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Identification and molecular profiling of DC-SIGN-like from big belly seahorse (*Hippocampus abdominalis*) inferring its potential relevancy in host immunity

Eunyoung Jo [a, b], Don Anushka Sandaruwan Elvitigala [a, c], Qiang Wan [a], Minyoung oh [a], Chulhong Oh [b], Jehee Lee [a, *]

[a] Department of Marine Life Sciences & Fish Vaccine Research Center, Jeju National University, Jeju Self-Governing Province, 63243, Republic of Korea
[b] Jeju International Marine Science Research & Education Center, Korea Institute of Ocean Science & Technology (KIOST), Jeju Special Self-Governing Province, 63349, Republic of Korea
[c] Department of Chemistry, University of Colombo, Colombo 03, 00300, Sri Lanka

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ABSTRACT

Dendritic-cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) is a C-type lectin that functions as a pattern recognition receptor by recognizing pathogen-associated molecular patterns (PAMPs). It is also involved in various events of the dendritic cell (DC) life cycle, such as DC migration, antigen capture and presentation, and T cell priming. In this study, a DC-SIGN-like gene from the big belly seahorse *Hippocampus abdominalis* (designated as *ShDCS-like*) was identified and molecularly characterized. The putative, complete ORF was found to be 1368 bp in length, encoding a protein of 462 amino acids with a molecular mass of 52.6 kDa and a theoretical isoelectric point of 8.26. The deduced amino acid sequence contains a single carbohydrate recognition domain (CRD), in which six conserved cysteine residues and two Ca^{2+}-binding site motifs (QPN, WND) were identified. Based on pairwise sequence analysis, ShDCS-like exhibits the highest amino acid identity (94.6%) and similarity (97.4%) with DC-SIGN-like counterpart from tiger tail seahorse *Hippocampus comes*. Quantitative real-time PCR revealed that *ShDCS-like* mRNA is transcribed universally in all tissues examined, but with abundance in kidney and gill tissues. The basal mRNA expression of *ShDCS-like* was modulated in blood cell, kidney, gill and liver tissues in response to the stimulation of healthy fish with lipopolysaccharides (LPS), *Edwardsiella tarda,* or *Streptococcus iniae*. Moreover, recombinant ShDCS-like-CRD domain exhibited detectable agglutination activity against different bacteria. Collectively, these results suggest that ShDCS-like may potentially involve in immune function in big belly seahorses.

1. Introduction

Organisms are constantly being exposed to pathogens, and thus they possess immunological systems for combatting the infections caused by such pathogens (Akira et al., 2006). In this regard, innate immunity plays a key role in the detection of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), peptidoglycan, and lipoteichoic acid (LTA) from bacteria cell walls and beta-glucan from fungal cell walls. These molecules are present exclusively in bacteria and fungi and are crucial for their survival (Medzhitov, 2007; Medzhitov and Janeway, 2000). Pattern recognition receptors (PRRs) recognize PAMPs, triggering various immune responses including opsonization, phagocytosis, and expression of antimicrobial peptides and inflammatory cytokines (Janeway and Medzhitov, 2002). Several PRRs have been identified to date, including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and NOD-like receptors (NLRs) (Takeuchi and Akira, 2010).

CLRs were originally termed Ca^{2+}-dependent (C-type) carbohydrate-binding lectins to distinguish them from other types

* Corresponding author. Marine Molecular Genetics Lab, Department of Marine Life Sciences, Jeju National University, 66 Jejudaehakno, Ara-Dong, Jeju 63243, Republic of Korea.
E-mail address: jehee@jejunu.ac.kr (J. Lee).

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of lectins. CLRs contain at least one carbohydrate-recognition domain (CRD), which binds carbohydrates in the presence of Ca^{2+} ions. CLRs exist as either soluble or transmembrane proteins (Vasta et al., 1994; Zelensky and Gready, 2005). Soluble CLRs cause infectious agents to be coated or opsonized, which allows them to be ingested through opsonic receptors. In contrast, transmembrane CLRs directly recognize pathogens and mediate their uptake (Cambi et al., 2005; Kerrigan and Brown, 2009; Zelensky and Gready, 2005).

DC-specific ICAM-3-grabbing non-integrin (DC-SIGN, CD209) is a transmembrane CLR expressed on the surfaces of dendritic cells (DCs). As antigen-presenting cells, immature DCs ingest pathogenic invaders, inducing their maturation and migration to lymphoid organs (Geijtenbeek et al., 2000b). Mature DCs stimulate resting T cells by expressing MHC and costimulatory molecules (Liu et al., 2001). In the process, DC-SIGN is involved in several important DC functions, including DC migration, antigen capture and presentation, and T cell priming (Van Kooyk and Geijtenbeek, 2002). Movement of DCs from the blood into peripheral tissues prompts continuous detection of antigens. At this time, DC-SIGN mediates rolling and transendothelial migration of DC though the binding of ICAM-2, expressed on endothelial blood vessels (Geijtenbeek et al., 2000a). DC-SIGN can facilitate the internalization of soluble ligands from the cell surface by forming a complex with the ligand, which is targeted to lysosomal compartments in order to process the ligand for MHC class II-mediated antigen presentation to T cells. In addition, ICAM-3 is found at substantial levels on resting T-cells and interacts with DC-SIGN with high affinity, mediating the initial interaction between DCs and resting T cells (Van Kooyk and Geijtenbeek, 2002).

The big belly seahorse (*Hippocampus abdominalis*) is a large seahorse species found in both temperate and tropical marine waters, especially in oceans near Southeast Australia and New Zealand (Lourie et al., 2004; Woods, 2000, 2007). The seahorse possesses unique features including a horse-shaped head, large eyes, curvaceous trunk, and prehensile tail. They are used in traditional medicines and tonics and are maintained as aquarium pets or ornaments (Koldewey and Martin-Smith, 2010; Vincent et al., 1996). Thus, the seahorse trade has increased recently. However, because of direct exploitation, including wild catches and destruction of their habitats, the seahorse population has declined. As a result, seahorses were listed in the "Convention on International Trade in Endangered Species of Wild Fauna and Flora" (CITES) Appendix on May 2004, indicating that wild populations are threatened or might become threatened (Lourie et al., 2004). Commercial aquaculture of seahorses has been proposed as one possible solution for meeting the increasing demand (Koldewey and Martin-Smith, 2010). Hence, aquaculture of these creatures has been attempted in several countries, such as New Zealand, Australia, and China (Woods, 2007). Recently, complete aquaculture and artificial reproduction of the big belly seahorse was successfully carried out in Jeju, Korea. However, intensive aqua-farming is generally accompanied by the threat of massive crop mortality due to pathogenic infections. Thus, the identification of molecular components involved in the immune defense strategies of the big belly seahorse may help in the management of diseases of these animals, contributing to the maintenance of a sustainable seahorse aquaculture industry.

In this study, a DC-SIGN-like gene was identified from the big belly seahorse and was designated as *ShDCS-like*. Tissue-specific basal mRNA expression levels, expression patterns following immune challenge, and the agglutination and sugar binding activities of the recombinant ShDCS-like-CRD domain were investigated.

2. Materials and methods

2.1. Seahorse cDNA database

A seahorse cDNA database was established using 454 GS-FLX sequencing technology (Droege and Hill, 2008). Briefly, total RNA was isolated from the tissues (blood, liver, kidney, gill, and spleen) of 18 healthy seahorses. Then, the extracted RNA was purified by RNeasy Mini kit (Qiagen, USA). The quality and quantity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Canada), giving an RNA integration score (RIN) of 7.1. For GS-FLX 454 sequence database preparation, the RNA was fragmented into segments with an average size of 1147 bp using the Titanium system (Roche 454 Life Science, USA). Sequencing was accomplished on half of a picotiter plate on a Roche 454 GS-FLX DNA platform at Macrogen, Korea. The raw 454 reads were trimmed to remove adaptor and low-quality sequences, and de novo-assembled into contigs using GS Assembler (Roche 454 Life Science, USA) with the default parameters. The Nile tilapia genome was obtained from NCBI Genbank database and used as a reference genome for mapping analysis.

2.2. Sequence characterization of ShDCS-like

A sequence homologous to that of a known DC-SIGN was identified using our constructed cDNA sequence database and the NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). A putative open-reading frame (ORF) was demarcated on the identified cDNA sequence, and the corresponding amino acid sequence was derived using DNAsist 2.2 (version 3.0) software. Characteristic domains and motifs in the protein sequence were predicted by ExPASy PROSITE (http://prosite.expasy.org/) and SMART online servers (http://smart.embl-heidelberg.de). TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the transmembrane localization of the protein. ExPASy PROSITE online tool was used to identify the conserved cysteine residues forming the disulfide bonds. Pairwise sequence alignment was performed using the EMBOSS needle program (http://www.Ebi.ac.uk/Tools/emboss/align). A multiple sequence alignment of DC-SIGN like gene in teleost was performed using the Clustal Omega program, and a phylogenetic tree was constructed by MEGA version 5.05 software using neighbor-joining method with the bootstrap support of 5000 replicates (Tamura et al., 2011).

2.3. Experimental animals

Healthy seahorses were obtained from the Korea Marine Ornamental Fish Breeding Center on Jeju Island and acclimatized in 300-L tanks under a controlled environment (salinity 34 ± 6 psμ and temperature 18 ± 2 °C). All seahorses were maintained for one week feeding with frozen mysis shrimps twice, daily, and seahorses were not provided any food during the challenge experiment.

2.4. Tissue collection for tissue-specific expression analysis in healthy animals

To determine tissue-specific expression profiles, six seahorses (3 males and 3 females) with an average body weight of 8 g were selected. Blood was collected by injuring the tail of each animal, and the peripheral blood cells were separated by immediate centrifugation at 3000×*g* for 10 min at 4 °C.

Various tissues (brain, gill, heart, intestine, liver, kidney, muscle, pouch, skin, spleen, and stomach) were extracted and immediately snap frozen in liquid nitrogen. Tissue samples were stored at −80 °C until total RNA isolation.

2.5. Immune challenge experiment

To determine the transcriptional response upon immune stimulation, seahorses with an average body weight of 3 g were intraperitoneally injected with 100 μL of LPS (1.25 μg/μL), *Edwardsiella tarda* (5×10^3 CFU/μL), or *Streptococcus iniae* (10^5 CFU/μL) dissolved or resuspended in phosphate-buffered saline (PBS). A control group was injected with 100 μL of PBS. A group of non-injected fish served as a baseline (0 h) control. The peripheral blood cells, gill, liver, and kidney tissues were sampled from five individuals at 3, 6, 12, 24, 48, and 72 h post-injection (p.i.).

2.6. RNA isolation and cDNA synthesis

Total RNA was isolated from a pool of tissue samples (n = 6 for tissue-specific expression; n = 5 for immune challenge experiment) using RNAiso plus (TaKaRa, Japan) reagent followed by clean-up with an RNeasy spin column (Qiagen, USA) according to the manufacturer's protocols. The RNA quality was examined by 1.5% agarose gel electrophoresis, and the concentration was determined at 260 nm using a μDrop Plate (Thermo Scientific, USA). Then, 2.5 μg of RNA was used to synthesize cDNA from each tissue sample using the PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Japan) according to the vendor's protocol. Finally, the synthesized cDNA was diluted 40-fold and stored at −80 °C until use.

2.7. Expression analysis by quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed to determine mRNA levels of *ShDCS-like* in different tissues and to assess transcriptional modulations after immune challenge using the Real Time System TP800 Thermal Cycler Dice (TaKaRa, Japan) with 10-μL reaction volumes containing 3 μL of diluted cDNA template, 5 μL 2 × TaKaRa Ex Taq™ SYBR premix, 0.5 μL each of the forward and reverse primers (10 pmol/μL), and 1 μL of nuclease-free H_2O (Table 1). The qPCR was performed under the following conditions: 95 °C for 10 s; 35 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s; and a final cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Each reaction was performed in triplicates. Expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The same qPCR cycle profile was used for detection of the reference gene, seahorse 40 S ribosomal protein S7 (Accession number: KP780177). Relative mRNA levels are expressed as means ± standard deviations (SD). Expression levels in non-injected animals (0 h) were determined to provide basal expression levels. With respect to challenge experiments, expression levels within each challenge were further normalized to those of the corresponding PBS-injected controls at each time point. Statistical analysis of control (0 h) and experimental groups was carried out by one-way ANOVA with a significance level of $p < 0.05$.

2.8. Cloning of the ShDCS-like CRD-coding DNA

The corresponding coding sequence for the CRD of ShDCS-like was amplified using the primers ShDCS-like-F and ShDCS-like-R, which were designed with *EcoR*I and *BamH*I restriction sites, respectively (Table 1). PCR was performed in a TaKaRa thermal cycler in a total volume of 50 mL with 5 U Ex Taq polymerase (TaKaRa, Japan), 5 μL 10 × ExTaq buffer, 4 μL 2.5 mM dNTPs, 80 ng template, and 10 pmol each primer. The reaction was performed according to the following program: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 53.5 °C for 30 s, and 72 °C for 1 min 30 s; followed by a final extension at 72 °C for 5 min. Amplified PCR products and pMAL-c2X vectors were digested with the corresponding restriction enzymes and purified using AccuPrep™ gel purification kit (Bioneer Co., Korea) after analysis on a 1% agarose gel. The digested pMAL-c2X vector (146 ng) and PCR product (56.5 ng) were ligated using Mighty Mix (TaKaRa, Japan) at 4 °C overnight, transformed into *E. coli* DH5α cells, and sequenced. After sequence confirmation, the recombinant expression plasmid was transformed into *E. coli* BL21 (DE3) competent cells.

2.9. Overexpression and purification of recombinant ShDCS-like CRD (rShDCS-like)

The recombinant ShDCS-like CRD (rShDCS-like) was overexpressed in *E. coli* BL21 (DE3) using isopropyl-b-galactopyranoside (IPTG). Transformed *E. coli* BL21 (DE3) cells were grown in 500 mL LB broth supplemented with 100 mM glucose and 100 μg/mL ampicillin at 37 °C with shaking. When the OD_{600} of the bacterial culture reached approximately 0.8, expression of rShDCS-like was induced by adding IPTG (final concentration 0.2 mM) and incubated for 8 h at 20 °C with shaking. Cells were harvested by centrifugation (3500 rpm for 30 min at 4 °C), and the pellet was resuspended in column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl). The following day, *E. coli* cells were thawed and lysed in column buffer using cold sonication. Subsequently, the recombinant protein was purified by the pMAL protein fusion and purification system (New England Biolabs, USA) and eluted with elution buffer (column buffer + 10 mM maltose). The integrity and purity of rShDCS-like were evaluated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of the purified protein was measured using the Bradford method (Bradford, 1976).

2.10. Agglutination assay

The bacterial agglutination activity of rShDCS-like was assayed following methods described previously (Huang et al., 2014). Gram-positive and gram-negative bacteria were used to determine the agglutination activity of the recombinant protein. Microorganisms were harvested by centrifugation at 5000 rpm for 5 min, washed with TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.5) twice, and then resuspended in TBS to an OD_{600} of 5.0. Next, 25 μL of bacteria culture was added to 25 μL of the recombinant protein (200 μg/mL) or maltose binding protein (MBP) in the presence or absence of 10 mM $CaCl_2$. The mixtures were incubated at room temperature for around 3 hs, and then bacterial agglutination was observed using a light microscope. The assay was conducted in triplicate to assure the credibility of the results.

Table 1
Primers used in this study.

Name	Purpose	Sequence (5'→3')
ShDCS-like-F	CRD amplification	$(GA)_3$gaattcTGCCAAAGTGGCTGGAAGAAGTTT-(*EcoRI*)
ShDCS-like-R	CRD amplification	$(GA)_3$ggatccCTACATTTCACAAACCCAATATCTCTGTGA-(*BamHI*)
ShDCS-like-qF	qPCR	GATGATGGGAATGGCGGCTACAA
ShDCS-like-qR	qPCR	GCAACACCCATCAACCCAAGGATAAG
ShRP7-qF	qPCR primer internal reference	GCGGGAAGCATGTGGTCTTCATT
ShRP7-qR	qPCR primer internal reference	ACTCCTGGGTCGCTTCTGCTTATT

2.11. Sugar-specific binding assay

The sugar specificity of rShDCS-like was determined according to methods described previously (Chen et al., 2013). A constant volume (25 μL) of serial dilutions (3.9, 7.8, 15.6, 31.3, 62.5, 125, and 250 mM) of various sugars (D-glucose, D-mannose, galactose, sucrose, and lactose) were mixed with 25 μL rShDCS-like (100 μg/mL) in the presence of $CaCl_2$ (10 mM) and incubated for 30 min at room temperature. Thereafter, 25 μL of previously prepared *E. coli* was added to each mixture, and the mixtures were incubated for 40 min at room temperature. Subsequently, the minimum concentration of each carbohydrate required to inhibit bacterial agglutination was determined. The assay was performed in triplicate to assure the credibility of results.

3. Results and discussion

3.1. Sequence characterization of ShDCS-like

Based on BLAST-X analysis of the cDNA sequence data from *H. abdominalis*, we identified a DC-SIGN-like gene from the big belly seahorse, designated as *ShDCS-like*. The ORF of *ShDCS-like* consists of 1386 bps and encodes a 462-amino acid sequence with a predicted molecular mass of 52.6 kDa and isoelectric point of 8.26 (Fig. 1). ShDCS-like contains a single CRD (residues 337–461) at the C-terminal end and a transmembrane domain (residues 47–69) at the N-terminal end, as predicted by the SMART program. Analysis with the TMHMM program also showed that ShDCS-like has a transmembrane domain. The neck region of ShDCS-like is located between the CRD and the transmembrane domain, and it is known to help the CRD region bind with a glycan on the pathogen surface rather than with glycans on the DCs themselves (Feinberg et al., 2005; Quan et al., 2009). There are tandem repeats in the neck region DC-SIGN of human. Moreover, it can be divided into two family DC-SIGN, and DC-SINGR according to difference in tandem-neck-repeat region. A constant size exists in the tandem repeat of DC-SIGN. On the contrary DC-SIGNR has considerable polymorphism (Khoo et al., 2008). However, no tandem repeat region has been found among teleostan proteins (Lin et al., 2009), and this is true for ShDCS-like as well. Thus, all the teleostan counterparts can be designated as DC-SIGN like. According to our multiple sequence alignment analysis, ShDCS-like harbors a conserved CRD, in which four cysteine residues (C365, C460, C434, and C452) form two disulfide bonds between C365–C460 and C434–C452 (Fig. 2). CLRs contain double-loop structures, where one loop forms an antiparallel β-sheet from N- and C-terminal β strands and the other forms a long loop region within the domain. The disulfide bridges connect the whole domain loop and the long loop region. Some CLRs have an extension of the N-terminal domain that forms a β hairpin, which is stabilized by two additional cysteine residues. The presence of the first two cysteine residues (C337, C348) suggests that ShDCS-like is a "long form" CLR. Moreover, four Ca^{2+}-binding sites were identified in the CRD domain, termed sites 1, 2, 3, and 4. Among these, site 2 is involved in carbohydrate binding, with two notable motifs of CLRs. The first motif consists of Glu-Pro-Asn (EPN) or Gln-Pro-Asp (QPD), which selectively binds mannose or galactose respectively, and the second motif is composed of Trp-Asn-Asp (WND). ShDCS-like contains two conserved motifs, a QPN and WND motif in the Ca^{2+} binding site (Zelensky and Gready, 2005). In general, among most CLRs, the first motif is either EPN or QPD. However, in some cases, these can be replaced by EPD, EPK, EPS, QPG, QPS, or QPN, as reported previously (Guo et al., 2013). With

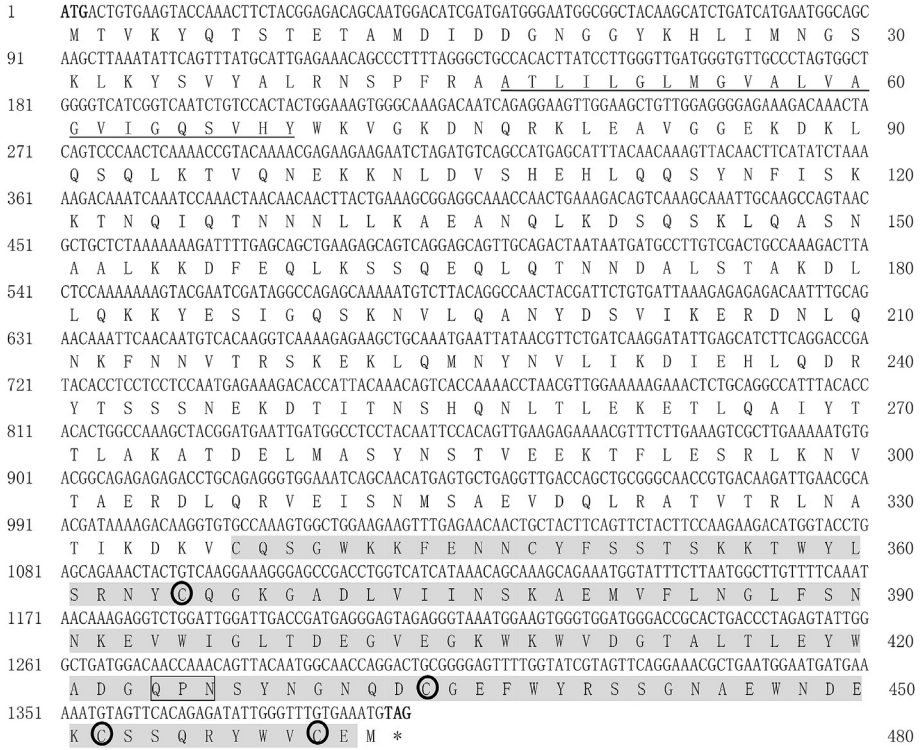

Fig. 1. Nucleotide and deduced amino acid sequences of ShDCS-like. Nucleotides are numbered on the left and amino acid positions are on the right. The start and stop codons are in bold. The transmembrane domain is underlined, and the putative CRD (337–461 aa) is shaded in gray. The conserved QPN motif is boxed and four cysteine residues forming disulfide bonds are circled.

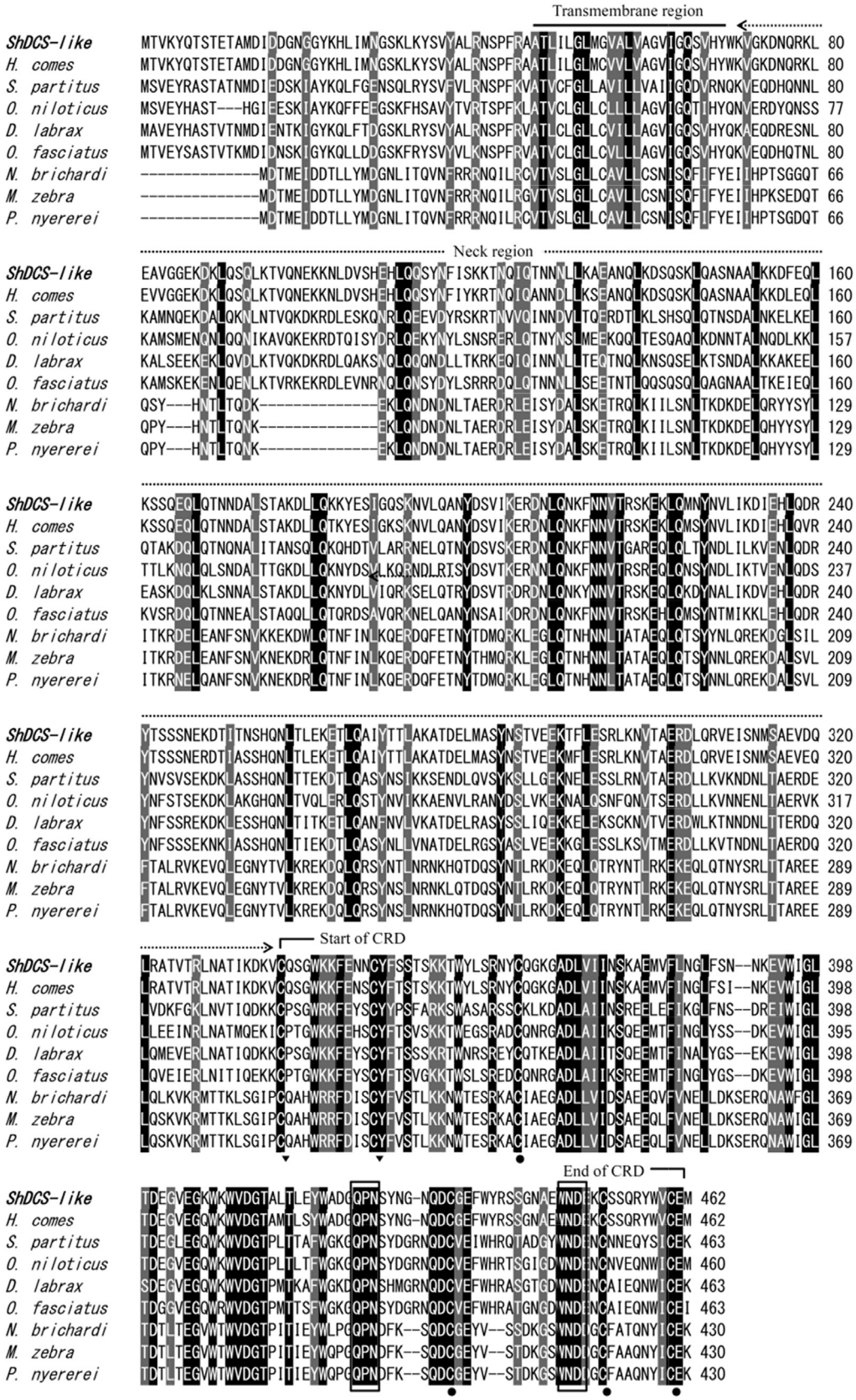

Fig. 2. Multiple alignment of ShDCS-like and its orthologs. Identical residues across all DC-SIGNs are in black, and similar amino acids are shaded in gray. Deleted amino acids in the alignment are indicated by dashes (–). Conserved cysteine residues involved in the formation of the CRD internal disulfide bridge are marked with ●, whereas two extra cysteine residues are marked with ▼. The two amino acid motifs are boxed.

respect to ShDCS-like, the presence of QPN motif was identified by *in-silico* analysis. Additionally some of the Pairwise sequence alignment showed that ShDCS-like exhibited the highest amino acid identity (94.6%) and similarity (97.4%) to a DC-SIGN-like protein from *Hippocampus comes* (Table 2). To evaluate the molecular evolutionary relationship of ShDCS-like with its homologs, a phylogenetic tree was constructed. As shown in Fig. 3, selected DC-SIGN like, DC-SIGN, DC-SIGNR molecules share a common vertebrate ancestral origin, forming a distinct main cluster in which the teleostan DC-SIGN-like proteins clustered independently. As expected, ShDCS-like clustered with the DC-SIGN-like counterparts. Collectins, known as soluble CLRs, formed a separate clade from vertebrate DC-SIGN or DC-SIGN-like proteins, showing a clear evolutionary distinction from these proteins. Collectively, our phylogenetic reconstruction reflects the vertebrate ancestral origin of ShDCS-like, affirming its clear homology with DC-SIGN-like counterparts from other species.

3.2. Tissue-specific expression of ShDCS-like

Relative basal expression levels of *ShDCS-like* were analyzed by qPCR in the blood, brain, gill, heart, intestine, liver, kidney, muscle, pouch, skin, spleen, and stomach in healthy big belly seahorses. The expression of *ShDCS-like* in each tissue was compared with that of the gene encoding big belly seahorse ribosomal protein S7 as an internal control, further normalizing the expression in each tissue to that in muscle. *ShDCS-like* mRNA was expressed in all tissues examined at different magnitudes, with the most pronounced *ShDCS-like* mRNA expression in the kidney and gill tissues and the weakest expression in muscle tissues (Fig. 4). Melanomacrophage centers (MMCs) consist of various pigments such as melanin, hemosiderin, and lipofuscin in an aggregated form (macrophage aggregate) and are found in hemopoietic tissues like the spleen and kidney. MMCs play many roles in immune functions, such as antigen recognition, by participating in antigen accumulation, migration of lymphocytes to MMCs, and association with antigen-presenting cells such as DCs (Agius and Roberts, 2003; Johansson et al., 2016; Lamers and Haas, 1985). Thus, *ShDCS-like* may be highly expressed in the kidney in order to recognize different pathogens effectively in combination with MMCs. Since the fish gill acts as the first barrier against infections (Uribe et al., 2011), high mRNA expression of *ShDCS-like* is likely to be detected in the gills. Similarly, *DC-SIGN* is highly expressed in lymphoid organs such as the thymus, bone marrow, lymph node, and spleen, in pigs (Huang et al., 2009). In various teleosts, the mRNA expression of *DC-SIGN* was predominantly detected in immune-related tissues including the blood, spleen, head kidney, and gills (Johansson et al., 2016; Yang et al., 2015).

3.3. The temporal expression profile of ShDCS-like following bacterial challenge

In order to determine changes in the expression of *ShDCS-like* upon infection, the mRNA expression levels of *ShDCS-like* were analyzed using qPCR in the liver, blood, gill, and kidney tissues after immune challenge with LPS, *S. iniae,* and *E. tarda.* In the kidney, the basal expression of *ShDCS-like* was significantly down-regulated at early and late phases of the experiment in response to all treatments, though a significant inductive transcriptional response was noted at 24 h post-infection (p.i.) upon LPS treatment (Fig. 5A). Similarly, in the gill, significant suppression of *ShDCS-like* basal transcription was observed at almost all time points p.i., upon each immune stimulation, with a single transcriptional up-regulation at 72 h p.i. upon *S. iniae* invasion (Fig. 5B). In contrast, blood cells showed inductive transcriptional responses at early phases post-treatment with LPS and *E. tarda*, along with late-phase transcriptional elevations at 72 h after *S. iniae* and LPS treatment (Fig. 5C). However, significant down-regulations in expression were noted at many time points after each treatment in blood cells. In contrast, mRNA expression of *ShDCS-like* was initially down-regulated and later up-regulated in liver cells upon LPS treatment, while it was initially and later down-regulated upon *E. tarda* invasion with a mid-phase transcriptional induction (Fig. 5D). Contrasting with the transcriptional modulation patterns observed in response to the above two stimuli, upon *S. iniae* treatment, *ShDCS-like* mRNA showed fluctuations in expression with consecutive up-regulations and down-regulations compared to the basal mRNA level. Collectively, the observations derived from our study clearly indicate that *ShDCS-like* is a potent responsive agent against bacterial infectious conditions.

Even though *ShDCS-like* was highly expressed in the kidney and gill tissues, mRNA expression levels were initially down-regulated under pathogen stress conditions. Interestingly, overall across all tissues, expression patterns of *ShDCS-like* seemed to fluctuate through down-regulation. According to extensive previous studies on DC-SIGN (CD209), regulation of C-type lectin expression was found to be complex and affected by various factors. IL-4, which acts through the JAK-STAT pathway with STAT6, is a positive factor for inducing *DC-SIGN* expression by inducing interactions between STAT 6 and *DC-SIGN* promoter region. However, IFN-γ inhibits the activation of IL-4-dependent genes by inhibiting tyrosine phosphorylation and nuclear translocation of STAT6, in turn suppressing *DC-SIGN* expression. In contrast, the uptake of antigens by DCs stimulates naive T cells to differentiate into T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 cells produce IFN-γ, causing inflammation and macrophage activation, whereas Th2 releases IL-4 to mount anti-inflammatory reactions (Relloso et al., 2002; Švajger et al.,

Table 2
Identity and similarity analysis of ShDCS-like with other DC-SIGN homologs.

Species	Identity (%)	Similarity (%)	Amino acids	Gene	Accession No.
Hippocampus comes	94.6	97.4	462	DC-SIGN like	XP_019745043.1
Oplegnathus fasciatus	61.6	78.0	463	DC-SIGN like	ACY66646.1
Dicentrarchus labrax	59.6	75.2	463	DC-SIGN like	ACF77004.1
Oreochromis niloticus	52.3	72.6	463	DC-SIGN like	XP_003448039.1
Stegastes partitus	51.2	72.6	463	DC-SIGN like	XP_008293368.1
Neolamprologus brichardi	30.9	48.8	469	DC-SIGN like	XP_006798969.1
Pundamilia nyererei	30.9	49.9	469	DC-SIGN like	XP_005726615.1
Maylandia zebra	30.5	50.3	469	DC-SIGN like	XP_004553763.1
Homo sapiens	22.4	35.0	532	DC-SIGN	AAK20997.1
Homo sapiens	21.6	34.9	518	DC-SIGNR	AAI10615.1
Rattus norvegicus	16.7	26.5	479	DC-SIGN	NP_001102319.1
Mus musculus	15.8	24.5	493	DC-SIGN	AAL13234.1

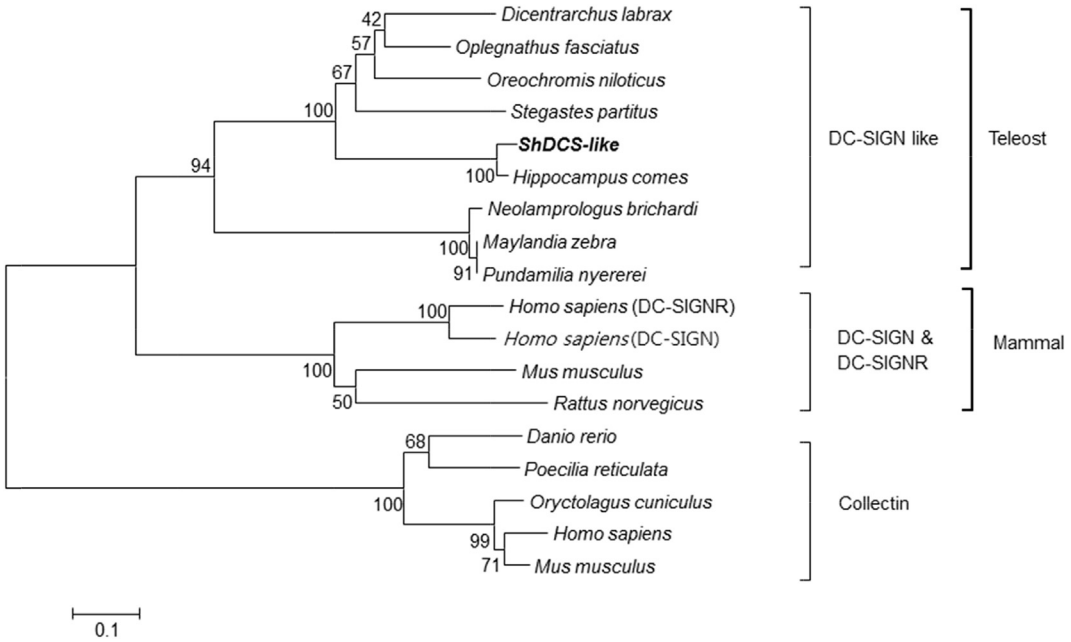

Fig. 3. Phylogenetic tree of DC-SIGN orthologs. The tree was constructed by MEGA 5.0 software using the neighbor-joining method with 5000 bootstrap replicates and included orthologs of DC-SIGN, collectin molecules. NCBI GenBank accession numbers are noted in Table 2 except for *Danio rerio* collectin sub-family member 11 (AAH85557.1), *Poecilia reticulata* collectin-11 isoform X2 (XP_008398081.1), *Oryctolagus cuniculus* collectin-11 isoform X3 (XP_008272779.1), *Homo sapiens* collectin (BAF79606.1), and *Mus musculus* collectin (AAI04382.2).

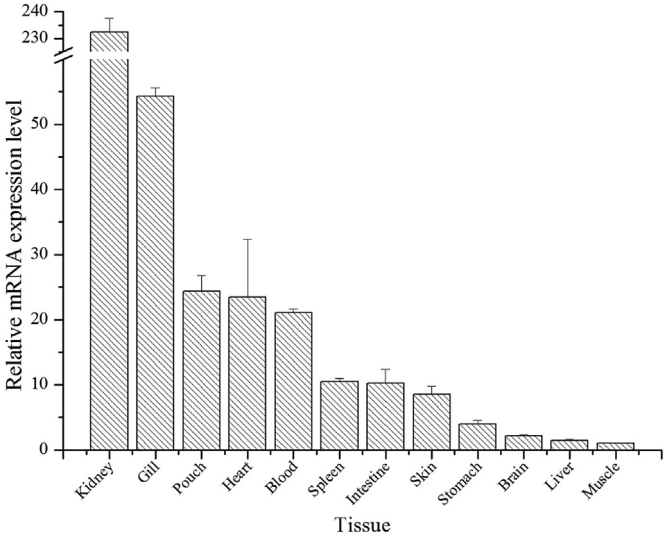

Fig. 4. Tissue-specific expression analysis of *ShDCS-like* mRNA detected by qPCR. Seahorse ribosomal protein S7 was used as a reference gene, and relative mRNA levels were normalized by those in muscle. The data are presented as mean ± SD (n = 3).

2010). However, following pathogen infections, the immune system of the host regulates Th1/Th2 development. In the event of host inflammation, either Th1 or Th2 development may occur, according to the infectious conditions. Thus, *DC-SIGN* expression may fluctuate according to IFN-γ expression or IL-4 expression based on T-cell development (Kitagishi et al., 2012). Similarly, the mRNA expression levels of the DC-SIGN-like gene of miiuy croaker also fluctuated in the spleen and kidney following *Vibrio anguillarum* challenge, resulting in decreased transcript levels at 6, 24, and 36 h p.i. and increased levels at 12 and 48 h p.i. (Shu et al., 2015). Overall, these data indicate that *ShDCS-like* plays a potential role in the seahorse innate immune system. However, extensive studies need to be carried out with cell fractions from different DC differentiation stages in order to confirm the fluctuations in the mRNA expression patterns detected in the current study.

3.4. Overexpression and purification of rShDCS-like

The coding sequence of the ShDCS-like CRD was ligated into a pMAL-c2X vector, overexpressed in *E. coli,* and purified using a pMAL purification system. The purified protein was analyzed by SDS-PAGE, showing the presence of ShDCS-like in induced cells and not in un-induced cells based on the intensity of the band corresponding to the expected size, further confirming that the fusion protein was successfully purified with high integrity. The molecular weight was observed to be approximately 57.5 kDa, consistent with the predicted mass (MBP 42.5 kDa + rShDCS-like 15 kDa) (Fig. 6).

3.5. Agglutination assay

C-type lectin can inhibit epithelial adhesion and colonization of bacteria by binding or causing agglutination (Sahly et al., 2008). To determine the potential agglutination activity of rShDCS-like, an agglutination assay was performed against gram-negative (*E. coli, E. tarda,* and *Aeromonas hydrophila*) and gram-positive (*S. iniae*) bacteria using rShDCS-like. As predicted, the bacterial agglutination activity of rShDCS-like depends on Ca^{2+}. In the presence of Ca^{2+}, rShDCS-like resulted in agglutination of *E. coli* and *S. iniae,* as shown in Fig. 7, confirming that Ca^{2+} is essential for agglutination. As expected, MBP-treated bacterial cultures did not show any detectable agglutination, even in the presence of Ca^{2+}, suggesting that there is no notable effect of the MBP in the rShDCS-like fusion protein on bacterial agglutination. However, rShDCS-like did not result in the agglutination of *E. tarda* or *A. hydrophila,* suggesting selectivity in its bacterial agglutination activity. The strength of agglutination differed with respect to the bacterial species. As observed by microscope, agglutination of *E. coli* was stronger than that of *S. iniae.*

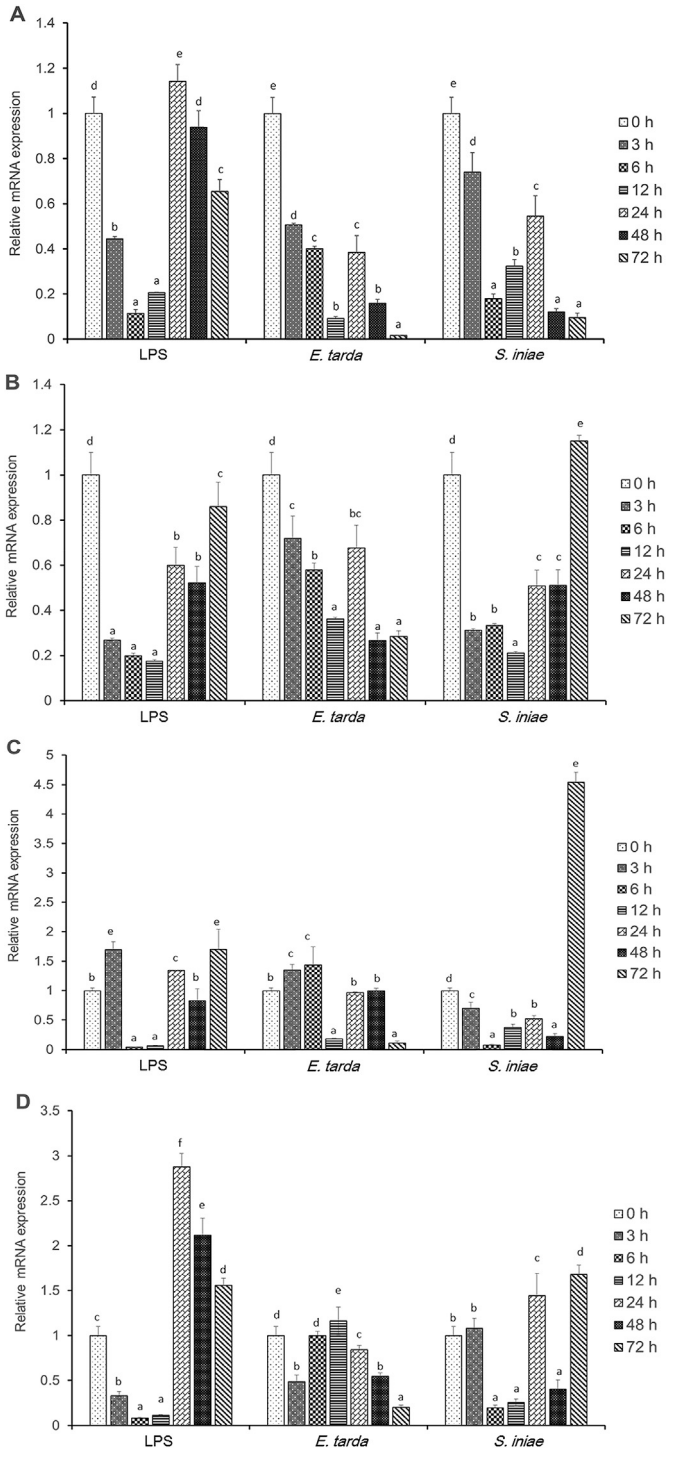

Fig. 5. Relative mRNA expression patterns of *ShDCS-like* in the kidney (A), gill (B), blood (C), and liver (D) upon stimulation with LPS, *E. tarda*, and *S. iniae* as determined by qPCR. The relative expression was calculated by the $2^{-\Delta\Delta CT}$ method using big belly seahorse ribosomal protein S7 as a reference gene and was normalized to that in PBS-injected controls. The data are presented as mean ± SD (n = 3), and significant differences at $P < 0.05$ are indicated with lowercase letters.

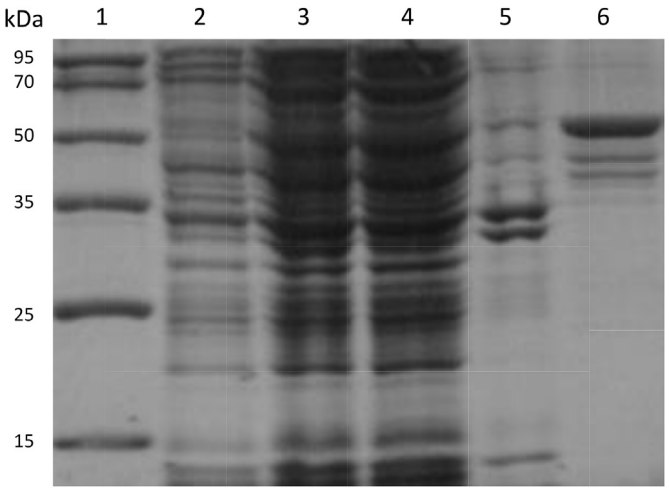

Fig. 6. SDS-PAGE analysis of the products of different steps in the expression and purification of rShDCS-like. Lane1, protein markers; lane 2, crude protein of un-induced *E. coli* cells; lane 3, crude protein of IPTG-induced *E. coli* cells; lane 4, soluble protein fraction of *E. coli* cells after IPTG induction; lane 5, insoluble protein fraction of *E. coli* cells after IPTG induction; lane 6, purified protein.

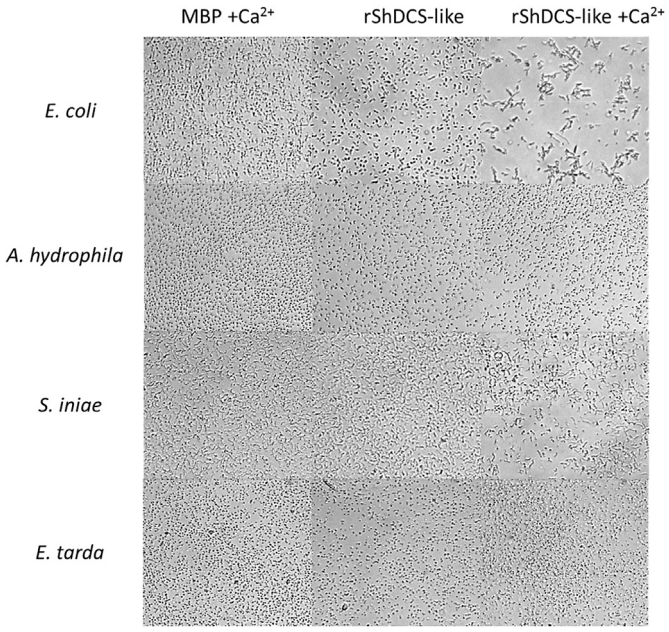

Fig. 7. The agglutination of bacterial species by rShDCS-like. Gram-positive (*S. iniae*) and gram-negative bacteria (*E. coli*, *A. hydrophila*, and *E. tarda*) were used for agglutination assays and were treated with MBP or rShDCS-like with or without Ca^{2+}.

Thus, this indicates that rShDCS-like has different agglutination activities against different bacterial species. Intriguingly, similar to our observations, differences in agglutination levels were also observed with previously reported recombinant CLRs. For example, the *Procambarus clarkii* C-type lectin has no agglutination activity with any bacteria tested (Zhang et al., 2013), while the *Haliotis discus* C-type lectin caused agglutination of all gram-positive but only some gram-negative bacterial species tested (Wang et al., 2008). Similarly, the *Eriocheir sinensis* C-type lectin showed stronger agglutination activity against *E. coli* than against *Staphylococcus aureus* (Guo et al., 2013).

3.6. Sugar specific binding assay

We attempted to analyze the affinity of rShDCS-like for sugar using various mono- or disaccharides. After adding *E. coli* into a mixture containing rShDCS-like and various sugar molecules, agglutination activity was detectably inhibited. Minimal inhibitory

Table 3
Minimal inhibitory concentration of rShDCS-like with respect to different sugar molecules.

Sugars	Minimum inhibition concentration (MIC) (mM)
D-glucose	>250
D-mannose	>250
Galactose	>250
Sucrose	125
Lactose	125

concentrations are shown in Table 3. Concentrations over 125 mM of sucrose and lactose inhibited bacterial agglutination, reflecting significant affinities to those sugar molecules. However, D-glucose, D-mannose, and galactose did not elicit any notable inhibition within the concentration range of sugar used in the experiment. As mentioned earlier, the CRDs of C-type lectins harbor conserved motifs in Ca^{2+}-binding site 2. CRDs with the EPN motif bind mannose or sugars with equatorial 3- and 4-OH groups, while those with the QPD motif bind galactose or sugars with 3- and 4-OH groups having an equatorial/axial arrangement (Kolatkar and Weis, 1996). The sugar specificity test showed that rShDCS-like has no detectable affinity to mannose or galactose. This may be because ShDCS-like has an atypical QPN motif rather than the standard QPD or EPN motifs. However, this observation deserves further investigation.

4. Conclusion

A DC-SIGN-like homolog was identified from the big belly seahorse and molecularly characterized. According to our *in-silico* analysis, ShDCS-like harbors most of the typical characteristics of the DC-SIGN domain architecture. The sequence homology of ShDCS-like with other DC-SIGN and DC-SIGN-like proteins was affirmed by comparative sequence analysis, and its close evolutionary relationship with teleostan counterparts was inferred by phylogenetic analysis. Universal tissue-specific mRNA expression of *ShDCS-like* in healthy animals was detected using qPCR, and we further observed significant transcriptional modulation upon immune stimulation with bacteria or their associated molecules. Moreover, the recombinantly expressed CRD domain of ShDCS-like resulted in the detectable and selective agglutination of bacteria and showed notable affinity to several disaccharides. Collectively, these results suggest that rShDCS-like may play an indispensable role in host immunity in the big belly seahorse, in particular facilitating immune responses against invading bacteria.

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