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Characterization of a tandem-repeat galectin-9 from Nile tilapia (*Oreochromis niloticus*) involved in the immune response against bacterial infection

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ABSTRACT

Galectin-9 is a < beta > -galactoside-binding lectin which could modulate a variety of biological functions including recognition, aggregation and clearance of pathogen. In this study, a galectin-9 homologue (*OnGal-9*) was identified from Nile tilapia (*Oreochromis niloticus*) and its expression model and biological effects on bacterial infection were analyzed. The open reading frame of *OnGal-9* sequence was 975 bp encoding 324 amino acids. It shares 45%–92% identities with other galectin-9 proteins. The deduced mature peptide of OnGal-9 possesses two conserved carbohydrate recognition domain (CRD) that connected with a linker peptide. Expression analysis indicated that OnGal-9 was distributed in all the tested tissues of healthy tilapia. The *OnGal-9* expression was significantly up-regulated in spleen, head kidney, and intestine after challenged by *Streptococcus agalactiae*. Meanwhile, the recombinant OnGal-9 (rOnGal-9) protein displayed strong binding and agglutination activity toward both *Streptococcus agalactiae* and *Aeromonas hydrophila*. Moreover, rOnGal-9 could promote phagocytosis of macrophages. Taken together, the results here indicate that OnGal-9 might be involved in the immune response of Nile tilapia against bacterial infection.

1. Introduction

Galectins are a class of proteins that bind specifically to b-galactoside sugars, which have varieties of functions including modulation of cell–cell interactions, cell–matrix adhesion and transmembrane signaling [1]. Galectins can recognize non-self glycans of microorganisms and be considered as pattern recognition receptors (PRRs) [2], which could regulate innate immune process activated by pathogen-associated molecular pattern (PAMPs) [3]. So far, 15 galectins of mammalian were identified and divided into three groups based on their conserved carbohydrate recognition domain (CRD): “prototype” (galectin-1, -2, -5, -7, -10, −11, −13, −14, −15), “chimera” (galectin-3) and “tandem repeat” (galectin-4, -6, -8, −9, −12) [4]. Galectin-9, a tandem-repeat galectin, has N- and C- terminal carbohydrate-binding domains connected by a link peptide [5]. Galectin-9 could stimulate bactericidal activity in infected macrophages by stimulating macrophage activation and IL1β secretion, which could restrict intracellular bacterial growth [6]. Further research have revealed that galectin-9 could expand regulatory T-cells and inhibits natural killer (NK) cell function following virus infection [7]. So far, teleost galectin-9 were only reported in Rainbow trout [8], Zebrafish [9], large yellow croaker [10], and Yellow catfish [11]. However, the information of galectin-9 in Nile tilapia was largely unknown.

Nile tilapia (*Oreochromis niloticus*) is one of the most important economically farmed fish species in China [12]. However, *Streptococcus agalactiae* has resulted in huge losses to tilapia culture in recent years [13]. So it is urgent to understand tilapia defense mechanism against pathogens. In the present study, a galectin-9 homolog from *O. niloticus* (*OnGal-9*) was identified and characterized. Moreover, the recombinant OnGal-9 was produced and its roles in immune system against bacterial

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Table1
Primers used in this study.

Primers	Nucleotide Sequence(5′→3′)	Comment
OnGal-9-F	ATGGCTTTTTATCAGCAGCCA	ORF amplification
OnGal-9-R	CTACACAATCACAGAGGTCAGGCT	ORF amplification
M13F	TGTAAAACGACGGCCAGT	Sequencing
M13R	CAGGAAACAGCT ATGACC	Sequencing
qOnGal-9-F	TGGGGCACTGAAGAGAGG	RT-PCR
qOnGal-9-R	TGGTGAAATGGCAGGCGA	RT-PCR
β-actin-F	CGAGAGGGAAATCGTGCGTGACA	RT-PCR Control
β-actin-R	AGGAAGGAAGGCTGGAAGAGGGC	RT-PCR Control
EOnGal-9-F	CCGGAATTCGCTTTTTATCAGCAG	Protein expression
EOnGal-9-R	CCGCTCGAGCACAATCACAGAGGTCA	Protein expression

EcoR I and *Xho* I sites are underlined.

infection were detected. These findings will help us to better understand the immune response of fish during bacterial infection.

2. Materials and methods

2.1. Fish preparation and bacterial challenge

Nile tilapia (50 ± 10g) were acquired from a local fish farm in Zhanjiang, Guangdong, China. The fish was cultured in 1000 L tank with aerated freshwater under 28 ± 2 °C for three weeks [14,15]. All experiments were conducted according to the principles and procedures of Guangdong Province laboratory animal management regulations.

In order to study the expression of *OnGal-9* in healthy tilapia, the tissues including brain, head kidney, gill, spleen, thymus, heart, liver, skin, and intestine were collected and frozen quickly by liquid nitrogen and stored in −80 °C until use.

S. agalactiae (ZQ1901) used in the experiment was isolated from Nile tilapia and kept in Guangdong Ocean University. The *S. agalactiae* was dissolved in phosphate-buffered saline (PBS) with a final concentration of 1×10^7 cells/mL. The stimulation groups were injected with 100 μL activated *S. agalactiae* [13,16]. The sample including spleen, head kidney, and intestine were collected at seven time points (0 h, 4 h, 12 h, 24 h, 48 h, 72 h, and 96 h), then frozen by liquid nitrogen and stored in −80 °C until use.

2.2. Cloning and sequence analysis of OnGal-9

In order to amplify the open reading frame [17] of OnGal-9, total RNA from spleen was extracted by using EasyPure RNA Kit (TransGen, China) according to the protocol. The first-stand cDNA was synthesized from total RNA by using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, China). The specific primer (OnGal-9-F, OnGal-9-R) were designed based on Nile tilapia transcriptome data (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA244908) and the prediction of NCBI Basic Local Alignment Search Tool. All the primers used in this study were designed by Primer 6.0 and showed in Table 1.

The potential open reading frame [17] of OnGal-9 was analyzed with ORF finder program (https://www.ncbi.nlm.nih.gov/orffinder/). Molecular weight, theoretical pI, amino acid composition of OnGal-9 were predicted by ProtParam tool (https://web.expasy.org/protparam/). Multiple sequence alignment of OnGal-9 protein sequences was

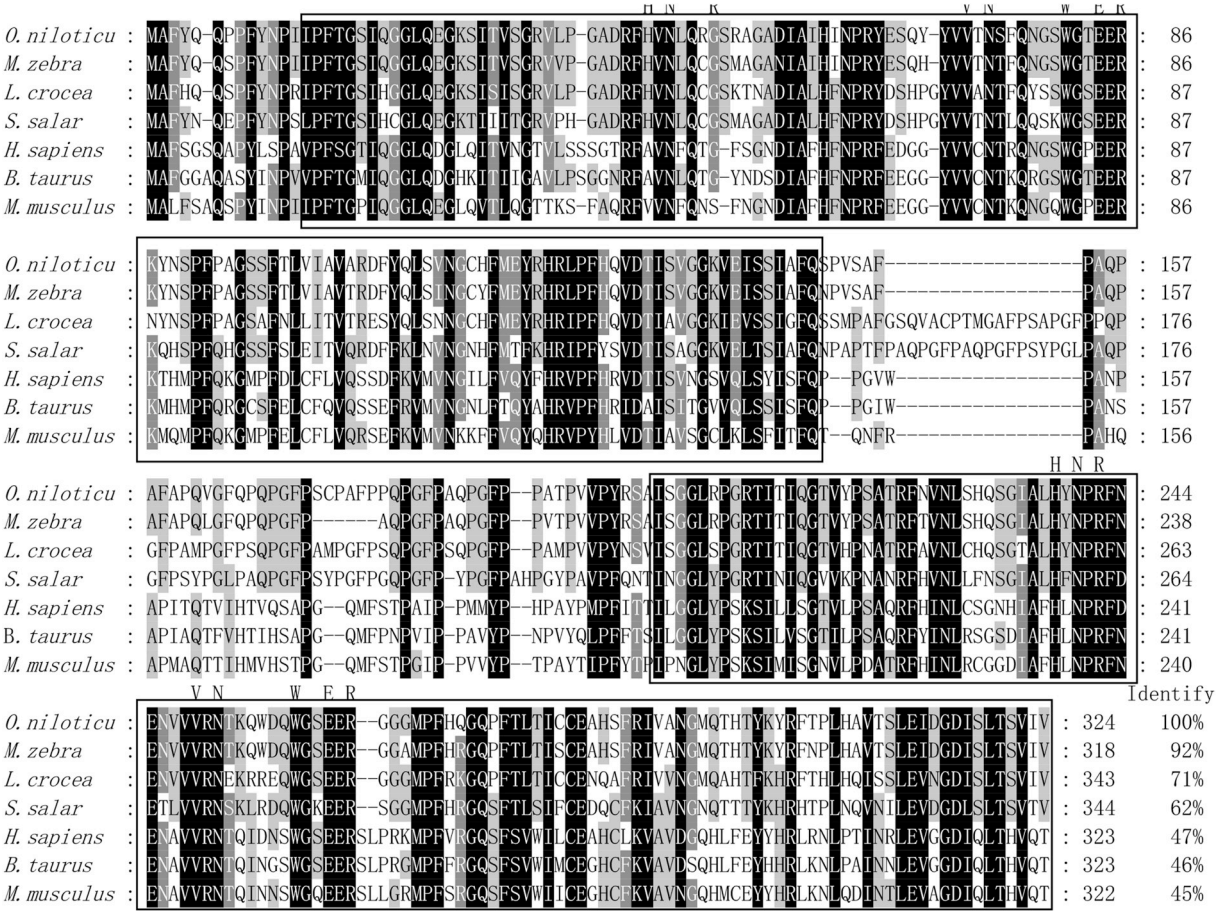


Fig. 1. Multiple sequence alignment of galectin-9 from various species. Conserved amino acid residues are shaded dark grey, and similar amino acids are shaded light grey. The CRD is in a black box. Conserved residues (H–N-R, V–N and W-E-R) for carbohydrate recognition and binding are shown above the alignment sequence.

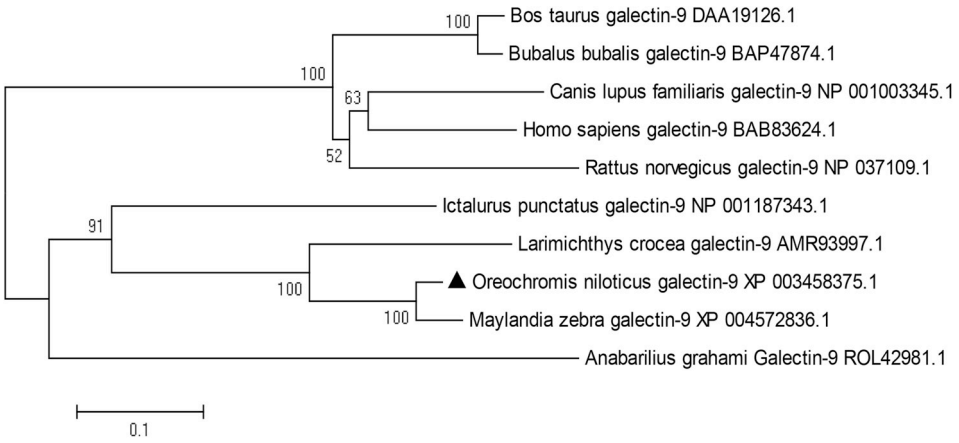


Fig. 2. Phylogenetic tree of OnGal-9 family members constructed using the NJ method by MEGA X program based on the alignment of 10 members of the Gal-9. The numbers at each branch indicates the bootstrap values (%).

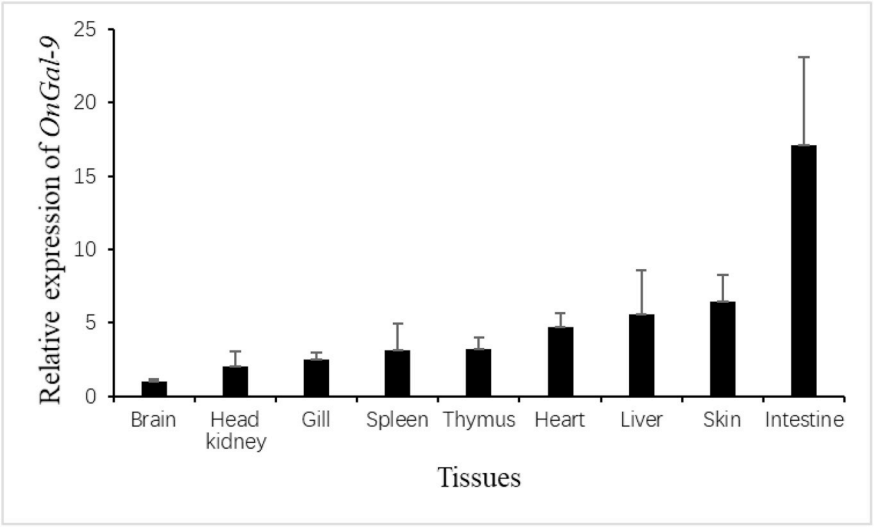


Fig. 3. Tissue distribution of OnGal-9 mRNA in healthy Nile tilapia. The ratio refers to the gene expression in different tissues relative to that in brain, and target gene expression is normalized to β-actin. Data are presented as means ± standard deviation.

performed by Clustal W program (http://www.clustal.org/clustal2/). The similarity analyses of the determined amino acid sequence were performed by UniProt programs (https://www.uniprot.org/). Phylogenic trees were constructed by the neighbor-joining method [18] using MEGA X software with 1000 bootstrap replications. The evolutionary distances were computed using the Poisson correction method [19] and were in the units of the number of amino acid substitutions per site.

2.3. Quantitative real-time PCR of OnGal-9 mRNA

The quantitative real-time RT-PCR analysis of *OnGal*-9 mRNA expression in different tissues and following bacterial injection were performed on Roche LC384 LightcyclerTM (Roche, Switzerland). First-strand cDNA was synthesized from total RNA as described in Section 2.1. The PCR reaction volume was 10 μL including 5 μL of FastStart Essential DNA Green Master (Roche, Switzerland), 1 μL of diluted cDNA, 0.5 μL of each primer (Table 1), and 3.5 μL of nuclease free water. The program was performed as follows: 1 cycle of 10 min at 95 °C, 40 cycles of 10 s at 95 °C, 15 s at 55 °C, and 15 s at 72 °C. The β-actin gene (housekeeping gene) was taken as internal control reference gene. The relative expression level of *OnGal*-9 mRNA was calculated using $2^{-\Delta\Delta Ct}$ method [20].

2.4. Expression and purification of rOnGal-9

A pair of primers (EOnGal-9-F and EOnGal-9-R) with restriction sites (*EcoR* I and Xho I) were designed (Table 1) to amplify the ORF sequence. The PCR products were purified and ligated into the pMD18-T vector. The recombinant pMD-18T plasmid and pGEX-4T-1 were digested with *EcoR* I and *Xho* I. The expression plasmid pGEX-4T-1-OnGal-9 was transformed into *E. coli* BL21 (DE3) (TransGen, China) and cultured in fresh ampicillin containing LB liquid medium. When OD600 reached 0.4–0.6, isopropyl- < beta > -Dthiogalactopyranpside (IPTG) was added to a final concentration of 1 mmoI/L and induced at 37 °C for 5 h. The bacterial solution was collected and washed three times with PBS. Lysozyme was added to a final concentration of 1 mg/ml, and placed on ice for 30 min, then centrifuged at 4 °C for 10 min. The supernatant was purified using a GST-tag protein purification kit (Beyotime, China), desalted and concentrated using an Amicon Ultra Centrifugal Filter (Amicon, USA). The purified protein was analyzed by 10% reducing SDS-PAGE and Western-Blot. In addition, the pGEX-4T-1 was also expressed and purified for subsequent experiments.

2.5. Binding of rOnGal-9 to microorganisms

The binding ability of rOnGal-9 to Gram-positive bacteria (*S. agalactiae*) and Gram-negative bacteria (*A. hydrophila*) was detected by the

method of Bai et al. [21]. In brief, The bacteria were cultured to an OD_{600} of 0.4–0.6, 10 μL of rOnGal-9 (1 mg/mL) was incubated with 500 μL of bacteria for 1 h at 37 °C, then washed three times with PBS and eluted with 7% SDS for 1 min, centrifuged at 12000 for 10 min. The supernatant was used to run SDS-PAGE as Section 2.3. Control bacterial cells were incubated with PBS or pGEX-4T-1 and performed the same treatment.

2.6. Bacterial agglutination assay

Briefly, *S. agalactiae* and *A. hydrophila* were cultured to an OD_{600} of 0.4–0.6 and washed three times with PBS. The bacteria were re-suspended in 0.1 M Na_2CO_3 and then FITC (Solaribo, China) was added to a final concentration of 0.1 mmol/L, incubated at 37 °C for 30 min. Then centrifuged three times to completely remove the FITC and incubated 10 μL of rOnGal-9(1 mg/mL) with the bacteria at room temperature for 1 h. The bacteria were applied to a grass slide and the results were observed with a fluorescent microscope.

2.7. Phagocytosis-promoting activity analysis

Macrophages were prepared as previous methods [22,23]. Briefly, healthy tilapia was anesthetized by MS222. The head kidney was carefully excised and transferred through a 40 μm stainless nylon mesh (Greiner Bio-OneGmbH, Germany). The cell suspension was suspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, US) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 25 U/mL heparin (Gibco). The cell suspension was slowly added into a 51%/34% percoll (GE Healthcare) density gradient, centrifuged at 400 g for 40 min, and the cell layer of the interface was carefully aspirated and then washed with PBS at 500 g for 10 min. Cell viability was measured using a trypan Blue staining kit (Sangon Biotech).

Here, 200 μL of FITC-labeled bacterial suspension was mixed with 180 μL of macrophages and 10 μL of rOnGal-9 (1 mg/mL) and incubated in the dark for 1 h with shaken every 5 min. In the control group, 10 μL of PBS or pGEX-4T-1 (1 mg/mL) was used instead of rOnGal-9. Then centrifuge at 500 g for 10 min to completely remove the non-ingested bacteria. The results were analyzed using flow cytometer. The fluorescence data for this experiment was limited to Gate and all data were repeated three times to ensure the accuracy of the analysis.

2.8. Statistical analysis

All data in this study were displayed as means ± standard deviation (SDs). Statistical analysis were performed by the LSD (least significant difference) test using SPSS 17.0 software. Differences were considered significant at $p < 0.05$ (*) and highly significant at $p < 0.01$ (**)

3. Results

3.1. Primary sequence analysis of OnGal-9

The ORF of *OnGal-9* was 975 bp and encoded 324 amino acid (aa) residues, including two CRD regions. The predicted molecular mass was 35.66 KDa with a theoretical isoelectric point of 9.23. Multiple alignments of galectin-9 from deduced amino acid sequence of Nile tilapia with other known galectin-9, and the result showed that the identity of OnGal-9 mature polypeptide with other species ranged from 45% to 92% (Fig. 1). Phylogenetic tree revealed that OnGal-9 was grouped together with other fish galectin-9 and formed a dependent clade, and the OnGal-9 was closely related to *Maylandia zebra* galectin-9 (Fig. 2).

3.2. The expression profiles of OnGal-9

Quantitative RT-PCR was used to examine the expression profiles of *OnGal*-9 mRNA in healthy tilapia. As showed in Fig. 3, *OnGal*-9 mRNA was detected in all the tested tissues with the highest expression in intestine, followed with skin, liver, heart, thymus, spleen, gill, head kidney, and brain (Fig. 3).

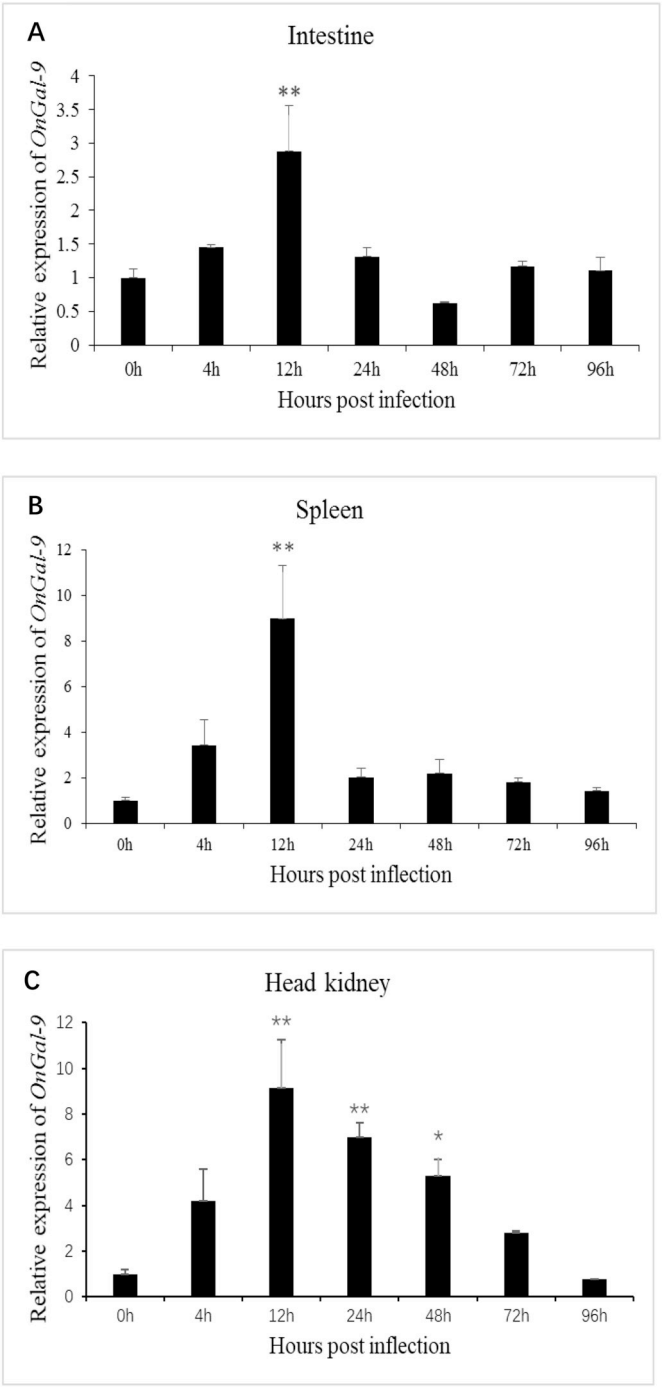


Fig. 4. Expression analysis of OnGal-9 in intestine(A), spleen9(B), and head kidney(C) after *S.agalactiae* challenge was performed by relative quantitative RT- PCR. Data are shown as mean ± SDs. Significant difference was indicated by asterisks as *$0.01 < P < 0.05$ and **$P < 0.01$.

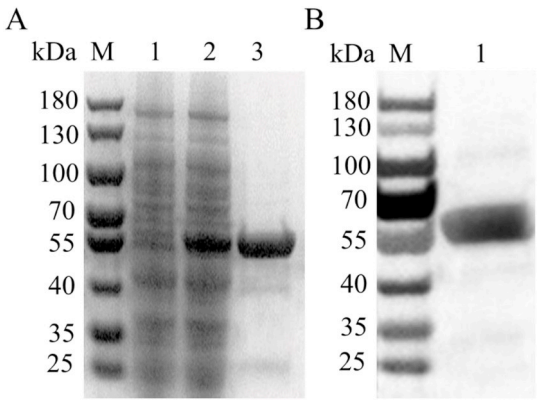


Fig. 5. SDS-PAGE and Western blot of rOnGal-9. (A)Lane M: markers (25–180 kDa); lane 1: bacteria liquid before IPTG induction; lane 2: bacteria liquid after IPTG induction; lane 3: purified rOnGal-9. (B)Lane M: markers (25–180 kDa); lane 1: Western blot analysis of rOnGal-9.

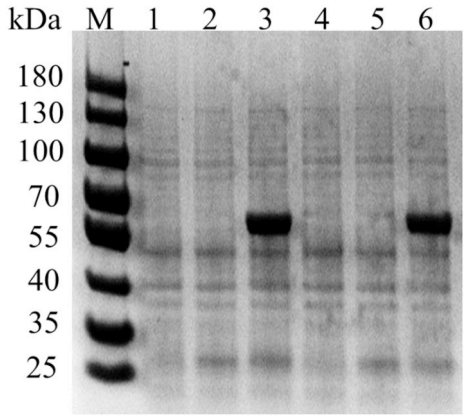


Fig. 6. Binding of rOnGal-9 to bacteria pathogens. Lane M: markers (25–180 kDa); lane 1: *S. agalactiae* included with PBS; lane 2: *S. agalactiae* included with pGEX-4T-1; lane 3: *S. agalactiae* included with rOnGal-9; lane 4: *A. hydrophila* included with PBS; lane 5: *A. hydrophila* included with pGEX-4T-1; lane 6: *A. hydrophila* included with rOnGal-9.

In order to explore the expression pattern of *OnGal-9* post infection with pathogens, tilapia was challenged with the most serious bacteria *S. agalactiae*, and the expression level of *OnGal-9* in spleen, head kidney, and intestine were tested by qRT-PCR. The 0 h tissues were used as control. In the intestine, the expression of *OnGal-9* was quiet slow in the first 4 h, peaked at 12 h, and reached the minimum level at 48 h. In addition, it presented another smaller but significant rising at 72 h post-infection in liver (Fig. 4A). Similar results were observed in spleen and head kidney. In spleen, the expression of *OnGal-9* was significant rised at 12 h post-infection and reached its peak, and then sharply down to a lower level at 48 h (Fig. 4B). However, the head kidney showed a much slower downward trend compare to that in spleen (Fig. 4C).

3.3. Recombinant OnGal-9 expression, purification and western blotting analysis

The ORF of OnGal-9 was cloned into pGEX-4T-1 vector, transformed into BL21 (DE3), and the recombinant protein fused with GST-tag was purified and analyzed by SDS-PAGE and western blotting. As shown in Fig. 5A, a band around 62 KDa was detected. At same time, the result of western blotting indicated that rOnGal-9 could be specific recognized by GST-tag antibody (Fig. 5B).

3.4. Binding of rOnGal-9 to bacteria pathogens

The binding ability of rOnGal-9 to *S. agalactiae* and *A. hydrophila* was investaged by SDS-PAGE. The result showed that rOnGal-9 was able to bind both *S. agalactiae* and *A. hydrophila*. While the pGEX-4T-1 and PBS could not bind neither of them (Fig. 6).

3.5. Agglutinating activity of rOnGal-9

FITC-labelled *S. agalactiae* and *A. hydrophila* were used to test the agglutinating activity of rOnGal-9, and the results were checked with inverted fluorescence microscope. As shown in Fig. 7, rOnGal-9 could agglutinate both *S. agalactiae* and *A. hydrophila*, while the pGEX-4T-1 and PBS could not agglutinate any bacteria.

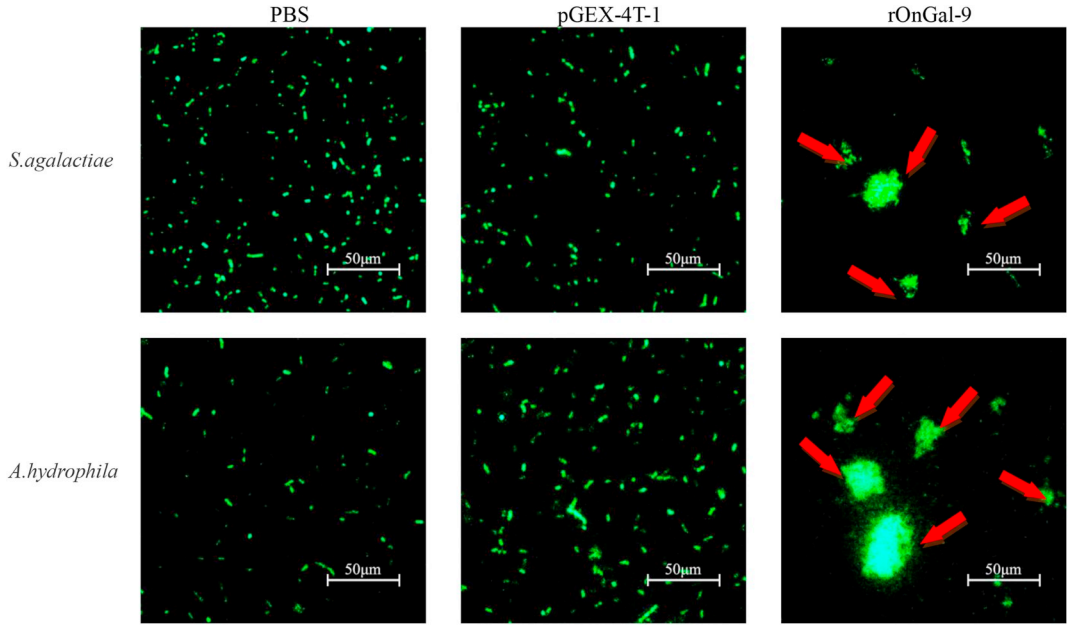
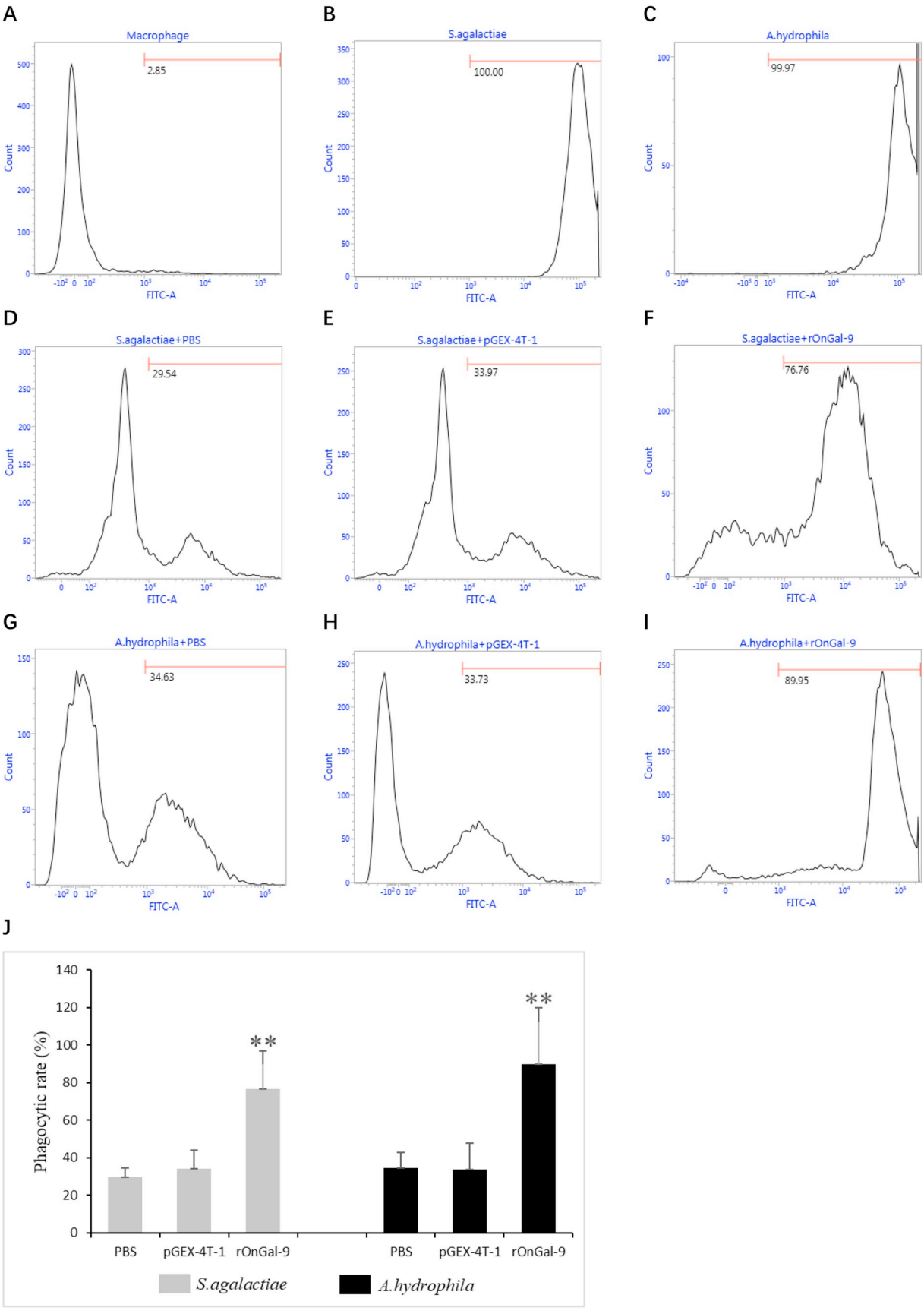


Fig. 7. Agglutinating activity of rOnGal-9 against FITC-labelled *S. agalactiae* and *A. hydrophila*. PBS or pGEX-4T-1was incubated with bacteria as a negative control. The agglutination was presented with red arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



(caption on next page)

Fig. 8. Effects of rOnGal-9 on phagocytosis of Nile tilapia macrophages. Flow cytometric analyses of the macrophages phagocytosing *S. agalactiae* and *A. hydrophila* which were treated as described in Section 2. Data show analyses of 10,000 events. The marker represented phagocytosis part. (A) The histogram of macrophages. (B and C) The histogram of FITC-labeled *S. agalactiae* and *A. hydrophila*. (D, E, F, G, H, and I) The histogram of flow cytometric analyses of the macrophages phagocytosing *S. agalactiae* and *A. hydrophila* pre-incubated with PBS, pGEX-4T-1, and rOnGal-9, respectively. The phagocytosis rates were shown near the marker. The results shown here were from one experiment out of three independent experiments. (J) The histogram of the phagocytosis rates. The average standard deviation was obtained from three experiments. The symbol * shows a significant difference from control ($p < 0.05$).

3.6. Enhancement of phagocytosis by rOnGal-9

The ability of rOnGal-9 promote the phagocytosis of bacteria by macrophages was determined by flow cytometer. As shown in histogram, there were almost no fluorescent signal of macrophages (Fig. 8 A). After labeled with FITC, a strong fluorescent signals of *S. agalactiae* and *A. hydrophila* could be observed in Fig. 8B and C. The phagocytosis rates of the macrophages were detected by phagocytosing the bacteria which were incubated with PBS, pGEX-4T-1, and rOnGal-9, respectively (Fig.8 D, E, F, G, H, and I). Statistical analysis of the data indicated that rOnGal-9-treated *S. agalactiae* and *A. hydrophila* had a significantly stronger fluorescent signal compare to those of PBS-treated and pGEX-4T-1-treated group (Fig. 8 J), hinting that rOnGal-9 could effectively promote phagocytosis.

4. Discussion

In this study, a tandem-repeat galectin-9 (*OnGal-9*) was identified and characterized from Nile tilapia (*Oreochromis niloticus*). The recombinant OnGal-9 protein showed significant binding, agglutination, and promoted phagocytosis of macrophages, suggesting that OnGal-9 might participate in tilapia immune response during bacterial infection.

The deduced amino acid sequence of OnGal-9 contained N- and C-terminal carbohydrate-binding domains connected by a link peptide. Both CRDs have the same typical sugar binding sites H–N-R, V–N, and W-E-R (Fig. 1), which is consistent with galectin family. Moreover, no signal peptide and transmembrane region were found in OnGal-9, so it could only be secreted via non-classical pathway [9]. Similar results were observed in the research of *Larimichthys crocea* [24], *Labeo rohita* [25], and *Pelteobagrus fulvidraco* [11]. Multiple alignments revealed that the two CRDs are highly conserved from fish to mammal (Fig. 1). The phylogenetic tree indicated that OnGal-9 was clustered with other teleost galectin-9s, which was in line with the traditional taxonomy (Fig. 2).

Tissue distribution analysis showed that *OnGal-9* distributed in all the checked tissues with the highest expression in intestine, which was consistent with the observation of galectin-9 in *Labeo rohita* [25]. Meanwhile, *OnGal-9* was also highly expressed in skin and liver. As we known, skin is the first barrier in fish mucosal immune system and is highly prone to infections [26], and the immune functions of liver in non-specific phagocytosis has been convinced [17]. The abundant presence of *OnGal-9* in various immune-related tissues revealed its multiple functions during immune response. Similar results were observes in the studies of Nile tilapia galectin-2, galectin-3 [27], and galectin-8 [28].

In order to examine the response of *OnGal-9* to bacterial infection, the temporal expression patterns of *OnGal-9* in different tissues (intestine, head kidney, and spleen) were investigated by qRT-PCR. The results showed that *OnGal-9* showed a similar time-dependent manner with distinct manifestations in the intestine, head kidney, and spleen after *S.agalactiae* infection, which was up-regulated and peaked at 12 h and then decreased, showing that these tissues were involved in the immune response of fish in a similar way. Similar results were observed in the study of mannose-binding lectin [16] and C-type lectin [29] from Nile tilapia. As we known, intestine serves as the major perpheral lymphoid organ [30], where is armored with various immune cell types, including B cells, macrophages, granulocytes, and T cells [31]. The sharp increase of expression in intestine hinted that possible roles of *OnGal-9* on the regulation of cell-mediated immune responses during bacterial infection. Additionally, although *OnGal-9* was mainly expressed in intestine, larger transcriptional responses were observed in spleen and head kidney following the *S. agalactiae* infection, probably because spleen and head kidney were two major hematopoietic organs in fish and main target organs attacked by *S. agalactiae* [32]. These data implied that OnGal-9 might possessed multiple functions in immune response against bacterial infection.

To explore the further functions of OnGal-9 during pathogen invasion, we prepared the recombinant OnGal-9 protein of Nile tilapia. Since OnGal-9 has two CRDs to bind sugar site, suggesting that OnGal-9 might combine pathogen by recognize the carbohydrates of their surface [33]. The binding experiment exhibited that rOnGal-9 could bind both *S. agalactiae* and *A. hydrophila* as expected. Furthermore, rOnGal-9 could also agglutinate both *S. agalactiae* and *A. hydrophila*, which was in accordance with other research of galectins [34–36]. The agglutinating activity of rOnGal-9 against bacteria can prevent these pathogens from entering cell and remaining extracellular surface or matrix [37], which may facilitate phagocytosis of bacteria by macrophages. Surprisingly, the significant promotion of rOnGal-9 on macrophages was observed in present study, similar finding was recorded in *Larimichthys crocea* galectin-9 [10]. These data indicated that OnGal-9 function as a PRR and an opsonin during bacterial infection.

Taken together, a tandem-repeat galectin-9 was identified and characterized from Nile tilapia (*Oreochromis niloticus*). It shared two CRDs with other galectin-9 species. The mRNA of *OnGal-9* in healthy tilapia was abundant in intestine. *OnGal-9* expression was up-regulated in a time-dependent manner in *vivo* following bacteria challenges. In addition, the recombinant protein could significantly bind and aggregate both Gram-positive and Gram-negative bacteria and promote phagocytosis of macrophages. These results suggested that OnGal-9 was involved in the immune response of bacterial infection.

Acknowledgement

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