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A novel C1q domain containing protein in black rockfish (*Sebastes schlegelii*) serves as a pattern recognition receptor with immunoregulatory properties and possesses binding activity to heat-aggregated IgG

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ABSTRACT

C1q-domain-containing (C1qDC) proteins, which are involved in a series of immune responses, are important pattern recognition receptors in innate immunity in vertebrates and invertebrates. Functional studies of C1qDC proteins in vertebrates are scarce. In the present study, a C1qDC protein (SsC1qDC) from the teleost black rockfish (*Sebastes schlegelii*) was identified and examined at expression and functional levels. The open reading frame of SsC1qDC is 636 bp, and the predicted amino acid sequence of SsC1qDC shares 62%–69% overall identity with the C1qDC proteins of several fish species. SsC1qDC possesses conserved C1qDC features, including a signal sequence and a C1q domain. SsC1qDC was expressed in different tissues and its expression was up-regulated by bacterial and viral infection. Recombinant SsC1qDC (rSsC1qDC) exhibited apparent binding activities against PAMPs including LPS and PGN. rSsC1qDC had antibacterial activity against *Vibrio parahaemolyticus*, and was able to enhance the phagocytic activity of macrophages towards *Vibrio anguillarum*. rSsC1qDC interacted with human heat-aggregated IgG. Furthermore, in the presence of rSsC1qDC, fish exhibited enhanced resistance against bacterial infection. Collectively, these results indicated that SsC1qDC serves as a pattern recognition receptor and plays a vital role in the defense system of black rockfish.

1. Introduction

The complement system, which consists of more than 30 soluble serum and cell-surface proteins, is important for the innate immune response [1,2]. C1q is a key recognition subcomponent of the complement C1 complex. Structurally, C1q contains a globular domain with remarkable ligand binding properties [3,4]. C1q also serves as a major link between innate immunity and adaptive immunity [5–7]. The C1q-domain-containing (C1qDC) family, a group of proteins with a globular C1q (gC1q) domain, can be divided into three subgroups based on their structural characteristics: (1) C1q with a signal peptide, a collagen-like region and globular domain; (2) C1q-like with a collagen-like region and globular domain; and (3) ghC1q with the globular domain only [8,9].

C1qDC proteins have been found in organisms ranging from bacteria to mammals [10]. However, the number of C1qDC genes has decreased dramatically during evolution. Reports have shown that there are 168 different C1qDC transcripts in the Mediterranean mussel (*Mytilus galloprovincialis*) and 337 in the Pacific oyster (*Crassostrea gigas*) [11,12]. In contrast; there are only 52 distinct C1qDC genes in the zebrafish (*Danio rerio*) genome and 31 in the human genome [13,14]. To date, numerous C1qDC proteins have been identified in invertebrates; some of them are involved in immune response functions, such as agglutination [15,16], phagocytosis [17], microbial recognition [18], and cell migration [19]. In teleost fish, recombinant C1qDC protein (sghC1q) of the half-smooth tongue sole (*Cynoglossus semilaevis*) exerted antimicrobial activities against Gram-negative and Gram-positive bacteria [20]. Japanese flounder C1qDC protein (C1ql3), and its gC1q domain C1ql3-part, were active against *Edwardsiella tarda*. C1ql3-part not only binds lipopolysaccharide and peptidoglycan, but also

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exerts antibacterial effects against *E. tarda in vivo*, which suggested that C1ql3 functions as a pathogen-recognition receptor [21]. However, the functional study of C1qDC in other teleost species is limited.

Black rockfish (*Sebastes schlegelii*), an important economic marine fish species, is extensively cultured in many countries, such as China, Korea, and Japan. This fish has suffered serious diseases, and the study of its responses to pathogen infection is limited. In this study, a novel C1qDC homolog from black rockfish (SsC1qDC) was characterized. The tissue distribution and expression pattern of *SsC1qDC* post-pathogen infection was examined. The binding activity and immunoregulation ability of recombinant SsC1qDC (rSsC1qDC) was investigated. Moreover, the role of rSsC1qDC in defense against bacterial infection was analyzed. These results will be helpful to further understanding of the biological functions of teleosts C1qDC proteins in innate immunity.

2. Materials and methods

2.1. Fish

Clinically healthy black rockfish (average weight 10.5 g) were purchased from a commercial fish farm in Shandong Province, China, and maintained at 20 °C in aerated seawater. Before experiments, fish were acclimatized in the laboratory for 2 weeks and verified to be free of pathogens in the liver, kidney, and spleen, as reported previously [22]. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma-Aldrich Corporation, St. Louis, MO, USA), as reported previously [23].

2.2. Bacterial and viral strains

E. tarda, *Vibrio alginolyticus, Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio ichthyoenteri*, *Aeromonas hydrophila*, *Pseudomonas putida*, *Micrococcus luteus*, and *Staphylococcus aureus* were preserved in the laboratory. *Escherichia coli* DH5α was purchased from Transgene (Beijing, China). All strains were cultured in Luria-Bertani broth (LB) medium at 37 °C (for *E. coli* DH5α, *Micrococcus luteus* and *Staphylococcus aureus*) or at 28 °C (all other microbes). Fish megalocytivirus, and infectious spleen and kidney necrosis virus (ISKNV) were kindly provided by Doctor Li of Pearl River Fishery Research Institute, Chinese Academy of Fishery Sciences, and were propagated in a continuous cell line established previously from the brain of *Siniperca chuatsi* [24].

2.3. Cloning of SsC1qDC

A cDNA library of black rockfish was constructed as reported previously [25]. The subsequent DNA sequence analysis showed that one of the clones contained the full-length cDNA of *SsC1qDC*, containing 5′- and 3′-untranslated regions (UTRs).

2.4. Sequence analysis

The cDNA and amino acid sequences of *SsC1qDC* were analyzed using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi), hosted in the National Center for Biotechnology Information (NCBI) website. Domain search was performed with the conserved domain search program of the NCBI website (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The theoretical molecular mass and the isoelectric point were predicted using EditSeq sequence editing software of the DNASTAR software package (DNASTAR, Inc., Madison, WI, USA). Multiple sequence alignments were created using the DNAMAN program (Lynnon Biosoft, Vaudreuil-Dorion, Quebec, Canada).

2.5. Quantitative real time reverse transcription-PCR (RT-qPCR) analysis of SsC1qDC expression under normal conditions

RT-qPCR analysis of *SsC1qDC* expression under normal conditions was determined as follows. Total RNA from the spleen, liver, kidney, blood, intestine, muscle, gill, heart, and brain (aseptically collected from five black rockfish), was extracted using the EZNA Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). RNA was digested with DNaseI. One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase kit (Invitrogen Corporation, Carlsbad, CA, USA). RT-qPCR was performed using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript RT-qPCR Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) [26]. The PCR reaction was performed in a 20 μl volume containing 10 μl SYBR® premix Ex Taq™ (Tli RNaseH Plus), 0.2 μM of each specific forward primer (5′– CAGGCGTTTATTACTTCACCATC –3′) and reverse primer (5′– GAACACTGCGTTTCCTCC ATTA –3′), and 2 μl diluted cDNA (50 ng/μl). The PCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 20 s. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified. The expression level of *SsC1qDC* was analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with beta-actin as an internal reference [27].

2.6. RT-qPCR analysis of SsC1qDC expression during pathogen infection

RT-qPCR analysis of *SsC1qDC* expression during bacterial infection was performed as reported previously [28]. *V. anguillarum* and *E. tarda* were cultured in LB broth at 28 °C to an optical density of 0.8 at 600 nm. Then, the cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS to a concentration of 1×10^6 CFU (colony forming units)/ml. ISKNV was prepared as described in section 2.2 and resuspended in PBS to a concentration of 1×10^6 copies/ml. Black rockfish were divided randomly into six groups (20 fish per group) and injected intraperitoneally with 50 μl V. *anguillarum, E.tarda,* ISKNV, or PBS. At 6, 12, 24, 48 and 72 h; or 1, 3, 5 and 7 d after infection, *SsC1qDC* expression was determined by RT-qPCR in the kidney and spleen, as described in section 2.3.

2.7. Construction of plasmids

To construct pColdC1q, which expresses the His-tagged recombinant SsC1qDC (rSsC1qDC), the coding sequence of *SsC1qDC* was amplified by PCR using primers SsC1qF1 (5′– GCCGCATATGCAAGAT GTTGGTAATGCTGCT –3′, the underlined sequence represents an *Nde*I site) and SsC1qR1 (5′– GCCGCTCGAGTCACATTTGAGTGACTAGAAA ACC –3′, the underlined sequence represents an *Xho*I site). The PCR products were ligated with the T-A cloning vector T-Simple (TransGen Biotech, Beijing, China) and the recombinant plasmid was digested with *Nde*I and *Xho*I to retrieve the *SsC1qDC*-containing fragment, which was inserted into pColdII (TaKaRa Biotechnology Co., Ltd., Dalian, China) at the corresponding sites, resulting in pColdC1q.

2.8. Expression, purification and reconstitution of recombinant protein

pColdC1q, and the control vector pColdII, were transformed into *E. coli Transetta* (DE3) (Trans, Beijing, China). The transformants were cultured in LB medium at 37 °C to the mid-log phase; the expression of His-tagged SsC1qDC was then induced by adding isopropyl-b-D-thiogalactopyranoside. Bacteria were incubated at 20 °C for 24 h RSsC1qDC was purified under denaturing conditions using nickel nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ, USA) according to the procedure recommended by the manufacturer. The purified protein was reconstituted as described previously [22]. The reconstituted proteins were dialyzed for 24 h against PBS at 4 °C and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA).

A previously reported turbot protein, recombinant suppressor of cytokine signaling 3 (rSmSOCS3) [29], was purified and reconstituted under the same conditions as rSsC1qDC, then used as a negative control. The purified protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining the gel with Coomassie brilliant blue. The concentration of the protein was determined using the Bradford method with bovine serum albumin as a standard.

2.9. Binding of rSsC1qDC with PAMPs

The binding of rSsC1qDC to LPS and PGN was determined according to a method reported previously [30]. Briefly, LPS or PGN (Sigma) was dissolved at a concentration of 50 μg/ml in 50 mM sodium bicarbonate (pH 9.8); 100 μl of the solution were then added to wells of a 96-well plate. The plate was incubated at 37 °C overnight and heated at 60 °C for 30 min. Each well was blocked with 50 μl of bovine serum albumin (BSA; 1 mg/ml) at 37 °C for 2 h and then washed three times with Tris buffer (50 mM Tris-Cl, pH 8, 50 mM NaCl). Different concentrations (7.5, 15 or 30 μg/ml) of rSsC1qDC solution (100 μl) were added to the wells and incubated at 18 °C for 2 h. The negative control wells were only filled with different concentrations of rSsC1qDC. The blank control wells were filled with 100 μl of Tris buffer. The plate was washed three times with Tris buffer. A mouse anti-His-tag antibody was then added, and the mix incubated at 37 °C for 1 h. Each well was washed four times and then incubated with 100 μl of horse-radish-peroxidase-conjugated rabbit anti-mouse Ig G antibody (diluted 1:1000) at 37 °C for 1 h. The plate was washed three times and color development was performed using the TMB Kit. The plates were read at 450 nm with a Precision microplate reader (Molecular Devices, Toronto, Canada). Positive readings were defined as absorbance values at least twice of that of the control.

2.10. Assay of antibacterial spectrum

Antibacterial spectrum assay was carried out as previously reported [31]. Bacteria were cultured to the mid-log phase. The cells were centrifuged, washed, and resuspended in PBS to 2×10^6 CFU/ml. Fifty μl of the suspension were plated on LB agar plates, sterile filter papers were slipped onto LB plates, and 5 μl of rSsC1qDC were added to the filter-paper. All plates were cultured for 24 h, and the antibacterial effect was determined according to the presence of an inhibition zone.

2.11. Killing kinetics

The killing kinetics were assayed as previously reported [31]. Suspensions of target bacteria were prepared as described above. The cells were centrifuged, washed, and resuspended in PBS at 1×10^6 CFU/ml. Then the cells were incubated with different concentrations of rSsC1qDC for 4 h. After incubation, the mixture was diluted in PBS, and 50 μl aliquots were plated on LB agar plates in triplicate. The inoculated plates were incubated for 24 h and clones visible clones on the plates were counted.

2.12. Heat-aggregated IgG interacting assay

Heat-aggregated IgG was prepared according to methods reported by Wang et al. [32] with little modifications. In brief, purified human IgG (Sigma) was prepared to a concentration of 500 μg/ml with TBS-NTC buffer (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 05% W/V NaN_3, 05% V/V tween-20 and 5 mM $CaCl_2$). After heating at 63 °C for 30 min, the IgG solution was cooled on ice immediately and stored at 4 °C for subsequent experiments. RSsC1qDC was coated overnight at 4 °C onto microtiter wells, in 0.2 M carbonate buffer (pH 9.6), at different concentrations (1.875, 3.75, 7.5, 15 and 30 μg/well). The same concentrations of rSmSOCS were used as control. The wells were washed and then blocked with PBS containing 3% (w/v) BSA for 2 h. After three rounds of washing, the wells were incubated with heat-aggregated human IgG (10 μg/well) in PBS-T at 18 °C for 2 h. Bound IgG was detected using goat-*anti*-human IgG-AP conjugate (Bioss, China) and pNPP (Sigma-Aldrich). Each experiment was repeated three times.

2.13. Preparation of head kidney macrophages

Black rockfish head kidney (HK) macrophages were prepared as reported previously [31]. Briefly, the HK of five fish was removed under aseptic conditions; it was then mixed and washed three times with PBS containing 100 U of penicillin and streptomycin (Thermo Scientific Hy Clone, Beijing, China). The tissues were placed on a metal mesh and pressed in 5 ml of L-15 medium (Thermo Scientific Hy Clone, Beijing, China) to generate cell suspensions. The suspensions were collected, washed twice, and resuspended in L-15 medium. HK macrophages were obtained from the cell suspensions by centrifugation in 34%–51% Percoll at $400 \times g$ for 30 min. The phagocytes appearing at the 34%–51% interface were collected, washed twice with L-15, and resuspended in L-15 supplemented with 15% calf serum and 1% penicillin and streptomycin (L-15S). The viability of the cells was examined using the trypan blue dye exclusion method. The cells were adjusted to 2×10^6 cells/ml in L-15S and distributed into 96-well cell culture plates.

2.14. Effect of rSsC1qDC on macrophages bactericidal activity

The phagocytic activity of macrophages was determined as reported previously [31]. Macrophages were incubated with 150 μM rSsC1qDC, rSmSOCS, or PBS in a total volume of 50 μl at 25 °C for 2 h. After incubation, the culture supernatant was discarded, and cells were washed with PBS three times. The cells were infected with *V. anguillarum* (10^5 CFU/well) and incubated at 28 °C for 5 h. Killing was stopped by adding 50 μl 0.2% Tween 20 to each well, followed by 50 μl LB medium (100 μl/well); the mixture was then plated on LB agar plates in triplicate. The plates were incubated at 28 °C for 48 h, and the visible colonies on the plates were counted. The killing index, which directly reflected the killing effect of macrophages, was defined as follows: 1 - (colonies incubated for 5 h/colonies incubated for 0 h). Experiments were repeated three times.

2.15. In vivo effect of SsC1qDC on bacterial infection

The *in vivo* effects of rSsC1qDC on pathogen invasion were determined by a method reported previously [33]. Briefly, *V. anguillarum* was prepared as above and incubated in TBS buffer (1×10^6 CFU) in the presence or absence of rSsC1qDC. Healthy black rockfish were randomly divided into four groups. The first group was the negative control and the other groups were injected with 9, 18, or 36 μg of rSsC1qDC or TBS. At 4 h after injection, fish in the four groups were injected intraperitoneally with 1×10^6 CFU of *V. anguillarum*. The kidney, liver, and spleen, the main organs infected by *V. anguillarum*, were collected aseptically from the fish at 12 h after infection. To examine bacterial loads in fish, the tissues were homogenized in TBS, and the homogenates were diluted in TBS and plated in triplicate on LB agar plates. The plates were incubated at 28 °C for 48 h, and the visible colonies on the plates were counted.

2.16. Statistical analysis

Statistical analysis was performed with SPSS software version 17 (Chicago, IL, USA). Data were analyzed with one-way analysis of variance, and statistical significance was defined as $P < 0.05$.

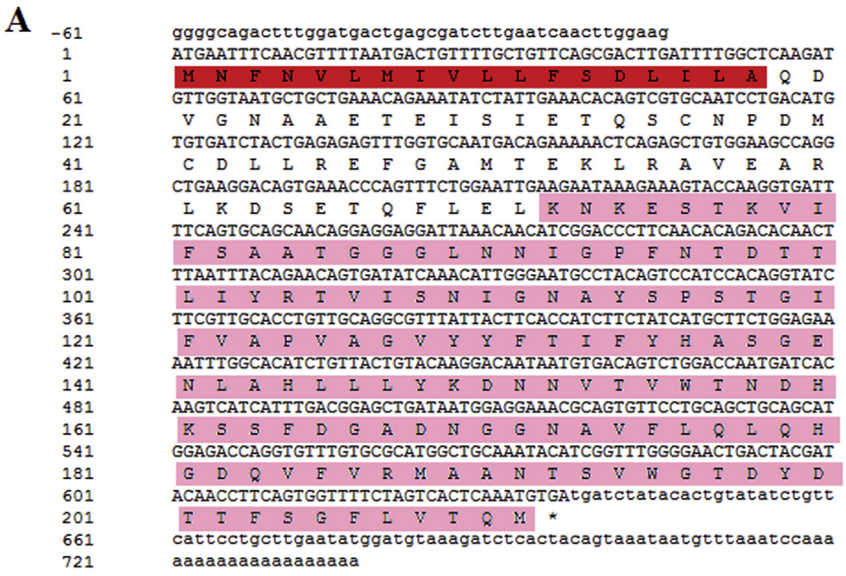

Fig. 1. Sequence analysis of *SsC1qDC*. (A) The nucleotide and predicted amino acid sequences of *SsC1qDC*. The nucleotides and amino acids are numbered along the right margin. The translation start (ATG) and stop (TGA) codons are in bold letters. The amino acid sequence of signal sequence and the C1q domain analyzed by SMART (http://smart.embl.de/) are boxed in red and pink, respectively. (B) Schematics of protein motifs of *SsC1qDC*. The signal sequence is indicated by a red rectangle, and the C1q domain is indicated by a pink hexagon. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Sequence analysis of SsC1qDC

The full-length cDNA of *SsC1qDC* contains a 5'-UTR of 45 bp, an open reading frame (ORF) of 636 bp, and a 3'-UTR of 106 bp (Fig. 1A). The ORF encodes a predicted protein of 211 amino acid residues with predicted molecular mass and theoretical isoelectric point of 23.1 kDa and 4.36, respectively. A signal peptide sequence (positions 1–18) and a C1q domain (positions 72–211) are found in SsC1qDC (Fig. 1B). BLAST analysis, using *SsC1qDC* as template against the available database, identified close matches in the C1q homologues of *Lates calcarifer*, *Larimichthys crocea*, *Labrus bergylta*, *Maylandia zebra*, *Kryptolebias marmoratus*, *Oreochromis niloticus*, and *Xiphophorus maculatus*, sharing 69%, 69%, 63%, 62%, 63%, 62%, and 62% overall amino acid sequence identities with SsC1qDC, respectively (Fig. 2). In contrast, SsC1qDC shares relative low sequence identity with C1qDC proteins from *Mus masculus* and *Homo sapiens* (17% and 33%, respectively) (Fig. 3). Phylogenetic analysis showed that SsC1qDC formed a separate cluster, which branched off from the group formed by C1qDC proteins from *L. calcarifer* and *L. crocea*.

3.2. Expression of SsC1qDC under normal physiological conditions

RT-qPCR analysis showed that under normal physiological conditions, *SsC1qDC* was expressed in all the examined tissues including blood, brain, muscle, kidney, spleen, intestine, heart, gills, and liver (Fig. 4). The difference in expression level between liver and blood was 12.4-fold.

3.3. Expression of SsC1qDC after pathogen challenge

To examine the effect of pathogen infection on *SsC1qDC* expression, black rockfish were challenged with the fish bacterial pathogens *V. anguillarum* and *E. tarda*, and viral pathogen ISKNV. *SsC1qDC* expression in kidney and spleen was analyzed by RT-qPCR at 6, 12, 24, 48 and 72 h post-infection (hpi) for *V. anguillarum* and *E.tarda*; or 1, 3, 5, and 7 d after infection (dpi) for ISKNV. After *V. anguillarum* infection, *SsC1qDC* expression in kidney increased at 6 hpi, peaked at 12 hpi (9-fold), and returned to the normal level at 72 hpi (Fig. 5A). The expression level of *SsC1qDC* in the spleen was significantly increased at all the examined time points, and peaked at 12 hpi (19.90-fold). Fig. 5B shows that *SsC1qDC* expression in kidney and spleen induced by *E.tarda* infection was remarkably increased at 6, 12, 24, and 48 hpi, with the highest expression occurring at 24 hpi (3.40-fold) and 12 hpi (13.32-fold). Fig. 5C shows that *SsC1qDC* expression in kidney induced by ISKNV infection significantly increased at 1 dpi and peaked at 3 dpi (14.52-fold). In spleen, the expression of *SsC1qDC* was significantly increased at 1, 3, 5 dpi, and peaked at 7 hpi (11.20-fold).

3.4. Binding of rSsC1qDC to PAMPs

rSsC1qDC, a recombinant protein expressed with His-tag *in vitro*, was purified from *E. coli* (Fig. S1). To characterize the PAMP recognition ability of rSsC1qDC, ELISA was used to measure the binding of rSsC1qDC to LPS and PGN, the major constituents of the outer membranes of Gram-negative and Gram-positive bacteria, respectively. The results showed that the binding activity of rSsC1qDC to LPS and PGN increased significantly as concentrations increased from 7.5 to 30 μg/ml, and rSsC1qDC exhibited higher affinity for LPS than for PGN (Fig. 6).

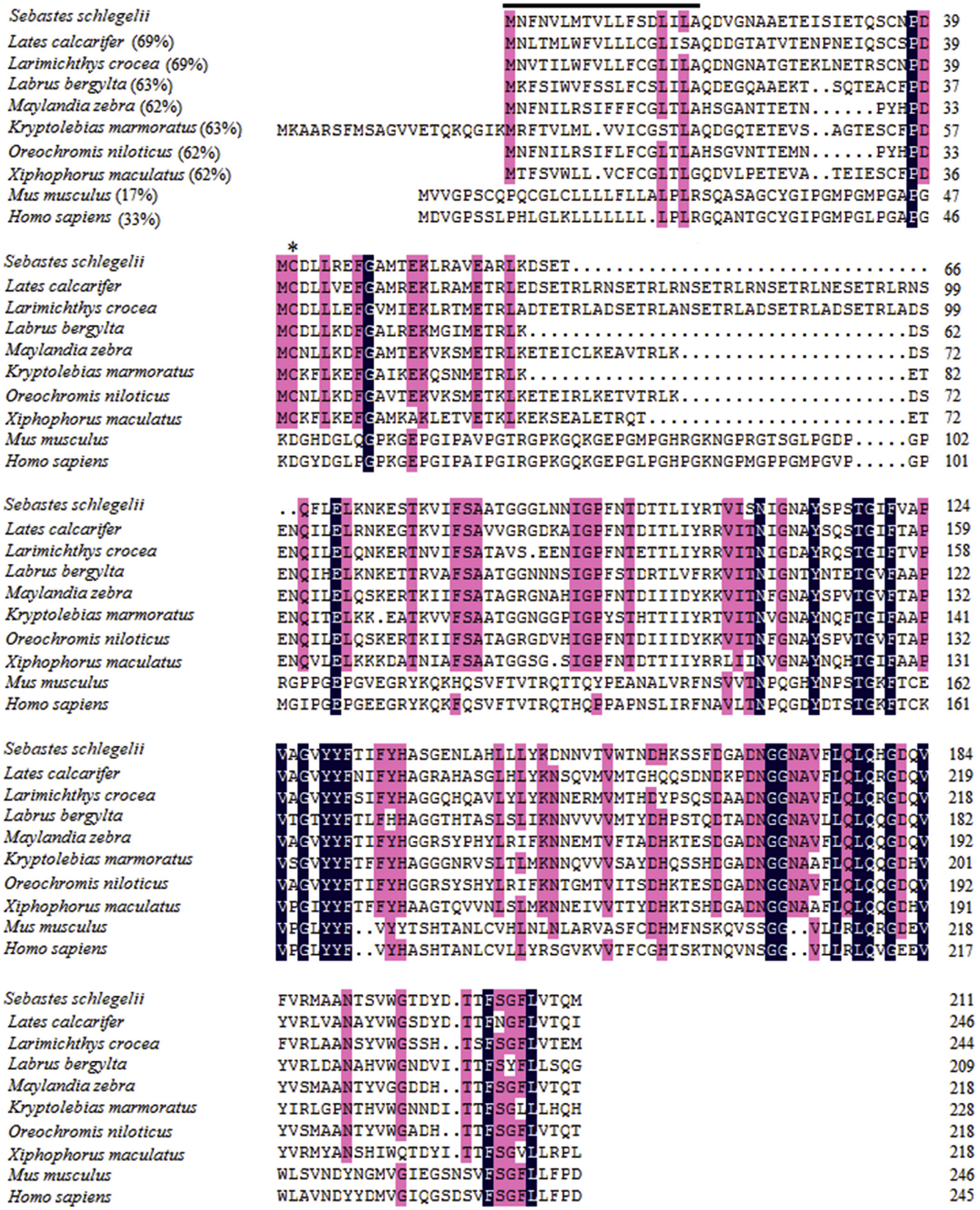

Fig. 2. Alignment of the predicted amino acid sequences of *SsC1qDC* homologues. The percentage number in the bracket following each species name represents the overall sequence identity between *SsC1qDC*and the specified species. The consensus residues are in blue, the residues that are ≥75% identical among the aligned sequences are in pink. The conserved cysteine residues are indicated by an asterisk, the signal sequence is indicated by "-". The Gen Bank accession numbers of the aligned sequences are as follows: *Lates calcarifer*, XP_018518064.1; *Larimichthys crocea*, XP_010754386.2; *Labrus bergylta*, XP_020504487.1; *Maylandia zebra*, XP_004563635.2; *Kryptolebias marmoratus*, XP_017287513.1; *Oreochromis niloticus*, XP_013132711.2; *Xiphophorus maculatus*, XP_023202885.1; *Mus musculus*, EDL29927.1; *Homo sapiens*, NP_758957.2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

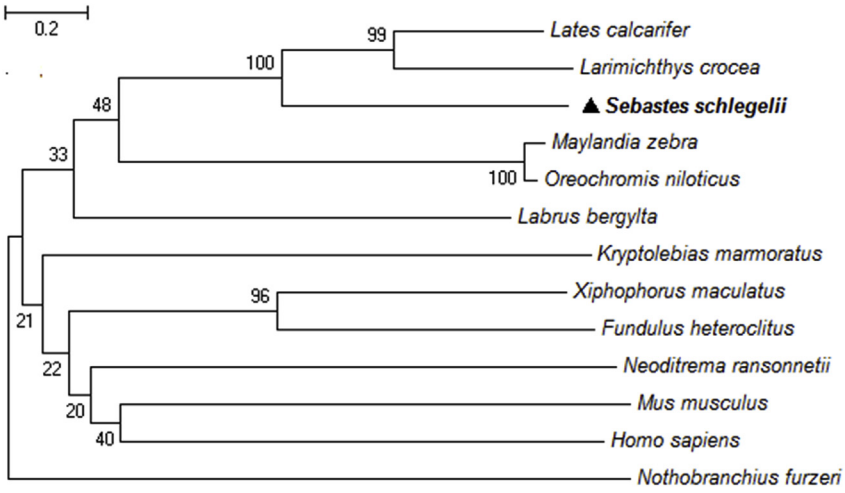

Fig. 3. Phylogenetic analysis of SsC1qDC and of other C1qDC proteins. The phylogenetic tree was constructed with MEGA 6 software (http://www.megasoftware.net/) using the neighbor-joining method. *SsC1qDC* is indicated by a triangle. Numbers beside the internal branches indicate bootstrap values based on 1000 replications. The accession numbers of the analyzed sequences are as follows: *Lates calcarifer*, XP_018518064.1; *Larimichthys crocea*, XP_010754386.2; *Labrus bergylta*, XP_020504487.1; *Maylandia zebra*, XP_004563635.2; *Kryptolebias marmoratus*, XP_017287513.1; *Oreochromis niloticus*, XP_013132711.2; *Xiphophorus maculatus*, XP_023202885.1; *Nothobranchius furzeri*, XP_015801800.1; *Neoditrema ransonnetii*, BAI40067.1; *Fundulus heteroclitus*, XP_012705358.1; *Mus musculus*, EDL29927.1; *Homo sapiens*, NP_758957.2.

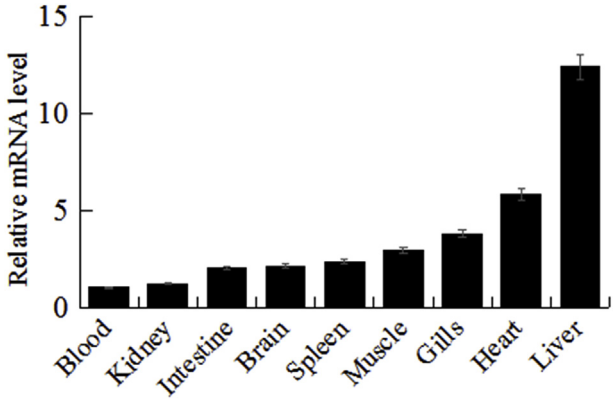

Fig. 4. *SsC1qDC* expression in fish tissues under normal physiological conditions. *SsC1qDC* expression in the blood, brain, muscle, kidney, spleen, intestine, heart, gills, and liver of black rockfish was determined by quantitative real time RT-PCR. The expression level of *SsC1qDC* in blood was set as 1. Values are shown as mean ± SEM (N = 3). N represents the number of times the experiment was performed.

3.5. Antimicrobial activity of rSsC1qDC

To detect the antimicrobial spectrum of rSsC1qDC, rSsC1qDC was incubated with the aforementioned bacterial strains on agar plates. RSsC1qDC exhibited antibacterial activity against the Gram-negative bacteria *V. parahaemolyticus*, the MIC (minimum inhibitory concentration) value was 60 mM. To further investigate the killing kinetics of rSsC1qDC against *V. parahaemolyticus*, *V. parahaemolyticus* was treated with different concentrations of rSsC1qDC or rSmSOCS for 4 h, and bacterial survival rates were determined by plate counting. The number of viable cells was dramatically reduced when the concentration of rSsC1qDC was above 1.875 μM. In contrast, treatment with rSmSOCS had no effect on bacterial survival (Fig. 7).

3.6. The interaction between rSsC1qDC and human heat-aggregated IgG

In order to determine whether rSsC1qDC could bind to human IgG, ELISA analysis was carried out and the results indicated that rSsC1qDC bound human heat-aggregated IgG in a dose-dependent manner, with the highest binding ability appeared when the concentration of rSsC1qDC was 7.5 μg/ml (Fig. 8).

3.7. Effect of rSsC1qDC on macrophage bactericidal activity

To investigate whether rSsC1qDC had any immune-regulating effect on fish macrophages, black rockfish macrophages were pretreated with rSsC1qDC before infection with *V. anguillarum*. The macrophage

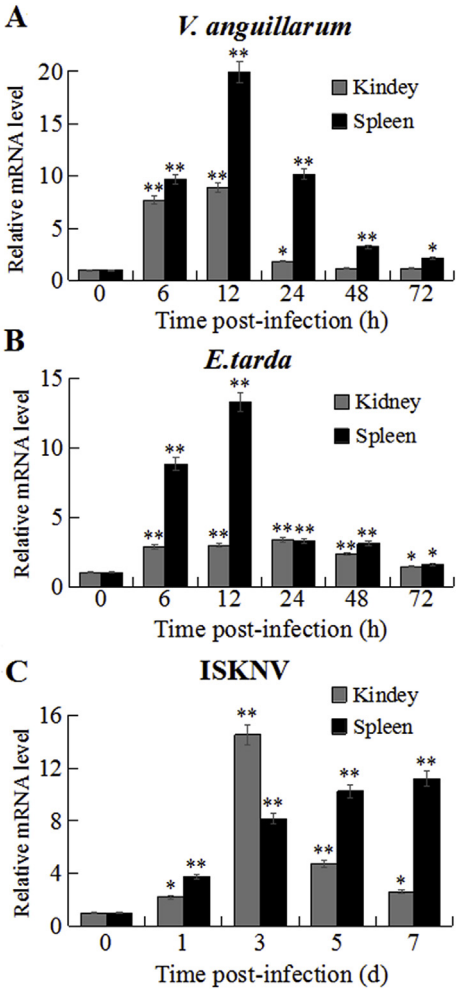

Fig. 5. *SsC1qDC* expression in response to pathogens challenge. Black rockfish were infected with *Vibrio anguillarum*, *Edwardsiella tarda*, or ISKNV and *SsC1qDC* expression in kidney and spleen was determined by quantitative real time RT-PCR at 6, 12, 24, 48, and 72 h post-infection (for *V. anguillarum* and *E. tarda*); or at 1, 3, 5, and 7 d post-infection (for ISKNV). In each case, the expression level of the control fish was set as 1. Values are shown as mean ± SEM (N = 3). N represents the number of times the experiment was performed. $^{**}P < 0.01$, $^{*}P < 0.05$.

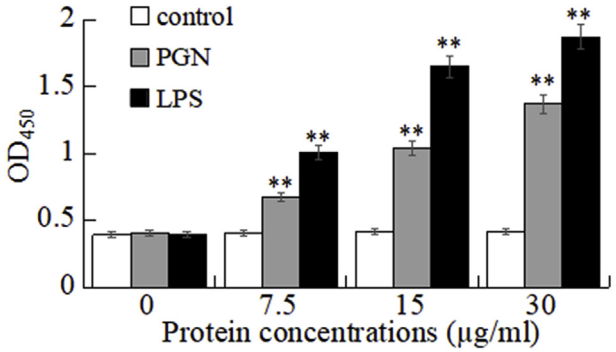

Fig. 6. Binding of rSsC1qDC to LPS and PGN. LPS or PGN were coated onto 96-well plates and then incubated with different concentrations of rSsC1qDC. The binding of rSsC1qDC to LPS and PGN was determined by ELISA. Values are shown as mean ± SEM (N = 3). N represents the number of times the experiment was performed. $^{**}P < 0.01$.

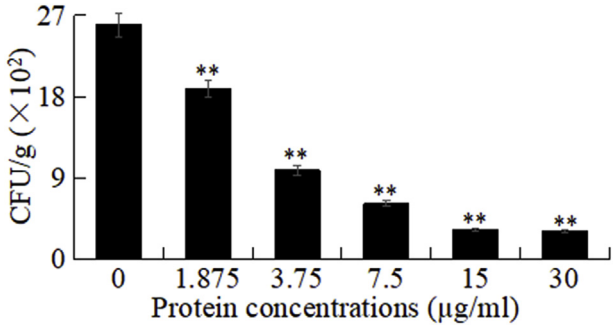

Fig. 7. Bacterial killing kinetics of rSsC1qDC against *Vibrio parahaemolyticus*. Bactericidal ability of rSsC1qDC at different concentrations against *V. parahaemolyticus* was detected. Values are shown as mean ± SEM (N = 3). N represents the number of times the experiment was performed. $^{**}P < 0.01$.

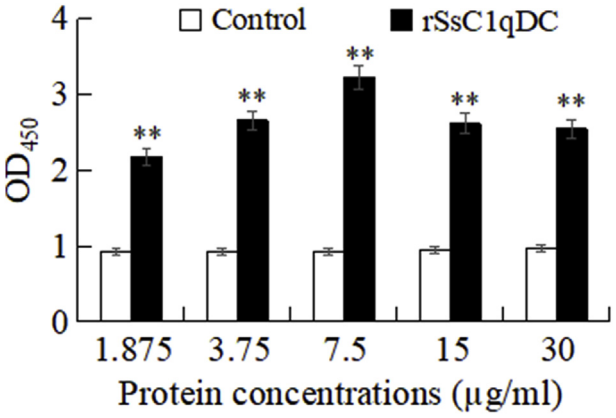

Fig. 8. Interaction of rSsC1qDC with human heat-aggregated IgG. Plates were coated with different concentrations of rSsC1qDC or rSmSOCS (control), and then incubated with human heat-aggregated IgG. The binding of rSsC1qDC to IgG was determined by ELISA. Values are shown as mean ± SEM (N = 3). N represents the number of times the experiment was performed. $^{**}P < 0.01$.

phagocytosis assay showed that the killing index of the macrophages treated with rSsC1qDC was significantly higher (2.28-fold) than that of macrophages incubated with rSmSOCS or PBS (Fig. 9), which indicated that rSsC1qDC had an immune-stimulating effect on macrophages.

3.8. *In vivo effect of rSsC1qDC on bacterial infection*

To investigate the *in vivo* effect of rSsC1qDC on bacterial infection,

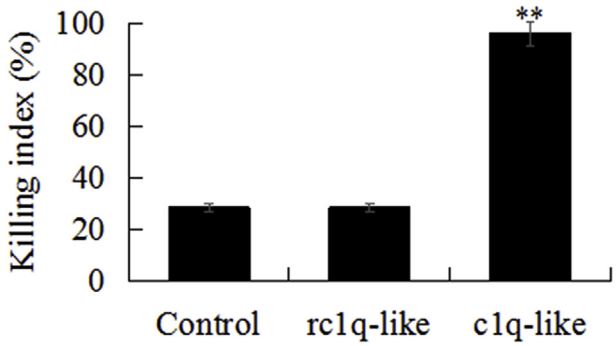

Fig. 9. Effect of rSsC1qDC on macrophage bactericidal activity. Black rockfish head kidney macrophages were treated with or without rSsC1qDC and incubated with *Vibrio anguillarum*. The killing index was determined after incubation. Data are presented as mean ± SEM (N = 3). N represents the number of times the experiment was performed. $^{**}P < 0.01$.

black rockfish were given different concentrations (9–36 μg/ml) of r*Ss*C1qDC before inoculation with *V. anguillarum*. Bacterial counts in the kidney, liver and spleen of the infected fish were determined at different times after infection. Twelve hours after infection, the numbers of *V. anguillarum* recovered from the kidney, liver and spleen of rSsC1qDC-treated fish were significantly lower than those from control fish (Fig. 10).

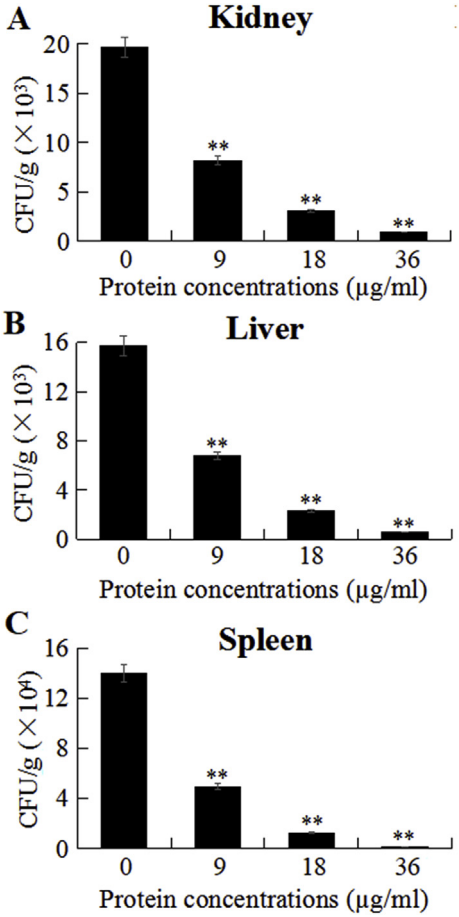

Fig. 10. *In vivo* effect of rSsC1qDC on pathogen infection. Black rockfish were given different concentrations of rSsC1qDC or PBS (control) before infection with *Vibrio anguillarum*. Bacterial loads in the kidney, liver, and spleen of the fish were determined at 12 h. Values are shown as mean ± SEM (N = 3). N represents the number of times the experiment was performed. $^{**}P < 0.01$.

4. Discussion

In this study, we identified and characterized a C1qDC gene, *SsC1qDC*, from the black rockfish, and examined its expression and biological properties. Structural analysis showed that SsC1qDC possesses conserved structural characteristics of C1qDC proteins, including a signal peptide and one typical complement component C1q domain, which is a common characteristic of C1q family members and is involved in modulating complement activation to contribute to the inflammatory process [34].

The amino acid sequence of SsC1qDC shares high identity (62%–69%) with C1qDC homologues of teleost fish, especially within the C1q domain, suggesting that it has conserved immune functions. A phylogenetic analysis indicated that SsC1qDC was clustered with the C1qDC proteins from *L. calcarifer* and *L. crocea.* The high sequence identity, together with the conserved C1qDC structural features, demonstrated that SsC1qDC is a new member of teleost C1qDC family.

In fish, C1qDC proteins were widely expressed in all of the tested healthy tissues, and the highest expression level was detected in the brain and the liver [20,21]. In this study, the expression of *SsC1qDC* occurred in all the examined tissues, and the highest expression was observed in liver, moderate expression was observed in heart and gills. Furthermore, the expressions of *SsC1qDC* in head kidney and spleen were significantly induced by fish pathogen *V. anguillarum* and *E. tarda*, which is similar to the results of other species. For example, in fish such as tongue sole and flounder, the expressions of *C1qDC* in kidney, liver, and spleen were induced by *V. anguillarum* and *E. tarda* [20,21]. In invertebrates, *C1qDCs* expression is enhanced by PAMPs or by bacterial challenge [35–38]. These results imply that *SsC1qDC* is involved in the immune response induced by bacteria and virus, and may play an important role in host defense against pathogen infection.

In vertebrates, complement C1q binds directly to PAMPs on the surfaces of many Gram-negative bacteria through the gC1q domain [39,40]. In the present study, rSsC1qDC bound LPS and PGN, indicating that SsC1qDC could serve as a PRR recognizing PAMPs.

Several C1qDC proteins have antibacterial effects and contribute to eliminate bacteria from the host. In Japanese flounder, PoC1ql3 exerted antimicrobial activity against *E. tarda* [21]. In tongue sole, CssghC1q (*Cynoglossus semilaevis*) was active against Gram-positive bacterium *Staphylococcus aureus* and Gram-negative bacteria *Vibrio harveyi, V. anguillarum*, and *Pseudomonas aeruginosa* [20]. Similarly, in this study, SsC1qDC was active against *V. parahaemolyticus*.

In vertebrates, the basis of the activation of the classical complement pathway is the interaction of complement C1q with immunoglobulins, via the gC1q domain [41,42]. In the present study, we found that rSsC1qDC binds heat-aggregated IgG in a dose-dependent manner, which strongly indicates that the SsC1qDC has the ability to bind immunoglobulins. The classical complement pathway is only present in species with immunoglobulins, however, rCfC1qDC, an invertebrate C1q protein from scallop *Chlamys farreri*, also has the ability to bind heat-aggregated IgG [32]. This phenomenon implies that the ancient invertebrate C1qDC proteins might have obtained the ability to bind immunoglobulins.

Several reports have demonstrated that C1qDC plays a crucial role in opsonization of macrophages, and in promoting elimination of pathogens. In mammals, complement C1q can coat bacteria and enhance the uptake of bacteria by phagocytic cells [43,44]. In scallop, rCfC1qDC could bind PAMPs from microbes and enhance the ability of hemocytes to phagocytize bacteria *in vitro* [32]. In *Crassostrea gigas*, a C1qDC protein CgC1qDC-1, significantly enhanced the phagocytosis of oyster hemocytes towards Gram-negative bacteria *E. coli* and *V. splendidus* [45]. Similarly, in this study, we found that rSsC1qDC could enhance the phagocytosis activity of black rockfish macrophages towards *V. parahaemolyticus*. These results collectively indicated that, in addition to a potential function as a PRR, C1qDC proteins work in opsonization, as complement C1q does. Consistent with above results, *in vivo* experiment showed that, compared to the control fish, fish administered with rSsC1qDC exhibited significantly reduced bacterial loads in the kidney, liver, and spleen, which implied a positive role of SsC1qDC in host defense against bacterial invasion. Similarly, in flounder, C1ql3-part exerted antibacterial effects against *E. tarda in vivo* [21]. At present, although numerous C1qDC proteins have been identified in other teleost fish, the study of their function in bacteria clearance is still scarce.

In conclusion, we identified and characterized a C1qDC homolog, SsC1qDC, from black rockfish. *SsC1qDC* expression was occurred ubiquitously in multiple tissues and significantly induced by bacterial or viral pathogens. *In vitro* experimental results indicated that rSsC1qDC not only bound LPS and PGN, but also displayed antibacterial activity against *V. parahaemolyticus*. Moreover, rSsC1qDC possessed the capability of interacting with heat-aggregated IgG and the capability of enhancing phagocytic activity of fish macrophages towards *V. anguillarum*. Results *in vivo* showed that rSsC1qDC promoted host clearance of bacterial pathogen.

Acknowledgements

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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.2019.01.004.

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