly, we counted the death of crayfish from the first day after *siPcReeler* injection. The results showed that, compared with the control group, the mortality of crayfish was significantly increased after *siPcReeler* injection (Fig. 4B). The survival rate of crayfish in the *siPcReeler*-injected group was only about 10% at the 6th day of infection, while in the control group, the survival rate exceeded 50%. We further carried out morphological observation on crayfish gills, which showed that, compared with the control group, the color of the gills becoming yellow or black when the RNAi-crayfish were treated by immersion infection (Fig. 4C). These data collectively suggested that *Pc*Reeler played a key role to resist external infection in crayfish.

To study the change of microbiota in gills after infection, the high-throughput sequencing of 16 s rDNA was performed. As shown in Fig. 4D, the phenotype prediction result showed that, after *Pc*Reeler knockdown, the proportion of bacteria with potential pathogenicity increased significantly. The microbiota composition was also analyzed. At the phylum level, the proportion of *Bacteroidetes* and *Proteobacteria* significantly increased, compared with the control group, while the proportion of most other bacteria decreased (Fig. 4E). Meanwhile, at the genus level, the proportions of *Chryseobacterium, Acinetobacter, Escherichia-Shigella* and *Delftia* were all significantly up-regulated. The variation of microbiota composition might be related to the disruption of commensal microbiota homeostasis, or due to the increased colonization of external bacteria. The above results further suggested that inhibition of *Pc*Reeler expression resulted in altered microbiota structure in gills and disruption of homeostasis.

3.4. *PcReeler interacts with bacteria and polysaccharides*

In order to explore how *Pc*Reeler functions in the antibacterial immunity of crayfish, we expressed and purified the recombinant protein (r*Pc*Reeler) (Fig. 5A). We tested whether r*Pc*Reeler can bind microbial polysaccharides. The results showed that r*Pc*Reeler could interact with LPS, PGN and zymosan in a dose-dependent manner (Fig. 5B). Since LPS and PGN are the main components of the cell walls of Gram-negative and Gram-positive bacteria, we tested the binding ability of r*Pc*Reeler to bacteria. As shown in Fig. 5C, r*Pc*Reeler can bind to Gram-negative bacteria, including *A. hydrophila* and *E. coli*, and to Gram-positive bacteria, including *S. aureus* and *M. luteus*. From the above results, we suppose that r*Pc*Reeler may interact with bacteria by binding polysaccharide to exert its antibacterial function. In order to demonstrate the effect of exogenous recombinant protein on the antibacterial immunity of crayfish, we injected r*Pc*Reeler into crayfish for overexpression. As shown in Fig. 5D, after bacterial infection, the survival rate of the overexpression-crayfish was continuously higher than that of the control group. These results suggested that r*Pc*Reeler could significantly reduce the crayfish mortality, and further confirmed the protective role of *Pc*Reeler in crayfish antibacterial immunity.

3.5. *PcReeler inhibits the formation of bacterial biofilms*

To further reveal the antibacterial mechanism of *Pc*Reeler, we isolated a variety of commensal bacteria from the gills and hemolymph of

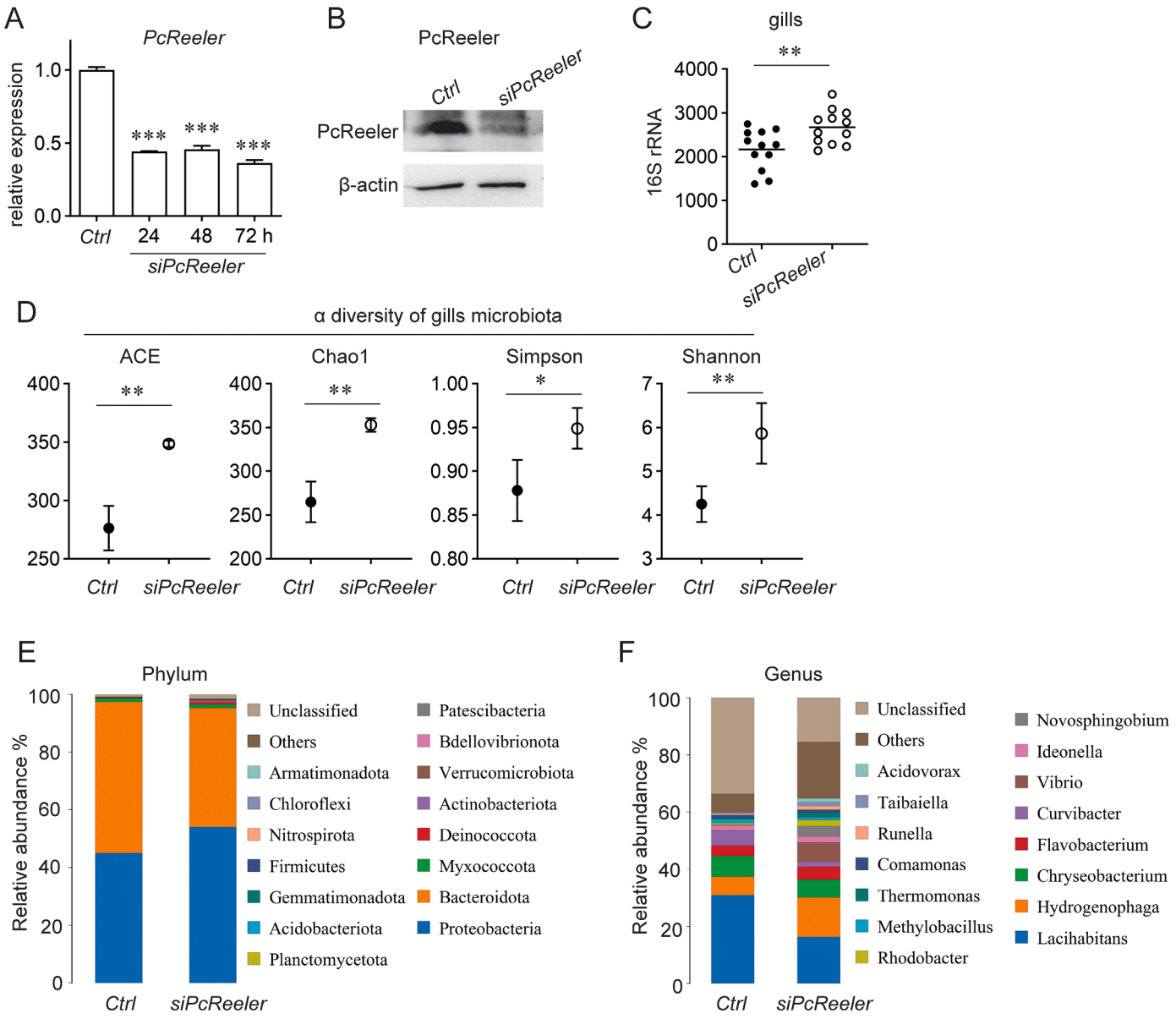

Fig. 3. Role of *Pc*Reeler to maintain the homeostasis of crayfish gill microbiota. (A) The RNAi efficiency of *Pc*Reeler. *siPcReeler* was injected into crayfish hemocoel. Crayfish were injected with the same amount of siGFP as control. The RNAi efficiency in gills was detected. The results were shown as mean ± SD. Student's t-test, ***, $p < 0.001$. Each sample was from at least four crayfish. (B) RNAi efficiency of *Pc*Reeler in protein level. The data were representative of three independent repeats. Each sample was collected from at least four crayfish. (C) The change of total bacterial abundance in gills after *Pc*Reeler silencing. Crayfish were injected with siRNA and raised in the absence of exogenous bacteria. The total DNA of the gills was extracted after 4 d. qPCR was used to analyze the total amount of bacteria in the gills. **, $p < 0.05$. (D) Alpha diversity in gill microbiota after *Pc*Reeler knockdown. The commercial 16S rDNA sequencing was performed, and Mothur software was used to calculate the α diversity. (E-F) Diagram of the relative bacterial abundance at the phylum (E) and genus (F) level after *Pc*Reeler knockdown.

healthy crayfish, including *Citrobacter freundii, Bacillus cereus, V. mimicvs, Acinetobacter haemolyticus, Comamonas aquatica, Acinetobacter tandoii, B. wiedmanii, A. veronii, Shewanella seohaensis, B. paramycoides* and *Acinetobacter johnsonii*. Because biofilm is a biological phenomenon in which bacteria adapt to the natural environment and is conducive to bacterial survival, we guessed whether the antibacterial immune function of *Pc*Reeler was related to its effect on bacterial biofilm formation. As shown in Fig. 6A, the biofilm formation of both *A. hydrophila* and *A. johnsonii* was significantly inhibited by the presence of r*Pc*Reeler. We then examined the effect of r*Pc*Reeler on the biofilm formation of other commensal bacteria. As shown in Fig. 6B, r*Pc*Reeler showed inhibitory effects on the growth of biofilms of most commensal bacteria, though the inhibitory effect on different bacteria varied. In order to verify this result obtained by using crystal violet staining, we observed the growth of biofilms of *A. johnsonii, B. wiedmanii, A. hydrophila, A. tandoii* and *B. paramycoides* under a confocal microscope. The results showed that the growth of bacterial biofilms was inhibited in the presence of different concentrations of r*Pc*Reeler (Fig. 8C). However, for different bacteria, the inhibitory effect of r*Pc*Reeler was not completely dose-dependent. Collectively, these data suggested that r*Pc*Reeler may exert an antibacterial effect by inhibiting the growth of bacterial biofilm.

4. Discussion

Gill is an important tissue for aquatic animals to interact with external environment. For example, fish gills, the mucus-secreting organ, are lined by epithelial cells with mucus-producing cells, and are important sites of innate immune defense [15]. Both *Atlantic salmon*

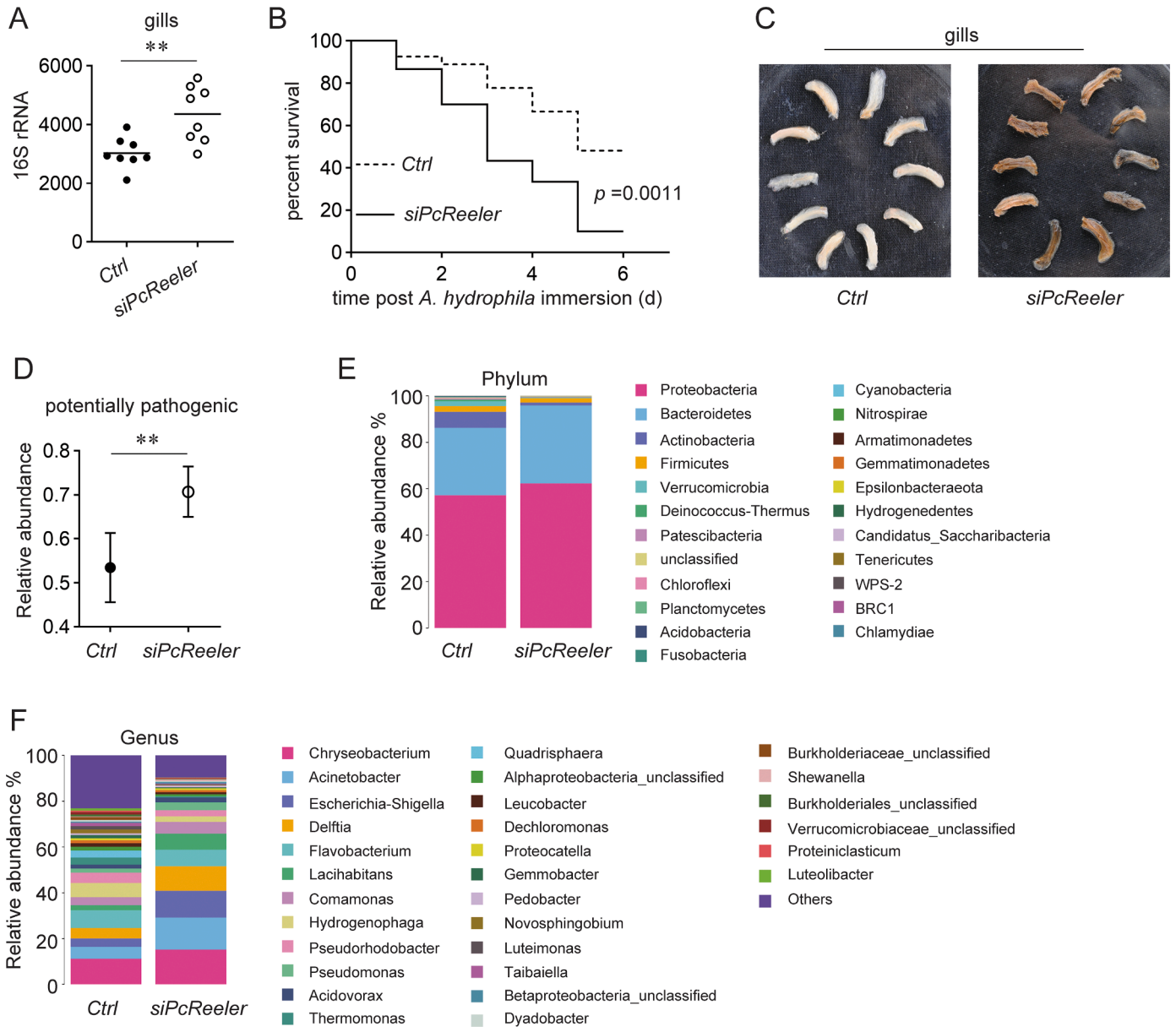

Fig. 4. Function of *Pc*Reeler to resist external infection. (A) The change in total bacterial abundance after RNAi and infection. Crayfish was injected with siRNA, and maintained in water containing *A. hydrophila*. The genomic DNA of the gill was collected after 5 d. The total abundance of bacteria were determined by detecting 16S rDNA using qPCR. **, $p < 0.05$. (B) Survival assay after RNAi and infection. Crayfish as treated as described above, and the death was recorded every day. The data was analyzed using Mantel-Cox in GraphPad-Prism. Each group consisted of 30 crayfish. (C) Morphological changes of gill filaments of crayfish after RNAi and infection. The crayfish were injected with siRNA and maintained in water containing *A. hydrophila*. The morphology of gill filaments was observed and photographed after 6 d. Each gill filament was from one crayfish. (D) Phenotype prediction of bacteria after RNAi. The phenotype was predicted through BugBase by using the 16 s rDNA sequencing data. (E-F) Diagram of the relative bacterial abundance at the phylum (E) and genus (F) level after *Pc*Reeler knockdown and immersion infection (For interpretation of the references to color in this figure, the reader is referred to the web version of this article).

and *Salmo trutta* have a systemic mucus response to amoebic gill disease [16,17], whereas *Oncorhynchus mykiss* showed only a localized response in gills [18,19]. Therefore, there is complex immune defense mechanisms in the gills of aquatic animals to help the body resist the invasion of pathogens. In this study, we found that the *Pc*Reeler was highly expressed in the gills of crayfish. Its expression was induced by bacteria immersion, and it played a role to maintain the microbiota homeostasis of gills. These findings would be helpful to develop strategies for disease prevention and control in aquaculture.

A. hydrophila is widely present in nature, especially in water, and has caused serious disease of aquatic animals. In this study, crayfish were stimulated by immersion infection by which the gills of crayfish were fully contacted with *A. hydrophila* in the water. Compared with intramuscular injection, this mode of infection can be closer to the state of bacterial stimulation in nature. After inhibiting the expression of *Pc*Reeler, the gills of the infected crayfish turned yellow and black, which intuitively proved that pathogenic microorganisms can invade the body through the gills in the water. Usually, in the absence of pathogens, the number of commensal microorganisms in the gills is tightly regulated to achieve a dynamic equilibrium state. The interaction between the host and its microbiota is critical for maintaining homeostasis [20]. In this study, inhibiting the expression of *Pc*Reeler increased the total abundance of bacteria, disrupted the homeostasis of the gill microbiota, and in particular, led to a significant increase in the abundance of Proteobacteria. Proteobacteria is currently the largest phylum in the bacteria, including many pathogenic bacteria such as *E. coli, V.*

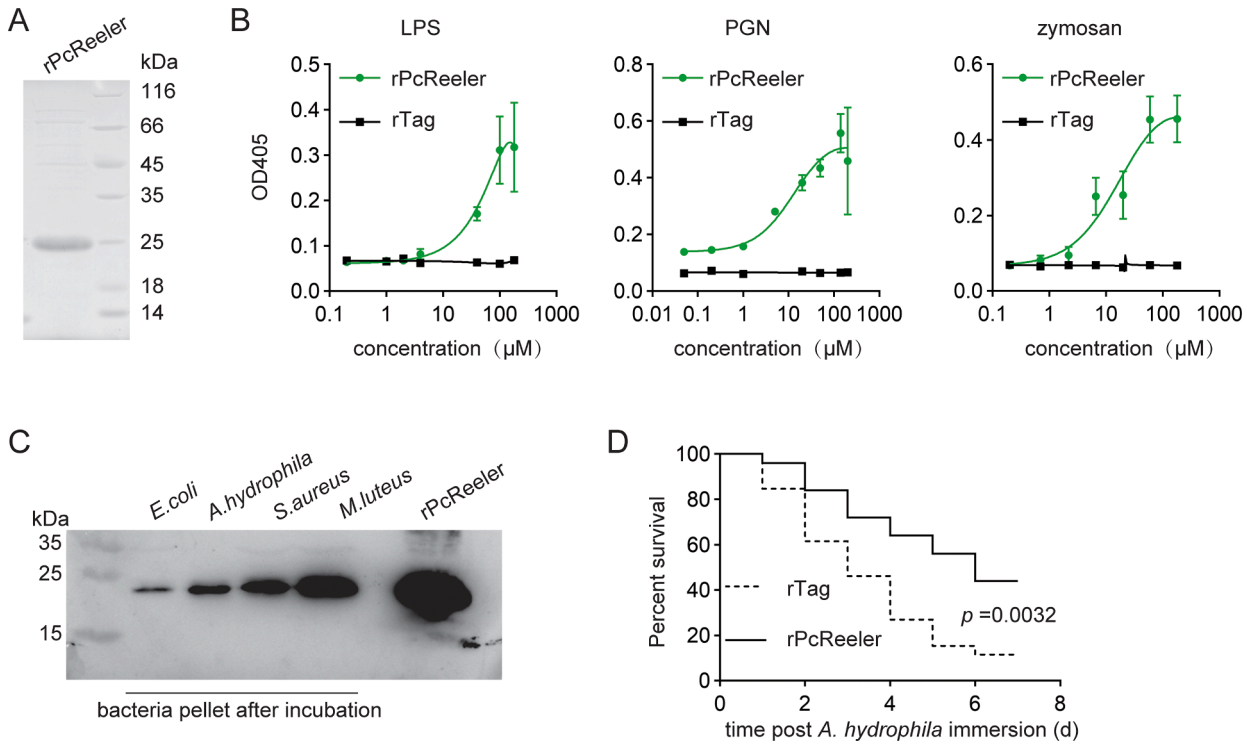

Fig. 5. The function of r*Pc*Reeler to interact with bacteria and polysaccharides. (A) The recombinant expression and purification of Reeler. The recombinant protein was expressed by pET-30a (+) vector in Rosseta (DE3) cells, and purified by using affinity chromatography. (B) The ability of r*Pc*Reeler to bind polysaccharides. The carbohydrates were added into 96-well plates. The protein solution of gradient concertation was added. The binding was analyzed by ELISA. Three independent replicates were performed. (C) Binding of r*Pc*Reeler to bacteria. The bacteria were incubated with r*Pc*Reeler. Binding was evaluated by analyzing the bacteria pellet using western blotting. (D) Survival assay after r*Pc*Reeler overexpression and infection. The crayfish were injected with proteins, and infected by *A. hydrophila* immersion. Crayfish death was recorded every day for about a week. Each group consisted of 30 crayfish. The experimental data was analyzed by Mantel-Cox detection in GraphPad Prism software.

cholerae and *Salmonella*. LPS is present in the outer membranes of these bacteria [21]. In studies of human intestinal disease, a link has been established between LPS-induced persistent inflammatory responses and the development of metabolic disorders [22,23]. Decreased expression of *Pc*Reeler also increased the relative abundance of *Vibrio* in the Proteobacteria phylum. *Vibrio* species are widespread in aquatic ecosystems, some of which can form pathogenic or symbiotic interactions with eukaryotic hosts [24]. The successful colonization of *Vibrio* species may result in the occurrence of host inflammatory responses and disruption of microbiota homeostasis, which are often associated with human and aquatic animal diseases [25,26]. Therefore, based on these results, we speculate that the inhibition of *Pc*Reeler expression leads to increase in the relative abundance of certain potentially pathogenic bacteria in crayfish gills and destroys the structure of the original microbiota. This consequence suppresses the host immunity and increases the risk of disease. When *Pc*Reeler expression is affected, the pathogenic microorganisms might easily enter the body through the gills and enter the circulatory system. This may lead to accelerated spread and replication of bacteria throughout the host.

The ability to form biofilms is one key feature for bacteria to survive and spread in changing environments. Biofilm is a beneficial manner of life for microorganisms, promoting colony growth and survival by providing nutrients and protection from antimicrobial agent [27]. Biofilms begin to form when bacteria enter the organism and attach to surfaces. Flagella-mediated movement often facilitates the initial stages of biofilm formation by enhancing the movement towards and along the epithelial surface [28]. Since bacterial adhesion to tissues is part of the infection process, the formation of bacterial biofilms can have an important impact on host infectious diseases [29]. This study demonstrates that *Pc*Reeler exerts antibacterial immunity by affecting the formation of bacterial biofilms in gills. *Pc*Reeler inhibits the aggregation and growth of pathogenic microorganisms, reducing their ability to infect host. Failure of biofilm formation of pathogenic microorganisms would reduce the ability of bacteria to adhere to tissues, limit the damage of invading bacteria to gills epithelium, and finally greatly reduce the risk of disease in crayfish.

The physical and chemical properties of the biofilm matrix components (protein, polysaccharide matrix, etc.) determine the formation of biofilm. In this study, we proved that *Pc*Reeler can bind to a variety of polysaccharide molecules, and speculated that *Pc*Reeler may inhibit the formation of bacterial biofilms by interacting with bacterial polysaccharides. One of the most distinctive features of biofilms is the presence of highly tolerant and persistent cells in their communities, and therefore, high resistance to drugs such as antibiotics [30]. Therefore, our discovery that the ability of *Pc*Reeler to inhibit biofilm formation also provides a new option for the development of aquatic fishery drugs and even human antibacterial drugs.

In summary, a Reeler domain containing protein from the gills of *P. clarkii* was identified. *Pc*Reeler maintains the homeostasis of microbiota in gills and reduces the risk of disease. Moreover, *Pc*Reeler plays an important antibacterial role by inhibiting the formation of bacterial biofilm and possibly by suppressing the adhesion and colonization of bacteria. This study provides new insights into the antibacterial immune mechanism of the gills in crayfish.

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.

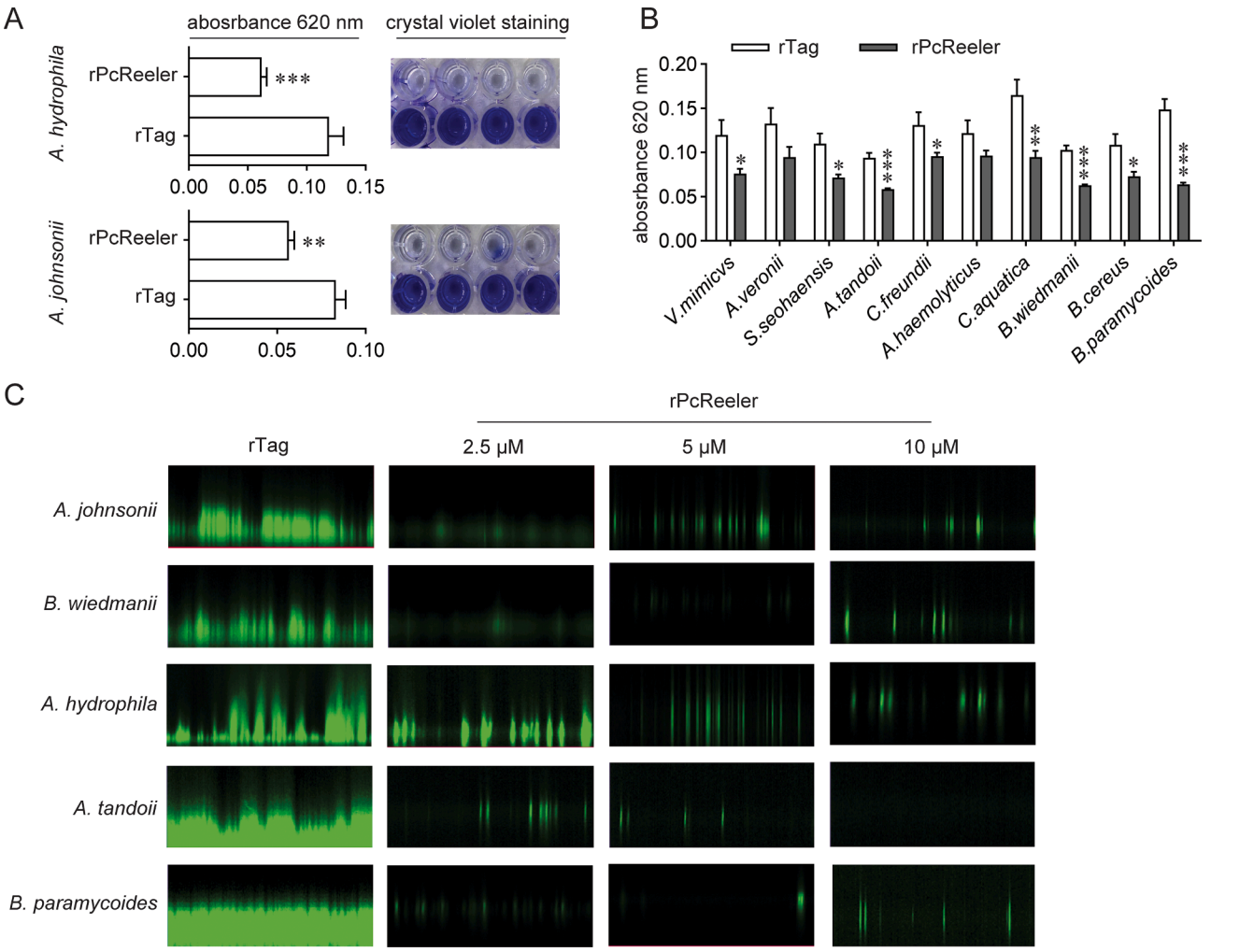

Fig. 6. The ability of r*Pc*Reeler to inhibit the formation of bacterial biofilm. (A) Inhibition of biofilm formation of *A. hydrophila* and *A. johnsonii*. The bacterial was mixed with tested proteins in 96-well culture plate to allow biofilm formation. Biofilms were stained with crystal violet, and the absorbance at 620 nm was measured by microplate reader. The experiment was repeated three times. (B) The influence of r*Pc*Reeler on the biofilm formation of other commensal bacteria. Student's t-test, ***, $p < 0.001$. **, $p < 0.01$. *, $p < 0.05$. (C) Inhibition of biofilm formation observed by confocal microscope. The biofilm was grown in the petri dish for 24–48 h, stained with acridine orange fluorescent dye, and observed using a spinning disk confocal microscope.

Data availability

Data will be made available on request.

Acknowledgments

This research was supported by the Open Fund of CAS Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences (No. KF2021NO03), the National Natural Science Foundation of China (No. 32173008).

References

[1] A. Kulkarni, S. Krishnan, D. Anand, S. Kokkattunivarthil Uthaman, S.K. Otta, I. Karunasagar, R. Kooloth Valappil, Immune responses and immunoprotection in crustaceans with special reference to shrimp, Rev. Aquac. 13 (1) (2021) 431–459.
[2] Y.H. Chen, J.G. He, Effects of environmental stress on shrimp innate immunity and white spot syndrome virus infection, Fish Shellfish Immunol. 84 (2019) 744–755.
[3] A. Tassanakajon, K. Somboonwiwat, P. Supungul, S. Tang, Discovery of immune molecules and their crucial functions in shrimp immunity, Fish Shellfish Immunol. 34 (4) (2013) 954–967.
[4] J.S. Miller, T. Nguyen, D.W. Stanley-Samuelson, Eicosanoids mediate insect nodulation responses to bacterial infections, Proc. Natl. Acad. Sci. 91 (26) (1994) 12418–12422.
[5] N. Madsari, S. Maskaew, S. Obchoei, P. Kwankaew, W. Senghoi, P. Utarabhand, P. Runsaeng, Determination of the efficacy of using a serine protease gene as a DNA vaccine to protect against Vibrio parahaemolyticus infection in Litopenaeus vannamei, Dev. Comp. Immunol. (2022), 104459.
[6] G. D'Arcangelo, G.G. Miao, S.-C. Chen, H.D. Scares, J.I. Morgan, T. Curran, A protein related to extracellular matrix proteins deleted in the mouse mutant reeler, Nature 374 (6524) (1995) 719–723.
[7] D.S. Rice, T. Curran, Role of the reelin signaling pathway in central nervous system development, Annu. Rev. Neurosci. 24 (1) (2001) 1005–1039.
[8] M. Kanost, J. Bradfield, K. Cook, J. Locke, M. Wells, G. Wyatt, Gene structure, cDNA sequence, and developmental regulation of a low molecular weight hemolymph protein from Locusta migratoria, Arch. Insect Biochem. Physiol. 8 (4) (1988) 203–217.
[9] A.S. Gandhe, S.H. John, J. Nagaraju, Noduler, a novel immune up-regulated protein mediates nodulation response in insects, J. Immunol. 179 (10) (2007) 6943–6951.
[10] S.W. Shin, S.S. Park, D.S. Park, M.G. Kim, S.C. Kim, P.T. Brey, H.-Y. Park, Isolation and characterization of immune-related genes from the fall webworm, Hyphantria cunea, using PCR-based differential display and subtractive cloning, Insect Biochem. Mol. Biol. 28 (11) (1998) 827–837.
[11] Y. Zhu, T. Johnson, A. Myers, M. Kanost, Identification by subtractive suppression hybridization of bacteria-induced genes expressed in Manduca sexta fat body, Insect Biochem. Mol. Biol. 33 (5) (2003) 541–559.
[12] Y. Bao, K. Mega, Y. Yamano, I. Morishima, cDNA cloning and expression of bacteria-induced Hdd11 gene from eri-silkworm, Samia cynthia ricini, Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 136 (4) (2003) 337–342.
[13] A.B. Veiga, J.M. Ribeiro, J.A. Guimarães, I.M. Francischetti, A catalog for the transcripts from the venomous structures of the caterpillar Lonomia obliqua: identification of the proteins potentially involved in the coagulation disorder and hemorrhagic syndrome, Gene 355 (2005) 11–27.

[14] Y.Y. Bao, J. Xue, W.J. Wu, Y. Wang, Z.Y. Lv, C.X. Zhang, An immune-induced reeler protein is involved in the Bombyx mori melanization cascade, Insect Biochem. Mol. Biol. 41 (9) (2011) 696–706.
[15] D. Gomez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens, Fish Shellfish Immunol. 35 (6) (2013) 1729–1739.
[16] H. Rodger, J. McArdle, An outbreak of amoebic gill disease in Ireland, Vet. Rec. 139 (1996) 348. -348.
[17] B.F. Nowak, J. Carson, M.D. Powell, I. Dyková, Amoebic gill disease in the marine environment, Bulletin 22 (2) (2002) 144–147. European Association of Fish Pathologists.
[18] B. Munday, C. Foster, F. Roubal, R. Lester, Paramoebic gill infection and associated pathology of Atlantic salmon, Salmo salar, and rainbow trout, Salmo gairdneri, in Tasmania, Pathology in marine science (1990) 215–222.
[19] S.D. Roberts, M.D. Powell, The viscosity and glycoprotein biochemistry of salmonid mucus varies with species, salinity and the presence of amoebic gill disease, J. Comp. Physiol. B 175 (1) (2005) 1–11.
[20] C.A. Thaiss, N. Zmora, M. Levy, E. Elinav, The microbiome and innate immunity, Nature 535 (7610) (2016) 65–74.
[21] G. Rizzatti, L. Lopetuso, G. Gibiino, C. Binda, A. Gasbarrini, Proteobacteria: a common factor in human diseases, Biomed. Res. Int. (2017) 2017.
[22] P.D. Cani, J. Amar, M.A. Iglesias, M. Poggi, C. Knauf, D. Bastelica, A.M. Neyrinck, F. Fava, K.M. Tuohy, C. Chabo, Metabolic endotoxemia initiates obesity and insulin resistance, Diabetes 56 (7) (2007) 1761–1772.
[23] P.D. Cani, R. Bibiloni, C. Knauf, A. Waget, A.M. Neyrinck, N.M. Delzenne, R. Burcelin, Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet–induced obesity and diabetes in mice, Diabetes 57 (6) (2008) 1470–1481.
[24] F.H. Yildiz, K.L. Visick, Vibrio biofilms: so much the same yet so different, Trends Microbiol. 17 (3) (2009) 109–118.
[25] C. Pruzzo, A. Huq, R.R. Colwell, G. Donelli, Pathogenic Vibrio species in the marine and estuarine environment. Oceans and health: pathogens in the marine environment, Springer, 2005, pp. 217–252.
[26] E. Rubio-Portillo, J.F. Gago, M. Martínez-García, L. Vezzulli, R. Rosselló-Móra, J. Antón, A.A. Ramos-Esplá, Vibrio communities in scleractinian corals differ according to health status and geographic location in the Mediterranean Sea, Syst. Appl. Microbiol. 41 (2) (2018) 131–138.
[27] R.M. Donlan, J.W. Costerton, Biofilms: survival mechanisms of clinically relevant microorganisms, Clin. Microbiol. Rev. 15 (2) (2002) 167–193.
[28] G. O'Toole, H.B. Kaplan, R. Kolter, Biofilm formation as microbial development, Annu. Rev. Microbiol. 54 (2000) 49.
[29] J.L. Del Pozo, Biofilm-related disease, Expert Rev. Anti Infect. Ther. 16 (1) (2018) 51–65.
[30] J. Yan, B.L. Bassler, Surviving as a community: antibiotic tolerance and persistence in bacterial biofilms, Cell Host Microbe 26 (1) (2019) 15–21.