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An LRR-domain containing protein identified in *Bathymodiolus platifrons* serves as intracellular recognition receptor for the endosymbiotic methane-oxidation bacteria

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ABSTRACT

As domain species in seep and vent ecosystem, Bathymodioline mussels has been regarded as a model organism in investigating deep sea chemosymbiosis. However, mechanisms underlying their symbiosis with chemosynthetic bacteria, especially how the host recognizes symbionts, have remained largely unsolved. In the present study, a modified pull-down assay was conducted using enriched symbiotic methane-oxidation bacteria as bait and gill proteins of *Bathymodiolus platifrons* as a target to isolate pattern recognition receptors involved in the immune recognition of symbionts. As a result, a total of 47 proteins including BpLRR-1 were identified from the pull-down assay. It was found that complete cDNA sequence of BpLRR-1 contained an open reading frame of 1479 bp and could encode a protein of 492 amino acid residues with no signal peptide or transmembrane region but eight LRR motif and two EFh motif. The binding patterns of BpLRR-1 against microbial associated molecular patterns were subsequently investigated by surface plasmon resonance analysis and LPS pull-down assay. Consequently, BpLRR-1 was found with high binding affinity with LPS and suggested as a key molecule in recognizing symbionts. Besides, transcripts of BpLRR-1 were found decreased significantly during symbiont depletion assay yet increased rigorously during symbionts or nonsymbiotic *Vibrio alginolyticus* challenge, further demonstrating its participation in the chemosynthetic symbiosis. Collectively, these results suggest that BpLRR-1 could serve as an intracellular recognition receptor for the endosymbionts, providing new hints for understanding the immune recognition in symbiosis of *B. platifrons*.

1. Introduction

Since firstly discovered in 1977, the ecosystem in deep-sea hydrothermal vents and cold seeps has attracted massive interests worldwide. Beyond our expectation, diverse lives such as bivalves, gastropods, shrimps, crabs and tubeworms could thrive in hydrothermal vents and cold seeps in lack of sunshine and sufficient food supply [1]. Continuous efforts are being made ever since hoping to reveal the secret of how megafauna survive there [2]. Unlike their offshore relatives, it turned out that the majority of deep sea invertebrates, including bivalves, shrimps and tubeworms, could obtain nutrition from their chemosynthetic symbionts. The symbiosis between megafauna and chemosynthetic bacteria is therefore suggested as the most important feature of hydrothermal vents and cold seeps ecosystem while the mechanism beneath it remained largely undisclosed [3,4].

As one of the dominant species inhabiting in both cold seeps and hydrothermal vents, Bathymodioline mussels (family: Mytilidae) have been regarded as model species in investigating the chemosynthetic symbiosis [5,6]. It was found that Bathymodioline mussels have evolved with specialized gill epidermal cell (bacteriocytes) bearing numerous chemosynthetic symbionts obtained horizontally from the environment [7,8]. Nevertheless, only two types of endosymbiotic bacteria (sulfur oxidation bacteria SOB and methane oxidation bacteria MOB) have been identified inside bacteriocytes of Bathymodioline mussel to date and both were found belonging to γ-proteobacteria [9,10]. Meanwhile, only few Bathymodiolinae species were in dual

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symbiosis harboring both types of bacteria while the others live merely with thiotrophic or methanotrophic bacteria [6]. It has also been demonstrated that Bathymodiolinae could obtain nutrition from either molecular leakage of bacteria (also known as “milking”) or degradation of bacteria in the lysosome (also known as “farming”) [11,12]. However, the cellular and molecular processes of host cell interacting with symbionts, especially the immune recognition between them remained largely unknown.

As suggested, the establishment of symbiosis in either invertebrates or vertebrates relies greatly on the innate immunity of the host while pattern recognition receptors (PRRs) plays irreplaceable roles in activating host innate system by recognizing symbionts’ microbial associated molecular patterns (MAMPs) [13]. Massive efforts have therefore been made attempting to reveal the participation of PRRs in innate immunity of Bathymodioline mussels, especially their role in recognizing symbiotic bacteria. For example, PRRs including Aggrecan, Carcinolectin (Cal), Galectin (Gal), Immune lectin receptor, Rhamnose binding lectin, Scavenger receptor cysteine-rich, Sialic acid-binding lectin (Sabl), c-type lectin, Toll-like receptor (TLR) 2 and peptidoglycan recognition protein (PGRPs) have been found vigorously modulated after *Vibrio* challenge while transcripts of Gal, Cal, Sabl, TLR2 and PGRPs were found decreased during long-term acclimatization or decolonization of symbionts [14–16]. However, due to the lack of cultivable symbionts, neither the identification nor expression profile of PRRs which could recognize or bind with symbionts directly has been well investigated.

As a dominant species in both cold seeps and hydrothermal vents of west Pacific, *Bathymodiolus platifrons* has been regarded as a model organism in investigating deep sea chemosymbiosis [5]. Results demonstrated that *B. platifrons* only harbored methanotrophic bacteria in their bacteriocytes and could obtained nutrition such as sterol from these symbionts [10]. Genome and transcriptome information suggested that PRRs including immunoglobin domain containing proteins, PGRPs, TLRs and C1qDC proteins were extensively expanded or positively selected in *B. platifrons* and might contribute crucially to the recognition of symbionts [5,17]. These findings have advanced our understanding about how Bathymodioline mussels interact with symbionts and provided us opportunity to further identify PRRs involved in the above processes. Therefore, the purpose of the present study is to [1] isolate and identify *B. platifrons* PRRs that could bind with symbiotic MOB directly using a modified pull-down assay [2], investigate the potential binding pattern of candidate PRRs (leucine-rich repeat protein BpLRR-1) against MAMPs, and [3] survey the expression pattern during decolonization and challenge of symbionts, hopefully providing new hints in decoding the immune recognition of symbiosis.

2. Material and methods

2.1. Animals collection, maintenance and MOB preparation

About two hundred of deep sea mussel *B. platifrons* were collected from the cold seep in the Formosa ridge of South China Sea (22°06′N, 119°17′E) during cruise 2017 dive 149 using remotely operated vehicle ROV Faxian operated from the R/V Kexue at depth of 1,100 m. Upon arrival at the main deck of the R/V, gill tissue from fifty mussels were dissected and frozen immediately for MOB pull-down assay or RNA extraction. The remaining individuals were transferred into an aquarium with circulating water system and acclimated at atmospheric pressure with filtered seawater (4 °C) and CH_4 supplement. Concentration of CH_4 was quantified with CH_4 sensor (Contros) and adjusted to the similar level *in situ* in cold seep. The seawater in the aquarium was half replaced every 24 h while mussels were employed for symbiont depletion assay and bacterial challenge at 48 h post acclimation.

MOB preparation was performed according to previous method with slight modification [10]. Briefly, gill tissue obtained during dive 149 were firstly homogenized on ice and filtered through sterilized gauze and nylon filters (pore size of 10 μm and 5 μm) subsequently. Enriched MOBs were then collected by centrifugation at 4000 g for 15 min at 4 °C and diluted using sterilized seawater. Part of the freshly obtained MOBs was subjected to genome DNA extraction using Mollusc DNA kit (Omega) while qRT-PCR of pmoA gene were further conducted using SYBR Green Premix Ex Taq II (Takara). Standard curve of MOB copy numbers was generated simultaneously using fragments of pmoA gene (diluted to 10^2-10^9 copy/μL) by qRT-PCR and employed to calculated copy numbers of enriched MOBs.

2.2. BpLRR-1 isolation and characterization

A modified pull-down assay using NHS Mag Sepharose (GE Healthcare) was conducted to isolate *B. platifrons* proteins potentially binding with symbiotic MOBs. In detail, magnetic beads were firstly equilibrated by equilibration buffer and incubated with lipid A monoclonal antibody (Santa Cruze, sc-57902) and LPS polyclonal antibody (Abcam, ab211144 and ab54089) overnight at 4 °C. The residual active groups of magnetic beads were then blocked and equilibrated according to the manual. A total of 10^7 copies of previously enriched MOBs were added and incubated with the beads for 60 min. Subsequently, a total of 3 mg gill proteins (1 mg/ml in PBS with PMSF) were supplemented into the mix and incubated overnight. The reaction mixture was then washed intensively by TBS-urea buffer for three times before eluted by 2 M urea and 0.1 M glycine-HCl. Three parallel trials were conducted while host proteins binding with MOB were finally separated by SDS-PAGE and further characterized by LC-MS/MS. All protein candidates identified were annotated according to the draft genome of *B. platifrons* originally reported by Sun et al. [5] and updated by Wang et al. (unpublished data).

2.3. RNA extraction, cDNA synthesis and SYBR green fluorescent quantitative real-time PCR (qRT-PCR)

Total RNA extraction, cDNA synthesis and qRT-PCR were carried out according to method described previously [18,19]. Gene specific primers used in the present study were listed in Table 1. Each trial contained three replicates and relative expression level of BpLRR-1 was calculated using $2^{-\Delta\Delta Ct}$ method with actin gene as internal control.

2.4. Cloning and bioinformatics analysis of BpLRR-1

Primers designed according to the nucleotide sequences of BpLRR-1 were employed for the cloning of coding region. The PCR amplification was conducted using cDNA template from gill tissue and verified using Vector NTI (Invitrogen). Homologues of BpLRR-1 protein were obtained from National Center for Biotechnology Information (NCBI) using blastp algorithm and aligned by Clustal W (Table 2). Signal peptide of BpLRR-1 was predicted by SignalP 4.1 program while protein motif features was annotated by SMART and pfam database. The presumed tertiary structure of BpLRR-1 was conducted by Swiss-Model and visualized by ESPript.

2.5. Prokaryotic expression and purification of recombinant BpLRR-1

The cDNA fragment encoding LRR domains of BpLRR-1 was cloned into pET-30a expression vector with 6×His tag and transformed into *Escherichia coli* BL21(DE3) cell. After validated by sequencing, recombinant BpLRR-1 protein (rBpLRR-1) was induced by IPTG and purified according to previous description under denaturing conditions. Renaturation of purified rBpLRR-1 proteins were then carried out after desalting by extensive dialysis. Concentration of rBpLRR-1 was subsequently quantified by BCA kit while protein purity was verified by SPS-PAGE and illustrated by an eStain 2.0 Protein Staining System.

Table 1
Primers employed in the present study.

IDs	Sequences(5′- 3′)	Amplicon Length	Reference
BpLRR-1_FL_F	GTAAAAAATAAGCACAAGAACGAAA	1461 bp	-
BpLRR-1_FL_R	TCAACTTACAGTTTGTCTTCTCACA		
rBpLRR-1_F	TGGCACTGTCTATCGCAATG	723 bp	-
rBpLRR-1_R	ACTAATCTTCGTTGTGGTTGGA		
rBpLRR-1_30a_F	GGATCCTCAGTAACTTGTTTG	704 bp	-
rBpLRR-1_30a_R	GCGGCCGCACTAATCTTCGTTG		
BpLRR-1_RT_F	AAATAAGCACAAGAACGAAACAGAGA	157 bp	-
BpLRR-1_RT_R	CTTTTTACTGCCACATTCAGAACAG		
BpActin_RT_F	GACGAAGCCCAGGTAAAACG	198 bp	[18]
BpActin_RT_R	CTTAGTCATCATTTCTCTGTTGCCT		
pmoA_RT_F	TGGACAGATTGGAAAGATAGACG	111 bp	[18]
pmoA_RT_R	GAAAGGCAGACGGTAACGG		

Table 2
Sequence similarity of BpLRR-1 and its homologues.

	BpLRR-1 vs MyLRR74A	BpLRR-1 vs CvLRR74A
Identical residues	204	211
Similar residues	70	74
Percent identify	39.8%	41.4%
Percent similarity	53.4%	55.9%

2.6. Surface plasmon resonance (SPR) analysis

SPR analysis in verification of binding activities of rBpLRR-1 against MAMPs was conducted according to methods reported previously [20]. In brief, monoclonal antibody of His tag (Abclonal, AE003) was firstly immobilized onto the CM5 sensor chip using Amine Coupling kit (GE Healthcare). A total of 40 μL rBpLRR-1 protein (0.5 mg/mL) was then loaded on the chip and bound with anti-His tag antibody reaching approximately 200 response units (RUs). A total of five MAMPs including LPS (1 mg/mL in DMSO, from *E. coli* 0111:B4, Sigma-Aldrich), PGN (0.2 mg/mL in PBS, from *Staphylococcus aureus*, Sigma-Aldrich), MAN (1 mg/mL in PBS, from *Saccharomyces cerevisiae*, Sigma-Aldrich), GLU (1 mg/mL in PBS, from *Euglena gracilis*, Sigma-Aldrich) and Poly I:C (1 mg/mL in PBS, Sigma-Aldrich) were injected into the reaction well afterward at 10 μL/min and RUs between each MAMP and rBpLRR-1 were recorded during 2 min's reaction. All data obtained were analyzed by converting to Langmuir binding model in the BIAcore T200 evaluation software according to the manual.

2.7. LPS pull-down assay

LPS pull-down assay was performed to isolate LPS binding proteins from *B.platifrons* gills according to method reported previously [21]. Briefly, LPS (5 mg/ml in PBS) was firstly immobilized with Epoxy-activated Sepharose 6B column at 4 °C and washed by using PBS. Total proteins extracted from *B.platifrons* gills (1 mg/ml in PBS with PMSF) was then loaded onto Sepharose 6B column and LPS- Sepharose 6B column successively and washed extensively with PBS buffer. Finally, host proteins binding with the column was eluted by LPS (7.5 mg/ml in PBS) and urea (8 mol/L) before subjected to SDS-PAGE and coomassie/silver staining. Protein candidates were further characterized by LC-MS/MS and annotated using genome information of *B.platifrons*.

2.8. Expression profiling of BpLRR-1 during symbiont depletion assay and bacterial challenge

A total of 30 Bathymodioline mussels in similar size (length ranging from 70 mm to 100 mm) were then transferred into a new aquarium and subjected to antibiotic treatment at atmospheric pressure (without CH_4 supplement). Ampicillin and streptomycin were supplemented into the aquarium at final concentration of 10 mg/L and refreshed every 3 days. Mussel gills were harvested at 0, 7, 14, 21, 28 days after the treatment for MOB quantification and qRT-PCR of BpLRR-1 transcripts. Each trial contains three replicates.

Symbiotic MOBs enriched from gill tissue and *Vibrio alginolyticus* (isolated from the macro fauna of Formosa ridge cold seep and kindly provided by Dr. Li Sun from Institute of Oceanology, Chinese Academy of Sciences) were employed for bacterial challenge. *V. alginolyticus* were cultured overnight using 2216E medium at 18 °C and collected during log phase. Both MOBs and *V. alginolyticus* were then suspended with sterilized seawater at concentration of 1×10^6 copy/mL and heated at 60 °C for 30 min to neutralize host protein or extracellular products. A total of 90 Bathymodioline mussels designated as seawater group, endosymbiont group and *V. alginolyticus* group were injected with 100 μL sterilized seawater, MOB suspension and *V. alginolyticus* suspension correspondingly at adductor muscle and collected randomly at 0, 6, 12, 24, 48 h post injection for qRT-PCR assay of BpLRR-1 transcripts. Each trial was conducted with three replicates.

2.9. Statistical analysis

Data obtained in the present study were given as means ± s.d. and subjected to one-way ANOVA analysis by SPSS v20, followed by a multiple comparison to detect significant differences between samples (* or a, b, c if $p < 0.05$).

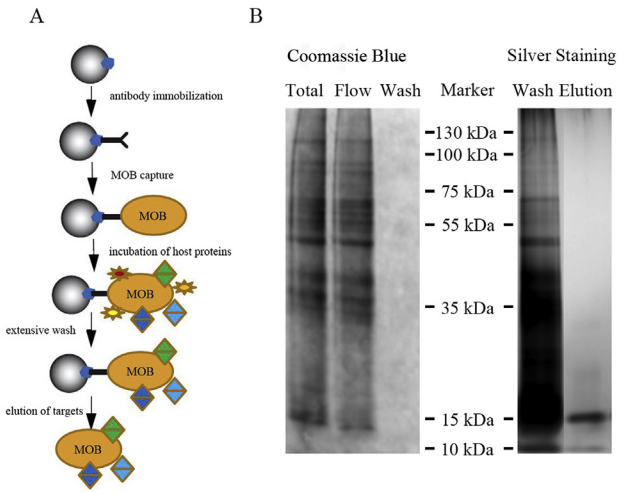
3. Results

3.1. Isolation and identification of BpLRR-1 by MOB pull-down assay

A modified MOB pull-down assay (Fig. 1A) was conducted to isolate host proteins bound with symbiotic MOBs and a total of along with 47 host proteins were identified by LC-MS/MS (Fig. 1B, Table 3). Among them, 10 peptide fragments of BpLRR-1 covering residues Met_1 to Asn_{13} and residues Met_{367} to Lys_{378} were characterized, demonstrating the reliability of MOB pull-down assay and suggesting its participation in recognizing symbionts (Fig. 1C).

3.2. Structure characteristics of BpLRR-1

The full length coding region of BpLRR-1 (1479 bp) was subsequently cloned using gene specific primer listed in Table 1. The open reading frame of BpLRR-1 encoded a polypeptide of 492 amino acid residues with molecular weight approximately 63,579 Da and isoelectric point of 5.67 (Fig. 2A). No signal peptide or transmembrane region was predicted in the deduced amino acid sequences while eight LRR motif and two EFh motif was annotated by SMART (Fig. 2B). Homologues of BpLRR-1 were investigated by blastp algorithm against non-redundant protein sequences in NCBI. It turned out that BpLRR-1 shared most similarity with CvLRR74A (XP_022312945.1) from *Crassostrea virginica* and MyLRR74A (XP_021365007.1) from *Mizuhopecten*



Start - End	Mr(expt)	Mr(calc)	Sequence
1 - 12	1374.6190	1374.6561	-.MDSSADVKNKHK.N
367 - 378	1474.7766	1474.7813	K.MLEVRNNLQTIK.V
367 - 378	1474.7766	1474.7813	K.MLEVRNNLQTIK.V
367 - 378	1474.7766	1474.7813	K.MLEVRNNLQTIK.V
367 - 378	1474.7766	1474.7813	K.MLEVRNNLQTIK.V
367 - 378	1474.7766	1474.7813	K.MLEVRNNLQTIK.V
367 - 378	1474.7796	1474.7813	K.MLEVRNNLQTIK.V
367 - 378	1474.7796	1474.7813	K.MLEVRNNLQTIK.V
367 - 378	1474.7796	1474.7813	K.MLEVRNNLQTIK.V
367 - 378	1474.7796	1474.7813	K.MLEVRNNLQTIK.V

Fig. 1. Modified MOB pull-down assay conducted for isolation of symbiosis-related PRRs. (A) A modified MOB pull-down assay was conducted herein with NHS Mag Sepharose (grey circle) using lipid A monoclonal antibody and LPS polyclonal antibody ("Y") for MOBs capture. Gill proteins were incubated with captured MOBs and PRRs binding with MOBs (rhombus) were eluted using urea after extensive wash. (B) Total proteins employed in the assay (designated as "Total") or after incubation with captured MOBs (designated as "Flow") or after extensive wash (designated as "Wash") were separated by SDS-PAGE and illustrated by coomassie blue staining. Silver staining was also conducted with proteins in wash buffer (designated as "Wash") and elution buffer (designated as "Elution"). Faint band could be observed in the elution besides the strong band at 15 kDa (antibodies immobilized previously). (C) The eluted proteins were identified by LC-MS/MS and a total of 10 fragments of BpLRR-1 were annotated.

yessoensis (Fig. 2A, Table 2). Tertiary structure of BpLRR-1 was further built using Swiss-Model and a total of ten α-helix and ten β-sheets were predicted, forming typical arc structures of LRR protein family (Fig. 2C).

3.3. High binding affinity of BpLRR-1 to LPS

Recombinant BpLRR-1 merely containing eight LRR domains (230 aa) with His tag was purified using Ni-NTA affinity chromatography and visualized by SDS-PAGE (Fig. 3A). A distinct band of rBpLRR-1 was found with molecular mass of approximately 29 kDa. The binding activity of rBpLRR-1 against different MAMPs was verified by SPR assay. As a result, a significant increase of RUs was observed during incubation with LPS, reaching nearly 580 RUs. Meanwhile, RUs remained almost unchanged when incubated with PGN, MAN, GLU or poly(I:C).

LPS pull-down assay was subsequently conducted for further verification of the binding affinity of BpLRR-1 against LPS (Fig. 3C). After extensive washing, B. platifrons proteins with moderate LPS binding affinity were firstly eluted with LPS buffer while proteins with higher binding affinity were eluted with 8 M urea. Among the LPS binding proteins identified, BpLRR-1 were found in both LPS (14 tryptic peptides from 367 to 378 aa and 478–487 aa) and 8 M urea (14 tryptic peptides from 367 to 378 aa) elution (Fig. 3D), confirming the reliability of result in SPR assay.

3.4. Expression pattern of BpLRR-1 during symbionts depletion and bacterial challenge

Quantification of MOB was carried out by qRT-PCR of pmoA gene and normalized by cytoplasmic DNA. It turned out that approximately 11568.23 copy/ng DNA of MOBs could be hosted by gill cells from freshly obtained *Bathymodiolus* and the copy number of MOBs inside gill

Table 3
Proteins identified in MOB pull-down by LC-MS/MS.

evm.TU.Super-Scaffold_464.6	evm.TU.Super-Scaffold_2122.4
evm.TU.Super-Scaffold_250.39	evm.TU.Super-Scaffold_626.6
evm.TU.Super-Scaffold_726.23	evm.TU.Super-Scaffold_105.21
evm.TU.scaffold865_size463771_obj.14	evm.TU.scaffold350_size778241_obj.9
evm.TU.Super-Scaffold_1688.6	evm.TU.Super-Scaffold_1127.2
evm.TU.scaffold3775_size111472_obj.1	evm.TU.Super-Scaffold_839.6
evm.TU.Super-Scaffold_12.16	evm.TU.Super-Scaffold_1985.1
evm.TU.Super-Scaffold_105.17	evm.TU.Super-Scaffold_414.16
evm.TU.Super-Scaffold_46.3	evm.TU.scaffold1362_size338679_obj.2
evm.TU.scaffold1334_size343687_obj.14	evm.TU.scaffold1447_size309492_obj.4
evm.TU.scaffold5134_size22328_obj.1	evm.TU.Super-Scaffold_449.19
evm.TU.Super-Scaffold_709.2	evm.TU.Super-Scaffold_209.38
evm.TU.scaffold1969_size200459_obj.7	evm.TU.Super-Scaffold_202.14
evm.TU.scaffold2578_size132567_obj.4	evm.TU.Super-Scaffold_1031.9
evm.TU.Super-Scaffold_606.26	evm.TU.Super-Scaffold_115.19
evm.TU.Super-Scaffold_481.29	evm.TU.Super-Scaffold_49.7
evm.TU.Super-Scaffold_195.39	evm.TU.Super-Scaffold_225.7
evm.TU.scaffold1227_size358460_obj.1	evm.TU.Super-Scaffold_14.20
evm.TU.Super-Scaffold_377.12	evm.TU.Super-Scaffold_40.57
evm.TU.Super-Scaffold_1538.2	evm.TU.Super-Scaffold_563.18
evm.TU.Super-Scaffold_13.67	evm.TU.Super-Scaffold_333.17
evm.TU.Super-Scaffold_166.3	evm.TU.Super-Scaffold_292.33
evm.TU.Super-Scaffold_206.41	evm.TU.Super-Scaffold_708.27
evm.TU.Super-Scaffold_1760.7	evm.TU.Super-Scaffold_2122.4

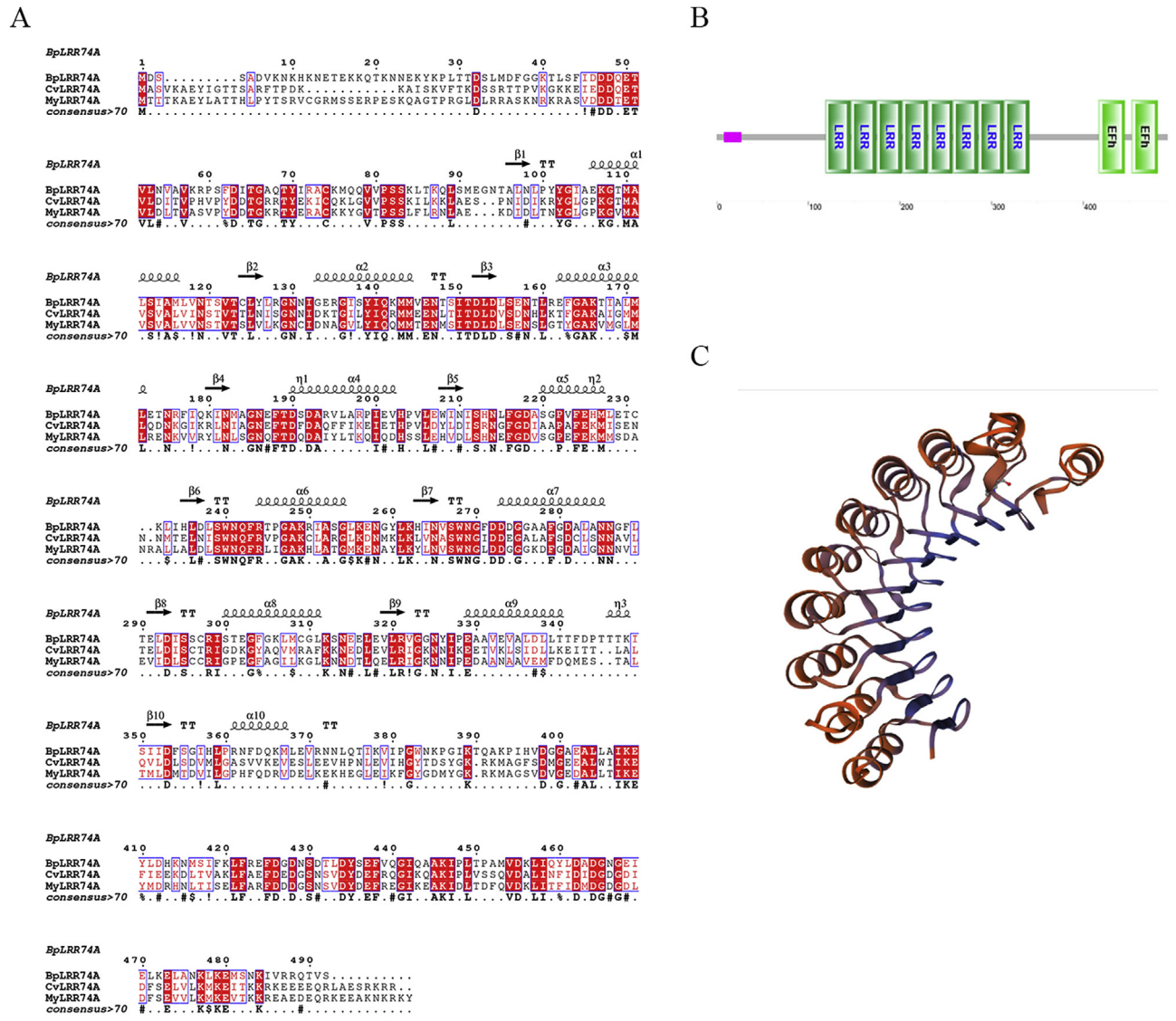


Fig. 2. Structure characteristics of BpLRR-1 and its homologues. (A) Sequence alignments of BpLRR-1 and its homologues in oyster and scallop. Identical amino acid residues are shaded in red while similar amino acids were illustrated in red characters. Conserved amino acids and secondary structures were also demonstrated. (B) Protein domains of BpLRR-1 were annotated by SMART. A total of eight LRR domains and two Ef hand domains were observed while no transmembrane region or signal peptides were found. (C) Tertiary structure of BpLRR-1 were built by Swiss-Model and found in arc shape in consistence with typical LRR proteins.

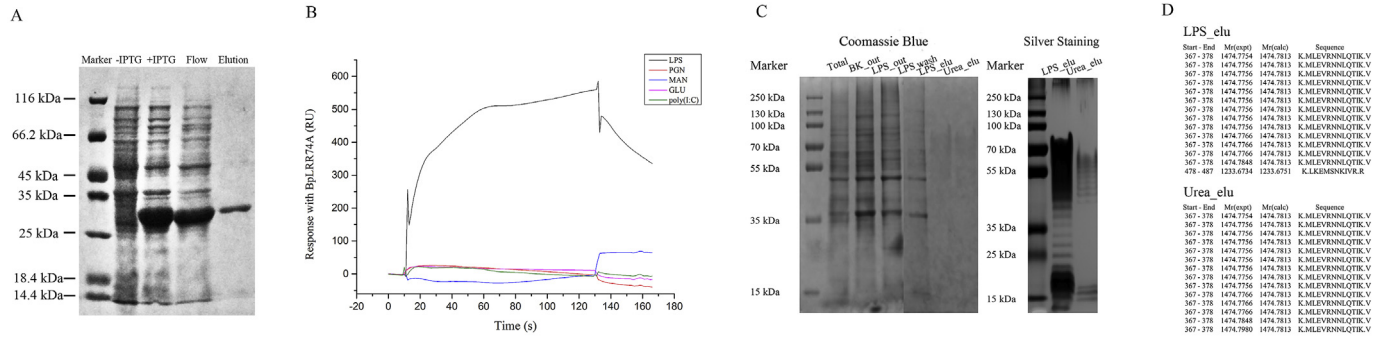


Fig. 3. MAMPs binding pattern of BpLRR-1. (A) rBpLRR-1 with His tag were expressed in *Escherichia coli* BL21(DE3) cell and purified with Ni-NTA affinity chromatography. Lysates of BL21(DE3) cell before IPTG induction ("-IPTG") or induced for 24 h at 20 °C ("+IPTG") or loaded onto Ni-NTA column ("Flow") along with purified rBpLRR-1 protein were separated by SDS-PAGE and illustrated by coomassie blue staining. (B) SPR assay were conducted using rBpLRR-1 proteins against LPS, PGN, MAN, GLU and poly(I:C). Significant increase of RUs could be found in LPS trial, suggesting high binding affinity. (C) LPS pull-down assay was conducted with gill proteins ("Total"). Proteins flowed though Epoxy-activated Sepharose 6B column ("BK_out") or LPS- Sepharose 6B column ("LPS_out") or after extensive wash ("LPS_wash") or eluted using LPS ("LPS_elu") and urea ("Urea_elu") were firstly subjected to SDS-PAGE and then stained by coomassie blue. Proteins in LPS_elu and Urea_elu were also stained with silver. As a result, significant bands could be observed in both lanes. (D) Proteins in LPS_elu and Urea_elu were identified by LC-MS/MS while fragments of BpLRR-1 were found in both LPS_elu and Urea_elu groups, demonstrating the binding capability with LPS.

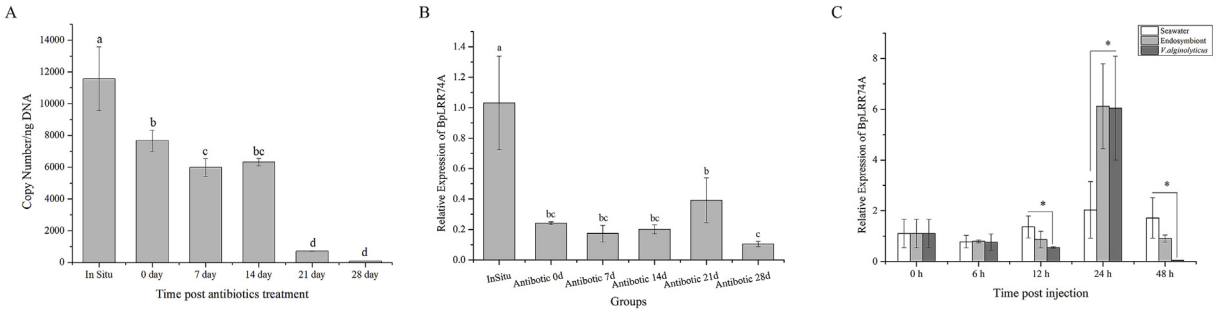


Fig. 4. Expression profile of BpLRR-1 during symbionts depletion and bacteria challenge. (A) Copy numbers of symbiotic MOBs during antibiotics treatment were quantified by qRT-PCR of pmoA gene in normalization with total DNA from the template. (B) Relative expression level of BpLRR-1 during symbionts depletion was surveyed by qRT-PCR with actin gene as internal control and was found down-regulated significantly. (C) Alternations of BpLRR-1 transcripts during enriched MOBs or nonsymbiotic *V. alginolyticus* challenge were investigated by qRT-PCR. Significant increases were observed in both groups at 24 h post challenge while BpLRR-1 transcripts also decreased at 6 h and 48 h post *V. alginolyticus* challenge.

descended rapidly during acclimation, merely remaining 0.66-fold of basal level after two days (Fig. 4A). The copy number kept decreasing after *Bathymodiolus* were treated with antibiotics and dropped to 94.61 copy/ng DNA at 28 days later. Consistently, transcripts of BpLRR-1 in *Bathymodiolus* gill also declined significantly during acclimation and antibiotics treatment, remaining 0.24-fold of basal level two days after acclimation and reaching 0.10-fold of basal level at 28 days after antibiotics treatment. Expression pattern of BpLRR-1 during symbiotic (MOB) or nonsymbiotic (*V. alginolyticus*) bacteria challenge was further investigated by qRT-PCR. As a result, expression level of BpLRR-1 was found increasing significantly in gill at 24 h post symbiotic MOB injection (6.12-fold of basal level at 0 h) yet remained unchanged at 6 h, 12 h, and 48 h. Transcripts of BpLRR-1 could also be markedly altered during *V. alginolyticus* stimulation and decreased firstly at 12 h post challenge before peaking to 6.04-fold of basal level at 24 h. The expression level of BpLRR-1 was then found depressed remarkably at 48 h post nonsymbiotic bacteria challenge.

4. Discussion

As the first step of host immune response, immune recognition mediated by PRRs is crucial in establishing symbiosis between host and symbionts [13]. However, the lack of cultivable symbionts has retarded the identification of Bathymodiolinae PRRs binding with them. Pull-down assay has been proven as effective method to characterize pathogen or symbiont binding proteins [22–24] while LPS (constituted by lipid A, core oligosaccharide and O polysaccharide) are found in the outer membrane of symbiotic MOBs. Therefore, a modified MOB pull-down assay was designed in the present study using lipid A monoclonal antibody and LPS polyclonal antibody for MOB capture (Fig. 1A). Due to the amount of gill proteins employed in the assay, only a faint band could be observed in the elution except for a sharp band at 15 kDa, which was suggested as antibodies immobilized previously (Fig. 1B). Nevertheless, results of LC-MS/MS demonstrated that a total of 48 proteins were identified from elution, confirming the reliability of the method. At the same time, a total of 10 fragment of BpLRR-1 were annotated from MOB pull-down assay, indicating that BpLRR-1 might be a recognition receptor for the endosymbiotic MOBs (Fig. 1C).

Since the first report in human, thousands of LRR proteins have been identified to date [25]. As found, LRRs are evolutionarily conserved in many species and encoded by plants, invertebrates and vertebrates with diverse functions including recognition with MAMPs [26]. Herein, coding region of BpLRR-1 was cloned and subjected to prokaryotic expression for functional verification. It is found that BpLRR-1 was structurally conserved with tandem LxxLxLxxN/CxL (L means Val, Leu, or Ile, N means Asn, Thr, Ser, or Cys and C means Cys or Ser) motif and adopt an arc shape constructed by β-strands and helices (Fig. 2) consistently with typical LRR proteins [27]. As suggested, the unique shape of LRR proteins has provided frameworks of protein-protein interaction as well as MAMPs recognition and diverse ligands including bacterial lipoprotein, lipopolysaccharide (LPS), peptidoglycan (PGN), oligodeoxynucleotides, flagellin, as well as other fungal MAMPs, could be recognized by LRR proteins [28]. To figure out binding pattern of BpLRR-1, SPR assay against LPS, PGN, MAN, GLU and poly(I:C) was conducted using rBpLRR-1 protein which merely contained LRR domain. As a result, only LPS was found bound by rBpLRR-1 (Fig. 3B). Subsequent LPS pull-down assay also demonstrated high affinity of BpLRR-1 against LPS (Fig. 3C and D). As demonstrated in other studies, majority of LRR proteins identified in bivalve were capable of binding with diverse ligands such as LPS, PGN, GLU and poly(I:C) [29–32]. The specified binding pattern of BpLRR-1 might result from the structural divergence in protein sequences of BpLRR-1 and its homologues (Fig. 2A, Table 2) and it was suggested that the bound with LPS by BpLRR-1 might play crucial role in recognition of symbiotic MOBs.

Massive reports have demonstrated that expression pattern of LRR proteins could be vigorously modulated under different MAMPs challenge, further facilitating the specificity of host immune response [26,33,34]. Herein, alternations of BpLRR-1 transcripts during decolonization of symbionts and bacteria challenge were surveyed and correlation between BpLRR-1 transcripts with symbionts was expected. As expected, copy number of symbionts decreased gradually during decolonization assay by antibiotics treatment (Fig. 4A). Meanwhile, BpLRR-1 transcripts were found down-regulated significantly after decolonization yet increased remarkably when *B. platifrons* was challenged with enriched MOBs (Fig. 4B and C). The coordination between symbiont copy number and BpLRR-1 expression level confirmed our speculation above and demonstrate the participation of BpLRR-1 in symbiosis. As demonstrated, members in the LRR family could be classified either as LRR-only proteins (only containing LRR domains) or LRR-containing proteins (containing both LRR domains and other domains) given their protein structure [27]. In the present study, two Ef hand domain were also found in BpLRR-1 protein while no signal peptide or transmembrane region was observed. As a classic Ca^{2+} binding motif, Ef hand domain has been found playing crucial role in intracellular Ca^{2+} signaling and modulating diverse immune processes. It was therefore suggested that BpLRR-1 could serve as an intracellular receptor of MOBs while the induction of BpLRR-1 transcripts during MOBs challenge might further modulate intracellular Ca^{2+} signaling pathway and downstream immune processes. Moreover, BpLRR-1 transcripts were also found vigorously modulated during *V. alginolyticus* challenge. However, the expression pattern differed significantly between MOB and *V. alginolyticus* challenge at 6 h and 48 h post challenge. The distinct expression pattern was further suspected to induce different immune response of host afterward, which might be decisive in symbiosis.

In conclusion, BpLRR-1 was identified from a modified MOB pull-

down assay and suggested as an intracellular recognition receptor for the endosymbiotic MOB given the MAMPs binding pattern and expression profile during decolonization of symbionts and MOB challenge. Although the subcellular location and downstream immune effects of BpLRR-1 in symbiosis remained undisclosed, our findings have shed new light in both understanding the innate immunity of *B. platifrons* and the immune recognition against symbiotic MOBs.

Ethics

Experiments conducted in this study were approved by the Ethics Committee of Institute of Oceanology, Chinese Academy of Sciences.

Author contributions

HC carried out the majority of experiment and data analysis, participated in the design of the study and drafted the manuscript. MXW, HZ and HW participated in the design of the study, helped with recombinant and molecular analysis of BpLRR-1, and discussed the results. ZL performed the SPR assay while LZ and ZSZ helped with symbiont depletion assay and bacterial challenge. CL and LC carried out the mussel sampling during the cruise. LCL conceived the study, coordinated the experiment and helped draft the manuscript. All authors gave final approval for publication.

Conflicts of interest

The authors declare no competing interests.

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References

[1] R. Danovaro, C. Corinaldesi, A. Dell'Anno, P.V.R. Snelgrove, The deep-sea under global change, Curr. Biol. 27 (11) (2017) R461–R465, https://doi.org/10.1016/j.cub.2017.02. 046.

[2] R. Danovaro, P.V. Snelgrove, P. Tyler, Challenging the paradigms of deep-sea ecology, Trends Ecol. Evol. 29 (8) (2014) 465–475, https://doi.org/10.1016/j.tree.2014.06.002.

[3] S.M. Sievert, C. Vetriani, Chemoautotrophy at deep-sea vents: past, present, and future, Oceanography 25 (1) (2012) 218–233.

[4] C.L. Van Dover, C.R. German, K.G. Speer, L.M. Parson, R.C. Vrijenhoek, Evolution and biogeography of deep-sea vent and seep invertebrates, Science 295 (5558) (2002) 1253–1257, https://doi.org/10.1126/science.1067361.

[5] J. Sun, Y. Zhang, T. Xu, Y. Zhang, H. Mu, Y. Zhang, et al., Adaptation to deep-sea chemosynthetic environments as revealed by mussel genomes, Nat. Ecol. Evol. 1 (5) (2017) 121, https://doi.org/10.1038/s41559-017-0121.

[6] S. Duperron, J. Lorion, S. Samadi, O. Gros, F. Gaill, Symbioses between deep-sea mussels (Mytilidae: Bathymodiolinae) and chemosynthetic bacteria: diversity, function and evolution, Comptes Rendus Biol. 332 (2–3) (2009) 298–310, https://doi.org/10.1016/j.crvi.2008.08.003.

[7] Y.J. Won, S.J. Hallam, G.D. O'Mullan, I.L. Pan, K.R. Buck, R.C. Vrijenhoek, Environmental acquisition of thiotrophic endosymbionts by deep-sea mussels of the genus *Bathymodiolus*, Appl. Environ. Microbiol. 69 (11) (2003) 6785–6792, https://doi.org/10.1128/aem.69.11.6785-6792.2003.

[8] K.M. Fontanez, C.M. Cavanaugh, Evidence for horizontal transmission from multilocus phylogeny of deep-sea mussel (Mytilidae) symbionts, Environ. Microbiol. 16 (12) (2014) 3608–3621, https://doi.org/10.1111/1462-2920.12379.

[9] R. Ponnudurai, L. Sayavedra, M. Kleiner, S.E. Heiden, A. Thurmer, H. Felbeck, et al., Genome sequence of the sulfur-oxidizing *Bathymodiolus thermophilus* gill endosymbiont, Stand. Genom. Sci. 12 (2017) 50, https://doi.org/10.1186/s40793-017 -0266-y.

[10] K. Takishita, Y. Takaki, Y. Chikaraishi, T. Ikuta, G. Ozawa, T. Yoshida, et al., Genomic evidence that methanotrophic endosymbionts likely provide deep-sea *Bathymodiolus* mussels with a sterol intermediate in cholesterol biosynthesis, Genome Biol. Evol. 9 (5) (2017) 1148–1160, https://doi.org/10.1093/gbe/evx082.

[11] E. Kádár, S.A. Davis, A. Lobo-da-Cunha, Cytoenzymatic investigation of intracellular digestion in the symbiont-bearing hydrothermal bivalve *Bathymodiolus azoricus*, Mar. Biol. 153 (5) (2008) 995–1004, https://doi.org/10.1007/s00227-007-0872-0.

[12] A. Fiala-Médioni, Z. McKiness, P. Dando, J. Boulegue, A. Mariotti, A. Alayse-Danet, et al., Ultrastructural, biochemical, and immunological characterization of two populations of the mytilid mussel *Bathymodiolus azoricus* from the Mid-Atlantic Ridge: evidence for a dual symbiosis, Mar. Biol. 141 (6) (2002) 1035–1043, https://doi.org/10.1007/s00227-002-0903-9.

[13] H. Chu, S.K. Mazmanian, Innate immune recognition of the microbiota promotes host-microbial symbiosis, Nat. Immunol. 14 (7) (2013) 668–675, https://doi.org/10.1038/ni. 2635.

[14] C. Detree, F.H. Lallier, A. Tanguy, J. Mary, Identification and gene expression of multiple peptidoglycan recognition proteins (PGRPs) in the deep-sea mussel *Bathymodiolus azoricus*, involvement in symbiosis? Comparative biochemistry and physiology Part B, Biochem. Mol. Biol. 207 (2017) 1–8, https://doi.org/10.1016/j.cbpb.2017.02.002.

[15] I. Barros, B. Divya, I. Martins, F. Vandeperre, R.S. Santos, R. Bettencourt, Post-capture immune gene expression studies in the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* acclimatized to atmospheric pressure, Fish Shellfish Immunol. 42 (1) (2015) 159–170, https://doi.org/10.1016/j.fsi.2014.10.018.

[16] I. Barros, S. Mendes, D. Rosa, *Vibrio diabolicus* immunomodulatory effects on *Bathymodiolus azoricus* during long-term acclimatization at atmospheric pressure, J. Aquac. Res. Dev. 7 (12) (2016), https://doi.org/10.4172/2155 -9546.1000464.

[17] P. Zheng, M. Wang, C. Li, X. Sun, X. Wang, Y. Sun, et al., Insights into deep-sea adaptations and host–symbiont interactions: a comparative transcriptome study on Bathymodiolus mussels and their coastal relatives, Mol. Ecol. 26 (19) (2017) 5133–5148, https://doi.org/10.1111/mec.14160.

[18] Y. Sun, M. Wang, L. Li, L. Zhou, X. Wang, P. Zheng, et al., Molecular identification of methane monooxygenase and quantitative analysis of methanotrophic endosymbionts under laboratory maintenance in *Bathymodiolus platifrons* from the South China Sea, Peer J. 5 (2017) e3565, , https://doi.org/10.7717/peerj.3565.

[19] H. Chen, Z. Zhou, L. Wang, H. Wang, R. Liu, H. Zhang, et al., An invertebrate-specific miRNA targeted the ancient cholinergic neuroendocrine system of oyster, Open Biol. (8) (2016) 6, https://doi.org/10.1098/rsob.160059.

[20] Z. Lv, L. Qiu, M. Wang, Z. Jia, W. Wang, L. Xin, et al., Comparative study of three C1q domain containing proteins from pacific oyster *Crassostrea gigas*, Dev. Comp. Immunol. 78 (2018) 42–51, https://doi.org/10.1016/j.dci. 2017.09.014.

[21] J. Xu, S. Jiang, Y. Li, M. Li, Q. Cheng, D. Zhao, et al., Caspase-3 serves as an intracellular immune receptor specific for lipopolysaccharide in oyster *Crassostrea gigas*, Dev. Comp. Immunol. 61 (2016) 1–12, https://doi.org/10.1016/j.dci.2016.03.015.

[22] Y. Liu, H. Zhang, Y. Liu, H. Li, X. Peng, Determination of the heterogeneous interactome between *Edwardsiella tarda* and fish gills, J. Proteom. 75 (4) (2012) 1119–1128, https://doi.org/10.1016/j.jprot.2011.10.022.

[23] H. Li, X. Huang, Z. Zeng, X.X. Peng, B. Peng, Identification of the interactome between fish plasma proteins and *Edwardsiella tarda* reveals tissue-specific strategies against bacterial infection, Int. J. Biochem. Cell Biol. 78 (2016) 260–267, https://doi.org/10.1016/j.biocel.2016.07.021.

[24] C. Wu, K. Soderhall, I. Soderhall, Two novel ficolin-like proteins act as pattern recognition receptors for invading pathogens in the freshwater crayfish *Pacifastacus leniusculus*, Proteomics 11 (11) (2011) 2249–2264, https://doi.org/10.1002/pmic.201000728.

[25] N. Matsushima, H. Miyashita, T. Mikami, Y. Kuroki, A nested leucine rich repeat (LRR) domain: the precursor of LRRs is a ten or eleven residue motif, BMC Microbiol. 10 (1) (2010) 235, https://doi.org/10.1186/1471-2180-10-235.

[26] Kedzierski Ł, J. Montgomery, J. Curtis, E. Handman, Leucine-rich repeats in host-pathogen interactions, Arch. Immunol. Ther. Exp. 52 (2) (2004) 104–112.

[27] P. Enkhbayar, M. Kamiya, M. Osaki, T. Matsumoto, N. Matsushima, Structural principles of leucine-rich repeat (LRR) proteins, Proteins: Struct. Funct. Bioinform. 54 (3) (2004) 394–403, https://doi.org/10.1002/prot.10605.

[28] B. Kobe, A.V. Kajava, The leucine-rich repeat as a protein recognition motif, Curr. Opin. Struct. Biol. 11 (6) (2001) 725–732, https://doi.org/10.1016/S0959 -440X(01)00266-4.

[29] M. Wang, L. Wang, Y. Guo, Q. Yi, L. Song, An LRR-only protein representing a new type of pattern recognition receptor in *Chlamys farreri*, Dev. Comp. Immunol. 54 (1) (2016) 145–155, https://doi.org/10.1016/j.dci.2015.09.006.

[30] W. Wang, T. Zhang, L. Wang, J. Xu, M. Li, A. Zhang, et al., A new non-phagocytic TLR6 with broad recognition ligands from Pacific oyster *Crassostrea gigas*, Dev. Comp. Immunol. 65 (2016) 182–190, https://doi.org/10.1016/j.dci. 2016.07.010.

[31] X. Wang, X. Zhao, C. Yan, Z. Jia, Z. Lv, C. Ma, et al., A novel LRR and Ig domain-containing protein could function as an immune effector in *Crassostrea gigas*, Fish Shellfish Immunol. 88 (2019) 318–327, https://doi.org/10.1016/j.fsi.2019.03.003.

[32] X. Wang, M. Wang, Q. Xu, J. Xu, Z. Lv, L. Wang, et al., Two novel LRR and Ig domain-containing proteins from oyster *Crassostrea gigas* function as pattern recognition receptors and induce expression of cytokines, Fish Shellfish Immunol. 70 (2017) 308–318, https://doi.org/10.1016/j.fsi.2017.09.023.

[33] M. Wang, L. Wang, L. Xin, X. Wang, L. Wang, J. Xu, et al., Two novel LRR-only proteins in *Chlamys farreri*: similar in structure, yet different in expression profile and pattern recognition, Dev. Comp. Immunol. 59 (2016) 99–109, https://doi.org/10.1016/j.dci.2016.01.013.

[34] M. Wang, L. Wang, Z. Jia, X. Wang, Q. Yi, L. Zhao, et al., The versatile functions of LRR-only proteins in mollusk *Chlamys farreri*, Dev. Comp. Immunol. 77 (2017) 188–199, https://doi.org/10.1016/j.dci.2017.08.005.