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A sialic acid-binding lectin with bactericidal and opsonic activities from *Ruditapes philippinarum*

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ABSTRACT

In the present study, a sialic acid-binding lectin was cloned and characterized from Manila clam *Ruditapes philippinarum* (designed as RpSabl). The open reading frame of RpSabl encoded a polypeptide of 162 amino acids with a calculated molecular mass of 17.7 kDa. Analysis of the conserved domain suggested that RpSabl was a new member of the sialic acid-binding lectins family. In non-stimulated clams, RpSabl transcripts were constitutively expressed in all five tested tissues, especially in hepatopancreas. After *Vibrio anguillarum* challenge, the expression of RpSabl mRNA in hepatopancreas was significantly up-regulated at 3 h (3.8-fold, $P < 0.05$), 6 h (4.9-fold, $P < 0.05$), 12 h (12.3-fold, $P < 0.01$) and 24 h (9.7-fold, $P < 0.01$), while RpSabl transcripts in hemocytes was only significantly up-regulated at 6 h (8.5-Fold, $P < 0.01$). RNAi-mediated knockdown of RpSabl transcripts affected the survival rates of Manila clam against *V. anguillarum*, perhaps mainly due to the inhibited expression of antibacterial effectors (e.g. lysozyme and defensin). Moreover, recombinant protein of RpSabl (rRpSabl) possessed binding activities towards lipopolysaccharides (LPS), peptidoglycan (PGN) and glucan *in vitro*. Coinciding with the Pathogen-associated molecular patterns (PAMPs) binding assay, rRpSabl displayed broad bacterial-agglutination properties towards *Vibrio harveyi*, *Vibrio splendidus*, *V. anguillarum*, *Enterobacter cloacae* and *Aeromonas hydrophila*. Meanwhile, the phagocytosis and encapsulation ability of hemocytes could be significantly enhanced by rRpSabl incubation. All these results showed that RpSabl could function as a versatile molecule involved in the innate immune responses of *R. philippinarum*.

1. Introduction

Complement system is a highly sophisticated and powerful defense mechanism composed of more than 30 soluble serum and cell-surface proteins in both innate and adaptive immunity [1]. Usually, the complement system can be activated through three pathways: the antibody-dependent classical pathway, the antibody-independent alternative pathway and the lectin pathway. C1q is a subcomponent of the complement C1 complex, which contributes to the recognition of pathogen surface structures and antibody-antigen complexes [2]. The C1q family comprises not only C1q subunits but also a diverse array of non-complement proteins. Its members include collagen, precerebellin, elastin microfibril interface-located protein (EMILIN), multimerin, and adiponectin [3]. All of them possess a globular C1q (gC1q) domain in the C-

terminal region, but vary in their N-terminal region [4]. Acting as a versatile pattern recognition protein (PRP), C1q could bind directly to a broad range of PAMPs of bacteria, viruses, and parasites, and thus enhance the phagocytosis of pathogens [5,6].

The C1q-domain-containing (C1q-DC) proteins are a family of proteins characterized by a globular domain of around 140 residues in the C-terminus with eight highly conserved residues, followed in most of them by a collagen-like region and a short amino-terminal region [7]. In vertebrates, C1q-DC proteins could act as mediators of immunological responses as well as homeostasis [3]. Presently, C1q-DC proteins have been found involved in the maintenance of immune tolerance via clearance of apoptotic cells, phagocytosis of bacteria, neutralization of retroviruses, cell adhesion, and modulation of dendritic cells (DCs), B cells and fibroblasts [8]. In marine invertebrates, several

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Sabls (containing C1q domains) and their functions have been reported in *Venerupis philippinarum* [9], *Limulus polyphemus* [10], *Crassostrea hongkongensis* [9], *Modiolus modiolus* [11] and *Solen grandis* [12]. For example, SgSABLs in *S. grandis* could agglutinate microbes, and mediate phagocytosis and encapsulation of hemocytes [13].

Ruditapes philippinarum is an economic species widely spread over many countries. The recent mass mortality of Manila clams has been attributed to pathogen invasion and environmental deterioration [14]. Therefore, it is urgently necessary to characterize immune-related molecules for disease control and the healthy management of clam aquaculture. Recently, a Sabl has been identified in Manila clam, but its immune functions still remain unclear [15]. In the present study, another sialic acid-binding lectin was identified from the clam *R. philippinarum* (designed as RpSabl), and the tissue-specific distribution, PAMPs recognition and opsonic activities were also investigated.

2. Materials and methods

2.1. Clams and bacterial challenge

Healthy Manila clams (shell length of 3.0–4.0 cm) were purchased from a local culture farm, and acclimatized in the aerated seawater at 20–22 °C for 10 days before processing. The clams were fed with an algae mixture of *Isochrysis galbana* and *Phaeodactylum tricornutum*, and the seawater was totally renewed daily.

Bacterial challenge experiment was performed according to our previous description [16]. Briefly, 300 clams were randomly divided into two groups and kept in six aerated tanks (50 L). Three tanks served as the control, while the other three tanks were immersed with *Vibrio anguillarum* at a final concentration of 1×10^7 CFU/mL. The *V. anguillarum* was cultured in 2216E medium at 28 °C for 24 h, harvested by centrifuging at 4000 g for 10 min, washed with filter-sterilized sea water (FSSW) for three times, and re-suspended in FSSW. The hemocytes of six individuals were randomly sampled at 0, 6, 12, 24 and 48 h post bacterial challenge.

2.2. Gene cloning and sequence analysis of RpSabl

A Sabl EST was identified through large-scale EST sequencing of the constructed cDNA library. Forward primers P1, P2 were used to clone the full-length cDNA of RpSabl with primer oligo (d_T) as the reverse primer, respectively (Table S1). The nucleotide sequence and deduced amino acid analysis were performed using the BLAST algorithm and the Expert Protein Analysis System. The protein domains were predicted with the simple modular architecture research tool version 4.0 [17]. Multiple alignments were performed with the ClustalW Multiple Alignment program and Multiple Alignment Show program.

2.3. Quantification analysis of RpSabl mRNA expression

The tissue distribution of RpSabl transcripts was analyzed in an Applied Biosystem 7500 Real-time PCR System. The RNA extraction, cDNA synthesis and quantitative PCR analysis were performed according to previously described [16]. Briefly, total RNAs from hemocytes, mantle, gills, hepatopancreas and adductor muscle were extracted from six individuals using Trizol reagent (Invitrogen, USA). After reverse transcription, a pair of gene-specific primers (P3 and P4, Table S1) was designed to detect the mRNA expression of RpSabl. β -actin primers (P5 and P6, Table S1) were used to amplify a 121 bp fragment as an internal control.

In addition, the mRNA expression of RpSabl in hemocytes and hepatopancreas after *V. anguillarum* challenge were also determined. At the end of each PCR, dissociation curve analysis of amplification products was performed to confirm the purity of PCR products. The $2^{-\Delta\Delta CT}$ method [18] was used to analyze the expression levels of RpSabl transcripts. All data were given in terms of relative mRNA expressed as

mean \pm S.D. (N = 6). Statistical analysis was performed by one-way analysis of variance (one-way ANOVA) with a Duncan test using SPSS 16.0 software, and *P* values less than 0.05 were considered statistically significant.

2.4. Synthesis and microinjection of dsRNA

Primers P7, P8 and P9, P10 (Table S1) were used to amplify the fragments of RpSabl and GFP (as control), respectively. The products with the T7 promoter were confirmed by sequencing and used as templates to produce the sense and anti-sense RNA strands for transcription *in vitro*. The products were purified using the Ribo-MAXTM Large-scale RNA production System-T7 (Promega, USA) according to the manufacturer's protocol. These dsRNAs were separately dissolved in DEPC-treated water to a final concentration of 1 mg/mL for subsequent experiments.

Natural healthy clams were collected and maintained in the aerated seawater for a week before processing. For the experiment, 300 clams were randomly selected, divided into 3 groups of 100 clams. About 60 μ g dsRNA was injected into the adductor muscle of each animal. Clams in blank and control treatments were injected with either PBS or dsRNA of GFP. After injection for 24 h, these tanks were immersed with *V. anguillarum* at a final concentration of 1×10^7 CFU/mL, and the mortality of Manila clams were recorded daily. At 72 h after bacterial infection, hemocytes were collected from six individuals of each treatment for total RNA extraction and reverse transcription. Then the cDNA templates were diluted 20-fold and used for subsequent qRT-PCR analysis. The mRNA expression of RpSabl and antibacterial effectors (P11, P12 for c-type lysozyme, P13, P14 for p-type lysozyme, P15, P16 for big defensin, P17, P18 for defensin, Table S1) was analyzed as described above.

2.5. Recombinant expression of RpSabl

The fragment encoding the mature peptide of RpSabl was amplified by two specific primers (P19 and P20, Table S1), cloned into pEASY-E1 simple vector (Transgen Biotech, Beijing, China), and transformed into *E. coli* competent cells (Trans1-T1 Phage Resistant Chemically Competent Cell, TransGen Biotech). The pEASY-E1-RpSabl plasmid was extracted and transformed into *E. coli* BL21 (DE3). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to test the positive transformants after induced by 1 mM IPTG for 4 h. The recombinant protein of RpSabl (rRpSabl) was purified by a Ni²⁺ chelating sepharose column, and refolded in gradient urea-TBS glycerol buffer. The concentration of refolded protein was measured by BCA method [19].

2.6. Preparation of antibody and Western blot analysis

Six weeks old mice were injected with the renatured protein rRpSabl in complete Freund's adjuvant (Sigma, USA) by hypodermic injection. Two weeks later, the rRpSabl in incomplete Freund's adjuvant (Sigma, USA) were hypodermically injected again. The next two injections were immunized at tail at one-week interval. The mice were sacrificed to collect immunized serum 7 days after the last immunization [11].

Samples of the recombinant protein were separated by SDS-PAGE. After electrophoresis, proteins were transferred onto a 0.45 mm nitrocellulose membrane at 300 mA for 1.5 h. The membrane was blocked with PBS containing 3% bovine serum albumin (BSA) at 37 °C for 1 h. After washed three times with PBS containing 0.05% Tween-20 (PBST), the membrane was incubated with anti-rRpSabl serum (1:1000 diluted in PBS). Then the membrane was washed with PBST for three times and incubated with goat-anti-mouse Ig-alkaline phosphatase conjugate (Southern Biotech, 1:5000 diluted in PBS) at 37 °C for 1 h. After washed for three times, the protein bands were stained with freshly prepared substrate solution containing nitroblue tetrazolium (NBT, Sigma, USA) and 5-bromo-4-chloro-3-indolylphosphate (BCIP, Sigma, USA) for 5 min

and stopped by washing the membrane with distilled water. Pre-immune serum was used as negative control.

2.7. PAMPs binding assay

The pathogen-associated molecular pattern (PAMP) binding activities of rRpSabl were performed by ELISA assay [16]. The microtiter plates were coated with 20 µg LPS, PGN, glucan or chitin (Sigma, USA) at 4 °C, respectively. After blocked with 3% BSA in PBS (200 µL/well) and washed three times with PBST, the plates were incubated with various concentrations of rRpSabl at 37 °C for 1 h, respectively. The plates were washed three times and incubated with 100 µL of rabbit anti-mouse serum dilution (1:1000 diluted in PBS) at 37 °C for 1 h, respectively. Then the plates were washed again and incubated with 100 µL goat-anti-mouse Ig-alkaline phosphatase conjugate (1:5000 diluted in PBS, Southern Biotech, USA) at 37 °C for 1 h. The wells were washed three times, and 100 µL of 0.1% (w/v) p-nitrophenyl phosphate (pNPP, Sigma, USA) was added to each well, and then incubated at room temperature in dark for 30 min. The reaction was stopped by 2 M NaOH and the absorbance was measured at OD₄₀₅. The PBS buffer was used as blank with pre-immune as negative control. Each experiment was carried out in triplicate. Samples with P (sample)–B (blank)/N (negative)–B (blank) > 2.1 were considered positive.

2.8. Microbe agglutination

The microbial agglutination assay was determined against Gram-positive bacterium (*Staphylococcus aureus*) and Gram-negative bacteria (*Vibrio harveyi*, *Vibrio splendidus*, *V. anguillarum*, *Enterobacter cloacae* and *Aeromonas hydrophila*) according to the previous report [13]. Briefly, the exponential bacteria were harvested and stained by crystal violet. The labeled microbes were then suspended in TBS-Ca²⁺ buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl₂, pH 7.5). Then 10 µL of microbe suspension was incubated with 25 µL of rRpSabl solution (final concentration of 1 µg/mL) at room temperature for 1 h, respectively. Microbes dissolved in TBS buffer were selected as the control. Thereafter, 10 µL of the mixture was mounted onto a glass slide and observed by a light microscopy (BX51, Olympus, Japan).

2.9. Swimming motility assay

Cell motility was examined on low-salt, soft-agar plates (1% tryptone, 0.25% NaCl and 0.3% agar) as described elsewhere [20,21]. Briefly, the Gram-positive bacterium (*S. aureus*) or Gram-negative bacteria (*V. harveyi*, *V. splendidus*, *V. anguillarum*, *E. cloacae* and *A. hydrophila*) were mixed with rRpSabl (final concentration of 1 µg/mL) and located on the soft-agar plates by puncturing inoculates halfway through the depth of the agar. Plates were incubated at room temperature for 8 h and monitored for the generation of bacterial halos as an indication of swimming motility. Plates were subsequently photographed with a digital camera to allow comparison of the relative swimming motilities of the different groups.

2.10. Antimicrobial activity

Bactericidal activity of rRpSabl against *E. coli* was evaluated by a liquid microdilution assay. Briefly, *E. coli* was incubated in liquid Luria-Bertani broth, and 100 µL of cell suspension (OD₆₀₀ = 0.01) was added to equal volume of rRpSabl (final concentration of 1 µg/mL). PBS alone was used as a negative control. Each sample was incubated at 37 °C and the OD₆₀₀ was measured at hourly intervals.

Attached biofilm formation was assayed in 96-well polystyrene plates (Corning Costar, USA) with crystal violet staining. Briefly, *E. coli* MG1655 was incubated with rRpSabl at concentrations of 0.1 and 1 µg/mL for 8 h. To remove growth effects, we normalized biofilm formation by dividing the total biofilm by the maximal bacterial growth as

measured by turbidity at 620 nm. Then the supernatant was poured out, and the plates were washed three times with room temperature water. After the plates were dried, 300 µL 0.1% crystal violet (completely dissolved in water solution) was added in each well for 20 min at room temperature. Then the staining solution was poured out, and the plates were washed three times. 300 µL 95% ethanol was added to each well and soaked for 5 min. The total biofilm was measured by turbidity at 540 nm. Ten replicate wells were repeated from two cultures independently.

2.11. Phagocytosis assay

Hemocytes from Manila clams were collected with equal volume of pre-chilled anticoagulant (Tris-HCl 50 mM; glucose 2%, NaCl 2%; EDTA 20 mM; pH 7.4). After harvested by centrifugation, hemocytes were re-suspended in TBS buffer (Tris-HCl 50 mM; CaCl₂ 5 mM) and incubated with 0.1 µg/mL or 1 µg/mL rRpSabl at room temperature for 30 min. Then 5 µL 3% fluorescent microsphere was added into each hemocytes suspension. The mixture was incubated for 1 h at room temperature, and phagocytosis was then analyzed using an Accuri C6 flow cytometer (BD).

2.12. Chemotaxis assay

Chemotactic properties of the rRpSabl were determined using PET cell culture inserts of 8.0 mm pore size (Becton & Dickinson, Franklin Lakes, NJ, USA) in 24-well plates [22]. Shortly, 250 µL of hemolymph from individual clams was added to the upper compartment, and 500 µL of rRpSabl (final concentration of 0.1 µg/mL or 1 µg/mL) or Tris-HCl (pH 8.0) were located in the lower compartment, respectively. After 4 h of incubation in the dark at room temperature, cells in the lower compartment were recovered, centrifuged and then counted using an Accuri C6 flow cytometer (BD).

2.13. In vitro encapsulation assay

In vitro encapsulation assay was performed according to the previous study [11]. Shortly, Ni-NTA agarose beads (Qiagen, Germany) were equilibrated in TBS buffer containing 10 mM CaCl₂, and then incubated with rRpSabl (the final concentration of 1 µg/mL) shaking at 4 °C overnight. Protein-coated beads were washed with TBS for three times, and then suspended in TBS. The hemolymph was withdrawn and diluted in anticoagulant, and then suspended in 200 µL of TBS. After the hemocytes were settled down into a 48-well cell culture plate, 1 µL of the protein-coated agarose beads (120–150 beads) was added and incubated at room temperature. Encapsulation of the beads was observed and counted after 6 and 24 h by a light microscopy (BX51, Olympus, Japan).

3. Results

3.1. Homologous analysis of RpSabl

The full-length cDNA of RpSabl was deposited in GenBank database under the accession no. [AZS54114](#). RpSabl exhibited high identities with Sabls (or C1q domain-containing protein) from other mollusks. For example, RpSabl shared 63% similarity with sialic acid-binding lectin from *Crassostrea gigas* (XP_011442511). The eight invariant amino acid residues (positions F²⁴, F⁴², N⁴⁸, F⁶⁰, G⁶⁷, Y⁶⁹, F¹⁴⁷ and G¹⁴⁹) were totally conserved in RpSabl (Fig. 1).

3.2. Tissue distribution and temporal expression profiles after *V. anguillarum* challenge

The distribution of RpSabl transcripts in various tissues of un-challenged clams was analyzed by qRT-PCR. RpSabl mRNA transcripts

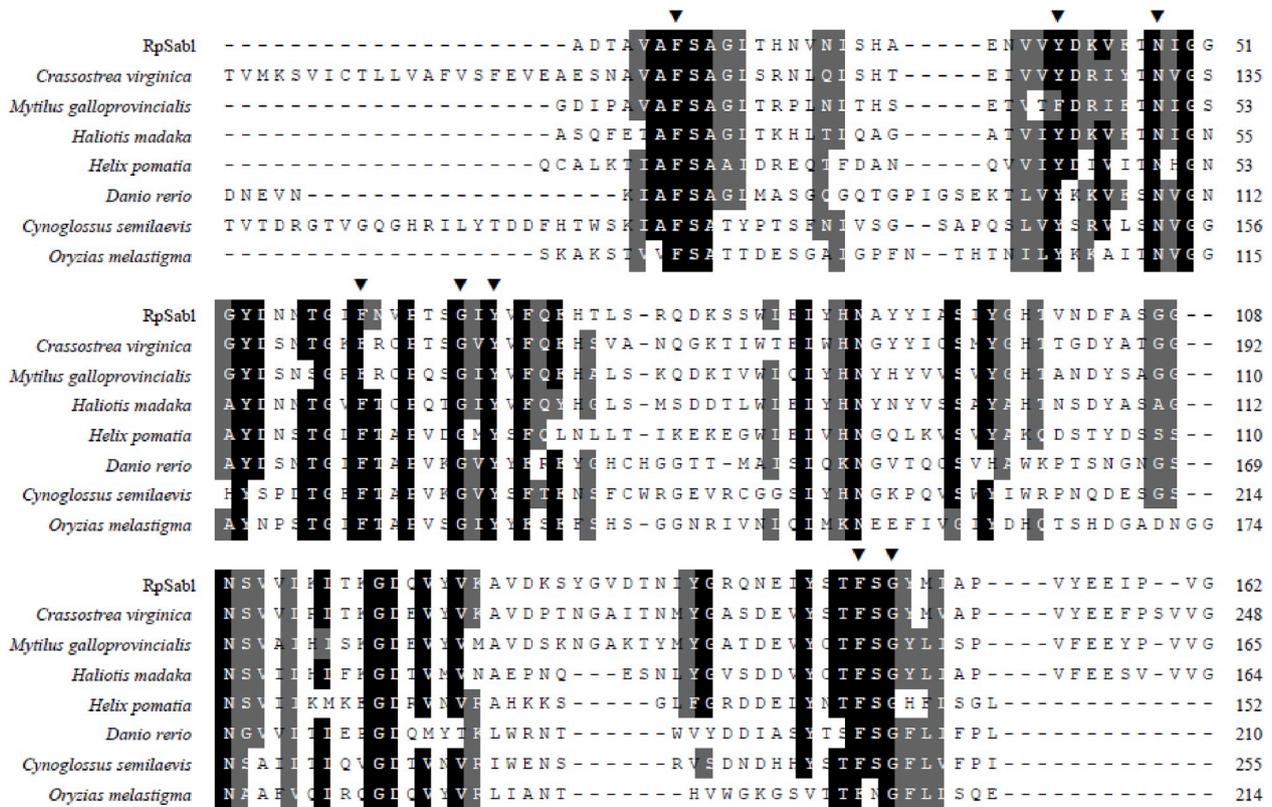


Fig. 1. Multiple alignments of RpSabl with Sabs from other animals, including *Crassostrea virginica* (XP_022323368); *Mytilus galloprovincialis* (CBX41690); *Haliotis madaka* (ALU63755); *Danio rerio* (AAI58180); *Cynoglossus semilaevis* (AIY69145); *Oryzias melastigma* (AEA51018). Conserved amino acid residues were shaded in dark, and similar amino acids were shaded in gray. Eight invariant amino acid residues were labeled by triangle (▼).

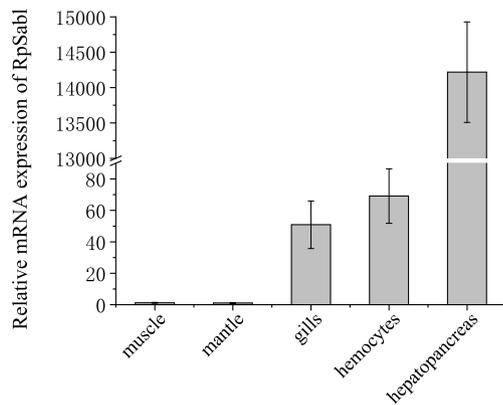


Fig. 2. Tissue distribution of RpSabl mRNA detected by qRT-PCR. Transcript level in mantle, gills, hemocytes, and hepatopancreas was normalized to that of muscle. Vertical bars represented the mean \pm S.D. (N = 6).

were predominantly expressed in hepatopancreas, moderately in hemocytes and gills, and marginally expressed in mantle and muscle (Fig. 2). The temporal expression profiles of RpSabl transcripts in hepatopancreas and hemocytes after bacterial challenge were shown in Fig. 3. After *V. anguillarum* stimulation, the mRNA expression of RpSabl in hepatopancreas was significantly up-regulated at 3 h (3.8-fold, $P < 0.05$), 6 h (4.9-fold, $P < 0.05$) and reached to the maximum value at 12 h (12.3-fold, $P < 0.01$). After that, the expression level of RpSabl transcripts decreased at 24 h (9.7-fold, $P < 0.05$) and 48 h (1.2-fold, $P > 0.05$) post challenge (Fig. 3A). However, the mRNA expression of RpSabl in hemocytes was up-regulated and reached the highest level at 6 h (8.5-fold of the control group, $P < 0.05$), then the expression level was down-regulated at 12, 24 and 48 h post stimulation (Fig. 3B).

3.3. Expression analysis and cumulative mortality after dsRNA injection

To assess the effect of silencing RpSabl on immune responses, 60 μ g of dsRpSabl was injected into the adductor muscle of each animal. The mRNA expression of RpSabl and several immune effectors (c-type lysozyme, p-type lysozyme, big defensin and defensin) were recorded at 72 h post *V. anguillarum* challenge. As revealed in Fig. 4A, *V. anguillarum* infection resulted in higher cumulative mortality in RpSabl-silenced clams than that of dsGFP- and PBS-injected groups. Silencing of RpSabl resulted in 30% mortality within a few days, whereas the dsGFP- and PBS-injected groups suffered little mortality. Meanwhile, the RpSabl transcripts in hemocytes were knocked down by 38% in clams after *V. anguillarum* challenge (Fig. 4B). During the period, the mRNA expressions of c-type lysozyme, p-type lysozyme, big defensin and defensin in RpSabl-silenced clams were significantly down-regulated to 0.48-fold ($P < 0.05$), 0.43-fold ($P < 0.05$), 0.58-fold ($P < 0.05$) and 0.36-fold ($P < 0.05$) of the control group (Fig. 4C), respectively.

3.4. Purification, refolding the recombinant protein and western blotting analysis

The purified protein of rRpSabl was analyzed on 15% SDS-PAGE with an apparent 18 kDa band visualized (Fig. S1, line 3), which was in accordance with the predicted molecular weight 17.6 kDa. The concentration of rRpSabl was determined to be 358.6 μ g/mL. Western blotting analysis was carried out to identify the specificity of antibodies. A clear reaction band was observed, supporting that the antibody could react with rRpSabl specifically (Fig. S1, line 4). Negative bands were not observed in the study (data not shown).

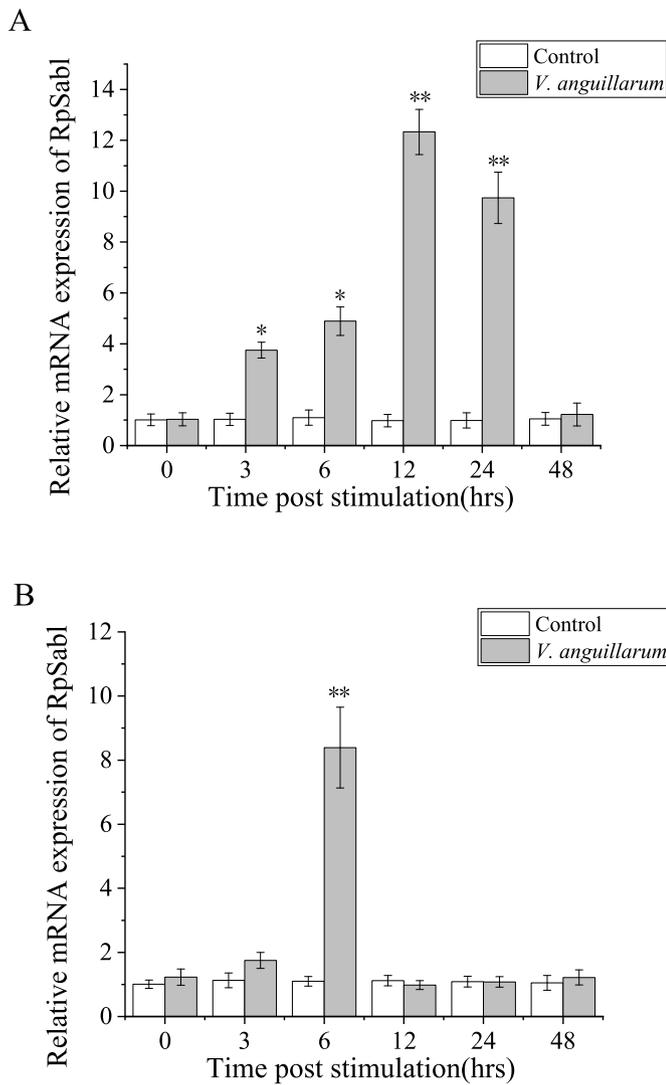


Fig. 3. Temporal expression of RpSabl mRNA in hepatopancreas (A) and hemocytes (B) of clams after *V. anguillarum* challenge. The values were shown as mean \pm S.D. (N = 6) (*: $P < 0.05$, **: $P < 0.01$).

3.5. PAMPs binding assay

PAMPs binding assays of rRpSabl were studied with some ligands. The binding activity was recorded as P/N value at 405 nm, and the samples with P/N > 2.1 were considered as positive. As revealed in Fig. 5, rRpSabl possessed obvious binding activities towards LPS, PGN and glucan in a dose-dependent manner. However, no binding activities towards chitin was observed in rRpSabl. The control groups could not bind any ligands.

3.6. Microbe agglutination assay

Several Gram-positive and Gram-negative bacteria were selected to test the microbial agglutination activity of rRpSabl. As revealed in Fig. 6A, rRpSabl showed obvious agglutinating activities towards *V. harveyi*, *V. splendidus*, *V. anguillarum*, *E. cloacae* and *A. hydrophila*, but not *S. aureus* (Fig. 6A). Meanwhile, swimming motility assay was examined on low-salt, soft-agar plates (1% tryptone, 0.25% NaCl and 0.3% agar), where the motility of Gram-positive bacterium (*S. aureus*) and Gram-negative bacteria (*V. harveyi*, *V. splendidus*, *V. anguillarum*, *E. cloacae* and *A. hydrophila*) was limited after incubated with rRpSabl (Fig. 6B).

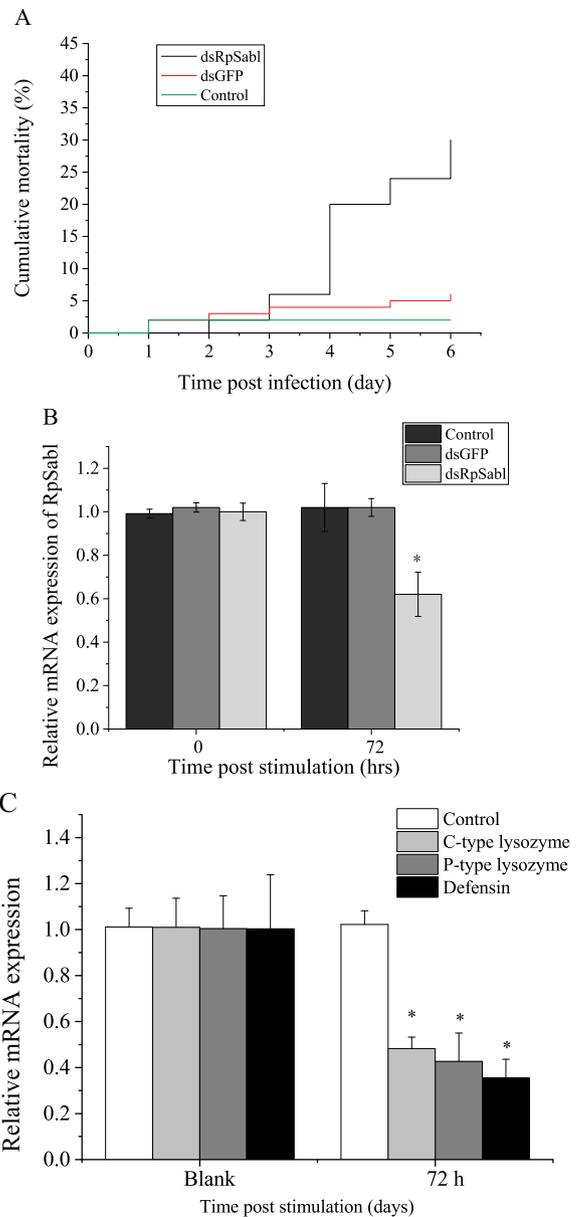


Fig. 4. Gene silencing of RpSabl in *Ruditapes philippinarum*. A. Cumulative percent mortality of clams post injection of dsRNA of RpSabl. The control and blank groups were injected with dsRNA of GFP and PBS, respectively. The mortality curve was determined using the Kaplan-Meier method. B. qRT-PCR analysis of RpSabl transcripts in hemocytes of clams post injection of dsRNA of RpSabl. C. qRT-PCR analysis of the expression of immune effectors transcripts in hemocytes of clams post injection of dsRNA of RpSabl.

3.7. Antibacterial activities

As revealed in Fig. 7, rRpSabl showed obvious inhibitory effect on the growth of *E. coli*. rRpSabl strongly suppressed the growth of *E. coli* during the exponential phase (2–6 h) and the stationary phase (6–8 h). Meanwhile, the incubation of RpSabl resulted in less biofilm dispersal of *E. coli* MG1655 on polystyrene surfaces, suggesting that RpSabl could dramatically reduce the biofilm formation in a concentration-dependent manner (Fig. S2).

3.8. Phagocytosis and chemotaxis assay

The phagocytosis assay was performed to test whether rRpSabl could enhance the phagocytosis ability of clam hemocytes. As revealed

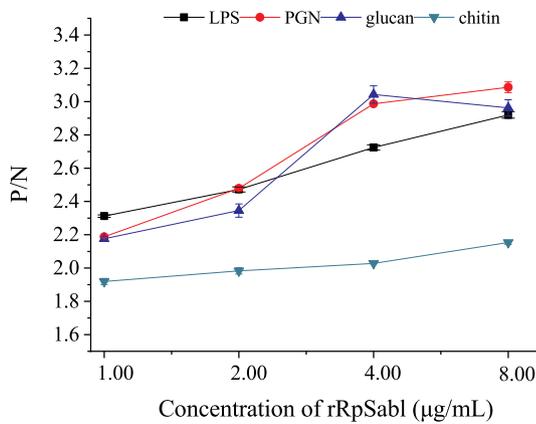


Fig. 5. PAMPs binding ability of rRpSabl. Plates were coated with LPS, PGN, glucan and chitin, and incubated with several concentrations of rRpSabl. After incubated with polyclonal antiserum against rRpSabl, the interaction was detected with goat-anti-mouse Ig-alkaline phosphatase conjugate at 405 nm. Samples with (sample – blank)/(negative – blank) > 2.1 were considered positive. Results were representative of average of three independent experiments.

in Fig. 8A, the phagocytic ability of hemocytes was 25.4% (1 µg/mL, $P < 0.05$) and 20.2% (0.1 µg/mL, $P < 0.05$) in the rRpSabl treatments compared with that of only 16.5% in the control group, indicating that rRpSabl could significantly enhance the phagocytic activity of clam hemocytes. However, rRpSabl elicited no remarkable chemotactic response from hemocytes, compared with the migration in control group (Fig. 8B).

3.9. In vitro encapsulation assay

rRpSabl-coated agarose beads were used to investigate the encapsulation ability of hemocytes towards non-self. After incubated with rRpSabl for 6 and 24 h, 45.0% and 89.0% beads were encapsulated by hemocytes from Manila clams compared with 6.0% in control group, respectively. After blocked by antibodies, only 10.0% protein-coated beads were encapsulated (Fig. S3), suggesting that rRpSabl could mediate the encapsulation ability of hemocytes.

4. Discussion

Sialic acid-binding lectins served as important pattern-recognition receptors (PRRs), which could specifically bind the terminal sugars, such as N-acetyl or N-glycolyl carbohydrates, glycoproteins and glycolipids [23]. To date, several Sabls have been reported in mollusks. However, the knowledge on their immune functions is still very limited in mollusks [9,15]. In the present study, a Sabl was identified from Manila clams, and the PAMPs recognition and binding activities, antibacterial activities, and opsonic activities were also investigated.

Generally, invertebrate Sabls exhibit a wide range of tissue expression distribution. For example, Sc-ghC1q from razor clam *Sinonovacula constricta* and AiC1qDC-1 from bay scallop *Argopecten irradians* distributed in all tested tissues including hemolymph, mantle, hepatopancreas and gills [24,25]. Some Sabls transcripts were found mainly expressed in host immune-related tissues, such as hepatopancreas and hemocytes [25]. In the present study, RpSabl transcripts were ubiquitously expressed in all tested tissues of healthy manila with higher expression level in hepatopancreas, hemocytes and gills. However, the transcripts of VpSABL previously detected in Manila clam was

dominantly expressed in mantle [15]. The different distribution of Sabls from Manila clam might serve for their various roles in immune functions.

Mollusks usually have open circulatory systems, and hemocytes are considered as the first line of defense system against invading pathogens. In the present study, the temporal expression pattern of RpSabl was investigated post pathogenic *V. anguillarum* challenge to clarify the involvement in the immune responses. The expression of RpSabl transcripts in hemocytes and hepatopancreas was significantly upregulated after bacterial challenge. The enhanced expression of Sabls upon induction was also detected in other species, such as *Crassostrea hongkongensis* [9] and *Mytilus galloprovincialis* [26]. The results suggested that RpSabl was an inducible acute-phase protein involved in immune responses of Manila clams. Moreover, knockdown of RpSabl transcripts caused significant increase in the cumulative mortality of manila clams post *V. anguillarum* challenge. In *Marsupenaeus japonicus*, the bacterial clearance ability of MjFREP2 was impaired by knocking down of MjFREP2. The cumulative mortality of MjFREP2-silenced shrimp was rather higher than that of the control group [27]. In *Hyriopsis cumingii*, knockdown of HcC1qDC6 inhibited the expression of tumor necrosis factor and whey acidic protein [28,29]. In the present study, reduced expression of RpSabl transcripts down-regulated the expression of several immune effectors in the hemolymph, indicating that clam mortality was attributable to the down-regulation of these immune effectors. The above results suggested that RpSabl protein was essential for the host survival from *V. anguillarum* infection.

In invertebrates, many lectins serve as PRRs that are involved in carbohydrate-mediated pathogen recognition, self/non-self discrimination, and regulation of the immune responses [9,30,31]. Recombinant Ch-sialectin from *C. hongkongensis* could bind to the sialic acid containing protein fetuin, and significantly inhibit the growth of both Gram-negative and Gram-positive bacteria [9]. In Pacific oyster, C1qDC-7 displayed strong binding affinity towards *V. splendidus* and *V. anguillarum* [32]. Recently, Sabls were known not only for their specific binding ability to sialic acid, but also for other non-self ligands [33]. For example, recombinant SgSABL-1 identified from the razor clam could recognize various ligands including LPS, PGN and β -glucan, and possess strong antibacterial activities against both Gram-positive and Gram-negative bacteria [13]. In the present study, rRpSabl showed obvious binding activities towards all the tested Gram-negative bacteria and several ligands, such as LPS and PGN, indicating that it might have broad PAMPs recognition spectrum. However, although rRpSabl possessed obvious binding activity towards PGN, no agglutinating abilities against *S. aureus* was observed. The reason may lie in the secretion of aminoglycosides modifying enzyme or repressor protein by *S. aureus* [34,35].

Mollusks were continuously exposed to a variety of pathogenic microorganisms in their surrounding environment, rendering the hosts possessed diverse lectins to protect themselves from the microbial invaders. Besides bacterial binding and agglutination activity, some mollusk lectins also showed inhibitory effect against the growth of bacteria. For example, oyster Ch-sialectin was able to inhibit the proliferation of Gram-negative bacteria *Vibrio alginolyticus*, *E. coli* and Gram-positive bacteria *S. aureus*, *Bacillus thuringiensis* [9]. In the present study, rRpSabl displayed obvious antibacterial activities towards *E. coli*, and such antibacterial abilities probably also caused the depression of *E. coli* biofilm formation even at low concentration. Likewise, the biofilm formation was limited by antibacterial peptide LL-37 at the very low and physiologically meaningful concentrations far below those required to kill or inhibit bacterial growth [36]. The reason might lie in the reduction of bacterial attachment or affection of their quorum-

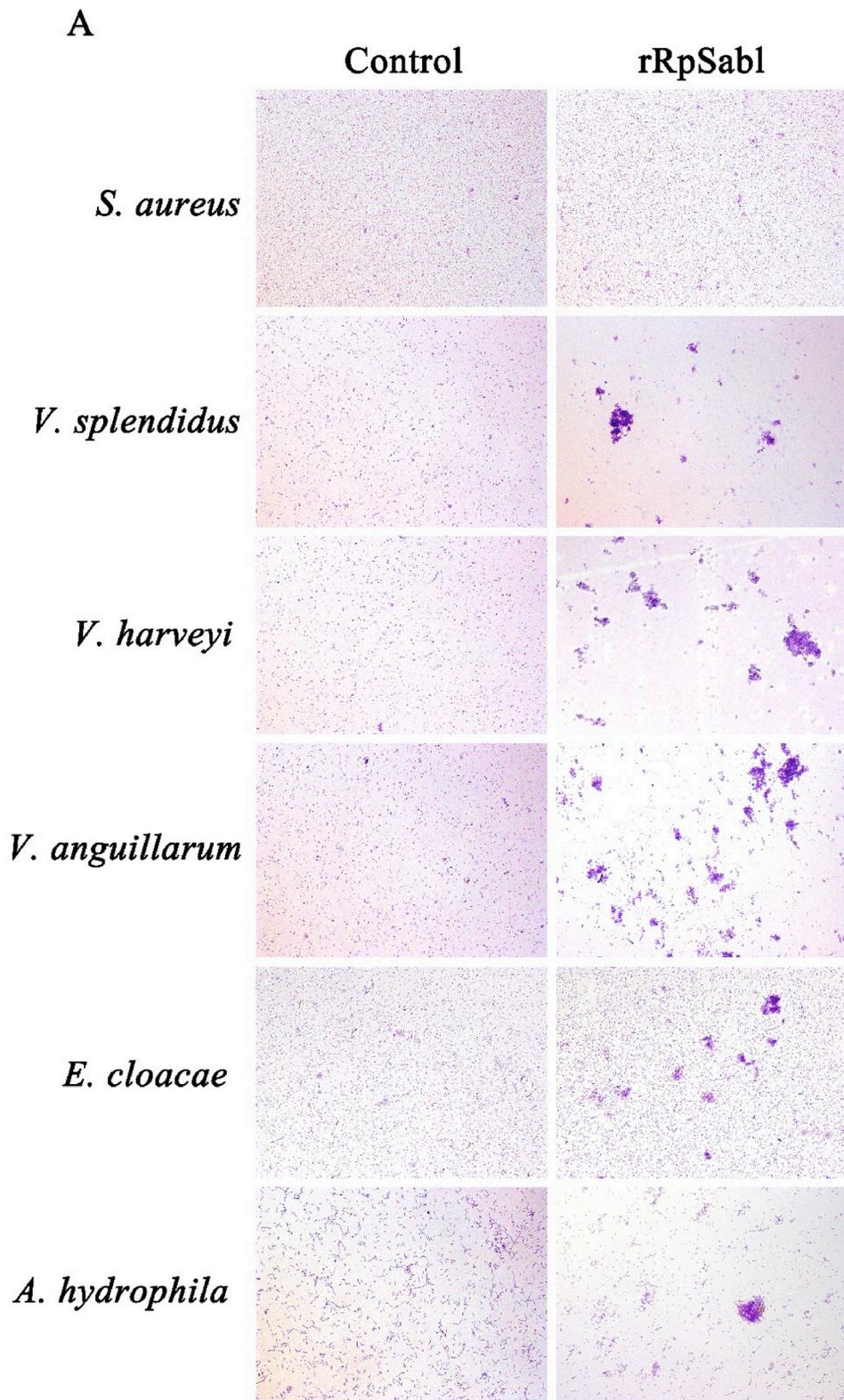


Fig. 6. The agglutination (A) and swimming motility (B) of Gram-positive bacterium (*Staphylococcus aureus*) and Gram-negative bacteria (*Vibrio harveyi*, *Vibrio splendidus*, *Vibrio anguillarum*, *Enterobacter cloacae* and *Aeromonas hydrophila*) induced by rRpSabl.

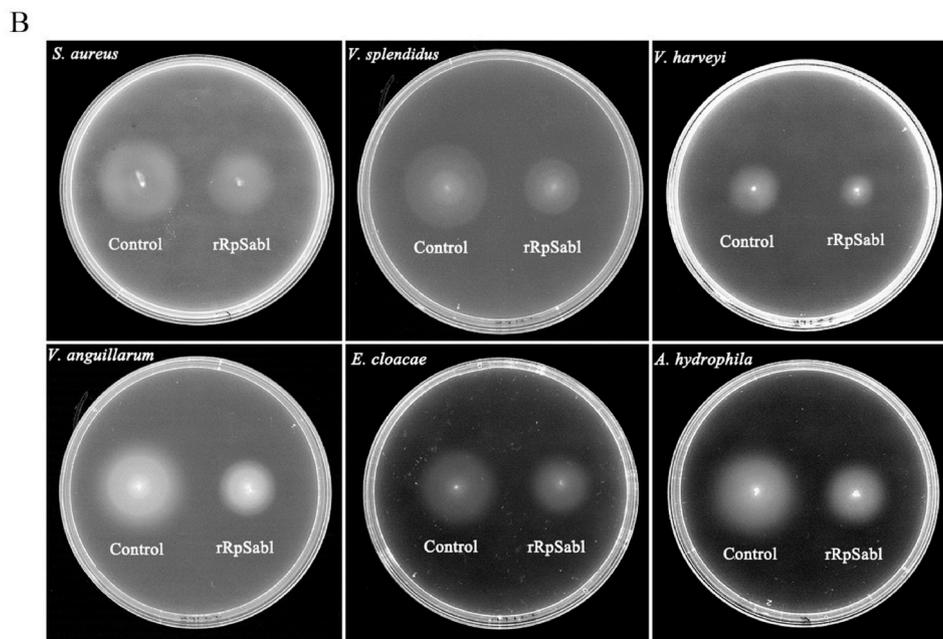


Fig. 6. (continued)

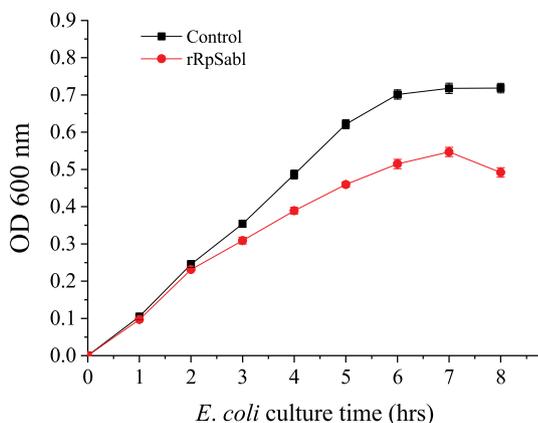


Fig. 7. Antibacterial activities of rRpSabl. Growth suppressive tests of rRpSabl against *E. coli* were measured at OD₆₀₀ every 1 h after starting the cultures. The data were the average ± S.D. of three independent cultures.

sensing systems by down-regulating the biofilm-development genes [34,37]. Overall, these data suggested that RpSabl had an important role in the host defense against invasive pathogens.

It is of prime importance to understand the opsonization activity of Sabs on hemocytes. In the present study, rRpSabl could enhance the phagocytic activity of hemocytes significantly. Similar results were reported in Zhikong scallop *Chlamys farreri* as well [38]. Moreover, the encapsulation ability of hemocytes could also be promoted by rRpSabl, which required the coordination from both cellular and humoral factors of the immune defense system [39,40]. For example, capsule formation involves cooperation between one or more classes of hemocytes and is likely mediated by cytokines and adhesion molecules [40]. Together,

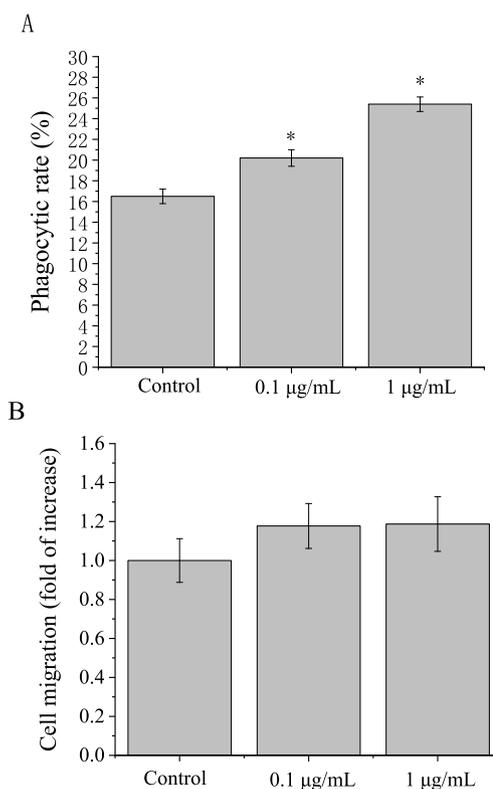


Fig. 8. Phagocytosis (A) and chemotactic activity (B) of hemocytes enhanced by rRpSabl. The values were shown as mean ± S.D. (N = 6) (*: $P < 0.05$, **: $P < 0.01$).

opsonization of hemocytes mediated by RpSabl, such as phagocytosis and encapsulation, was verified in the immune defense of Manila clam.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2019.08.074>.

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