Identification of an amphioxus intelectin homolog that preferably agglutinates gram-positive over gram-negative bacteria likely due to different binding capacity to LPS and PGN

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**A B S T R A C T**

Intelectin is a recently described galactofuranose-binding lectin that plays a role in innate immunity in vertebrates. Little is known about intelectin in invertebrates, including amphioxus, the transitional form between vertebrates and invertebrates.

We cloned an amphioxus intelectin homolog, AmphITLN-like, coding 302 amino acids with a conserved fibrinogen-related domain (FRD) in the N-terminus and an Intelectin domain in the C-terminus. In situ hybridization in adult amphioxus showed that AmphITLN-like transcripts were highly expressed in the digestive tract and the skin. Quantitative real-time PCR revealed that AmphITLN-like is significantly up-regulated in response to *Staphylococcus aureus* challenge, but only modestly to *Escherichia coli*. In addition, recombinant AmphITLN-like expressed in *E. coli* agglutinates Gram-negative and Gram-positive bacteria to different degrees in a calcium-dependent manner. Recombinant AmphITLN-like could bind lipopolysaccharide (LPS) and peptidoglycan (PGN), the major cell wall components of Gram-negative and Gram-positive bacteria, respectively, with a higher affinity to PGN.

Our work identified and characterized the first time an amphioxus intelectin homolog, and provided insight into the evolution and function of the intelectin family.

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**1. Introduction**

Innate immunity constitutes the first line of defense against pathogens depending on the pattern recognition. Host pattern recognition receptors (PRRs) can recognize microbial molecules (also known as the pathogen-associated molecular patterns, PAMPs) that are unique to groups of related microorganisms [1]. These microbial molecules are often the cell wall components of microbes, primarily carbohydrate chains such as lipopolysaccharides, peptidoglycan, lipoteichoic acids, and β-glucan [2,3]. Lectin, as a key member of PRRs, could function as phagocytosis receptors, soluble opsonins, and agglutinins to mediate pattern recognition [4,5].

Intelectin is a recently described lectin that was first discovered in *Xenopus laevis* (*oocyte lectin XL35*) forms the fertilization membrane to block polyspermy [6]. Then homologs have been identified in *Homo sapiens*, *Mus musculus*, *Ictalurus punctatus*, *Salmo gairdnerii*, *Ctenopharyngodon idellus*, *Lampetra fluviatilis*, *Danio rerio*, and *Halocynthia roretzi*, etc. [4,7–12].

The expression patterns of intelectins differ both within and among species. For example, human intelectin-1 (HL-1) is widely distributed in several tissues whereas human intelectin-2 is only found in the small intestine [13]. In the BALB/c mouse strain, intelectin1 presents in small intestine paneth cells [7] [14], while intelectin2 is restricted in the ileum of uninfected mice but appears throughout gut after nematode *Trichinella spiralis* infection [7]. In the 129S7 mouse strain six intelectin genes were identified with distinct sequences and site-specific expressions in the gastrointestinal tract [15]. In channel catfish, intelectin1 occurs in all tissues while intelectin2 is predominantly expressed in the liver and weakly in the intestine and trunk kidney.

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The up-regulation of intelectin expression has been observed in the small intestine of mice after *Trichuris muris* infection [7], in liver of rainbow trout after infection with *Listonella* [16], in grass carp *Ctenopharyngodon idella* following LPS injection [10], and in channel catfish injected with *Edwardsiella ictaluri* [4], implying its role in immune response. HL-1 has a Ca2+-dependent affinity to α-pentose and α-galactofuranosyl, and can bind to *Mycobacterium bovis* bacillus Calmette-Guérin, but exhibits no agglutination activities toward some bacteria or erythrocytes [17,18]. However, XLS5 agglutinates trypsinized and glutaraldehyde-fixed rabbit erythrocytes [6] and bacterial agglutinating activity has been identified in catfish *Silurus asotus* [19]. The phagocytosis-enhancing property of intelectin has been reported in humans and ascidians [12,18]. Furthermore, sheep intelectin-2 co-purifies with mucin Muc5ac from gastric mucus suggesting that intelectin may play a role in modifying the rheology of mucus [20]. However, in invertebrates, the physiological function of intelectin is still poorly understood.

The basal chordate amphioxus is becoming a new model organism for studying the origin of the vertebrate immune system [21]. Here we report the cloning of one intelectin, AmphiITLN-like, in amphioxus and examined its tissue expression pattern in adults. *Staphylococcus aureus* and *Escherichia coli* challenge cause significant increase of AmphiITLN-like expression within 8–12 h, suggesting AmphiITLN-like may be involved in acute phase response. The bacterial binding, bacterial agglutinating and polysaccharide binding indicated that AmphiITLN-like is likely to recognize the major components of bacteria cell walls (LPS and PGN) and then acts as agglutinin. Therefore, our results provide insight into the evolution and function of the intelectin gene family.

2. Materials and methods

2.1. Animal

Adult amphioxus (*Branchiostoma japonicum*, also known as *Branchiostoma belcheri tsingtauense*) was collected from the sandy sea floor at Shazikou near Qingdao, China. Prior to the experiments, they were starved for three days in sterilized filtered seawater to clear the gut.

2.2. Cloning of AmphiITLN-like cDNA

Total RNA was extracted from adult amphioxus using Trizol (Takara), following the manufacturer’s instructions. The specific primers (*AmphiITLN*-like-F: 5’-ATGGAACATCAATGCACTAC-3’ and *AmphiITLN*-like-R: 5’-TTAGCCGATAAAAAGATGACGG3’*) were designed based on a hypothetical protein jgi:Braflf17/469 in JGI and used for RT-PCR cloning. The PCR parameters were as follows: 1 cycle at 94°C for 5 min, 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 15 s, and 1 cycle at 72°C for 10 min. PCR products were cloned into the pEASY-T3 vector (Transgene) for sequencing.

2.3. Sequence analysis and phylogenetic tree construction

The intelectin homolog sequence for *Branchiostoma floridana* was obtained using blastp at the Joint Genome Institute website (JGI, [http://www.jgi.doe.gov](http://www.jgi.doe.gov)) or Genbank (NCBI, [http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) with amino acid sequences of zebrafish Intelectin1 (NP_001070900); 2 (NP_001153015) and 3 (NP_001153056). Intelectin homolog sequences in other organisms were obtained from NCBI, JGI, and the Ensembl Genome Browser ([http://www.ensembl.org](http://www.ensembl.org)) by blastx, tblastn, or blastn using the NCBI, JGI, and the Ensembl Genome Browser (http://www.ensembl.org) as query. The multiple alignments were constructed using Clustal X with p-distance correction. A phylogenetic tree was created using neighbor-joining method in MEGA4.0 program and the topological stability of the tree was evaluated by 1000 bootstrap replicates. Deduced protein sequence was performed using ExPASy ([http://www.expasy.ch](http://www.expasy.ch)). The FRED domain was determined with SMART ([http://smart.embl-heidelberg.de](http://smart.embl-heidelberg.de)). N-glycosylations were predicted by NetNGlyc 1.0 ([http://www.cbs.dtu.dk/services/NetNGlyc](http://www.cbs.dtu.dk/services/NetNGlyc)).

2.4. In situ hybridization histochemistry

The DIG-labeled *AmphiITLN*-like riboprobes of ~750 bp were synthesized in vitro following the DIG-UTP supplier’s instructions (Roche). Adult animals were fixed in 4% paraformaldehyde in 100 mM phosphate-buffered saline (PBS, pH 7.4) at 4°C overnight then immersed into 30% sucrose for at least 24 h. Samples were embedded in paraffin and cut into 7 μm serial sections that were mounted onto poly-l-lysine-coated slides, dried at 37°C for 24 h, de-waxed in xylene for 20 min, then immersed in absolute ethanol for 10 min. The hybridization procedure was conducted as described previously in Ref. [22].

2.5. Quantitative real-time PCR

Healthy adult amphioxus was challenged by immersion exposure to *E. coli* or *S. aureus* at a concentration of 10⁹ CFU/ml in filtered seawater. PBS was used as negative control. Gut samples were separated and collected 0, 2, 4, 8, 12, or 24 h following the exposure.

Quantitative real-time PCR was performed following manufacturer’s instructions (Takara) using primers *AmphiITLN*-like-qPCR-F (5’-AAACCCCGGCGCTAACCT-3’), *AmphiITLN*-like-qPCR-R (5’TGGAA-CCGCGGACTGC3’), β-actin-qPCR-F (5’TCTCTCAACCTCCTACC-3’), and β-actin-qPCR-R (5’CAACACACAGGGCCTTC3’). The β-actin gene was chosen as the reference for internal standardization. Samples were analyzed in triplicate. The expression level of *AmphiITLN*-like was analyzed using 2-∆∆Ct methods in which the discrepancy between Ct for *AmphiITLN*-like and β-actin was calculated to normalize the variation in the amount of cDNA in each reaction. Statistical analyses were performed using SPSS 13.0 with the level of significance set at p < 0.05.

2.6. Expression and purification of recombinant *AmphiITLN*-like protein in E. coli system

The complete open reading frame (ORF) of *AmphiITLN*-like was amplified using primers pET29bITLN-F (5’-GGAATTCCTATGCAACGGCATTCTAC-3’) and pET29bITLN-R (5’-CTCGAGGAGAACAGCTGCTCA-3’), anchoring the Ndel and Xhol restriction sites, respectively. The amplified products were cloned into the pET29b vector and transformed into *E. coli* BL21 (DE3) cells for expression.

Isopropyl-β-D-thiogalactoside (IPTG, 0.5 mM) was added when the optical density (OD) 600 of the bacterial culture reached 0.6. After 4 h induction, the cells were collected by centrifugation at 4500 g for 10 min and resuspended in resuspension solution (25 mM Tris, pH 8.0 and 200 mM NaCl). Following sonication and centrifugation, the precipitate was washed as described by Du et al. [23], and then purified by His-Bound resin (Bio-Rad) under denaturing conditions following the manufacturer’s instructions. The purified protein was refolded in 100-fold volume dialysis buffer (0.1 M Tris, pH 8.0; 5 mM EDTA and 5 mM Cysteine) for 16 h at 4°C and then in resuspension solution for another 16 h. Each fraction was analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained using Coomassie brilliant blue R250. Protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as the standard.

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2.7. Preparation of AmphiITLN-like mouse antiserum and western blotting

Purified AmphiITLN-like protein (20 μg) was diluted with saline to a final volume of 50 μl and then mixed with 50 μl QuickAntitoday adjuvant (Kbqbio). The emulsified mixture was subcutaneously injected into C57Bl-6j mice hosted at an SPF facility in Sandong University. The mice were each immunized twice (one injection/week) and blood serum was collected three weeks thereafter. Western blotting was conducted to test antiserum specificity and antiserum titer was determined by enzyme-linked immunosorbent assay (ELISA).

Western blotting analysis was conducted as follows: the protein was separated by SDS-PAGE, transferred to a PVDF membrane (Millipore), then blocked with blocking solution (5% nonfat milk in TBS (25 mM Tris, pH 7.6, containing 0.15 M NaCl)) for 1 h at room temperature. The primary antibodies (rabbit anti-His antibody purchased from Sigma and the homemade mouse serum against AmphiITLN-like) was diluted 1/2000 in blocking solution and incubated for 1 h at room temperature. After washing three times with TBS buffer (0.1% Tween-20 in TBS), the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Sigma, 1/20000 diluted in blocking solution) followed by washing three times with TBS. Protein was visualized with Chemiluminescent HRP Substrate (Millipore).

2.8. Bacteria-binding assays

A total of 10^7 bacteria were mixed with 10 μg recombinant protein in TBS by gentle orbital rotation for 1 h at room temperature. Bacteria were pelleted and washed five times with 1 ml TBS buffer. Afterward, the pellet was rapidly suspended in 100 μl reducing sample buffer (250 mM Tris–HCl, pH 6.8; 10% (W/V) SDS, 2.5% bromophenol blue, 50% glycerol, and 5% β-mercaptoethanol) and denatured by heating at 100 °C for 15 min. The solution was washed and the final pellet was analyzed by 12% SDS-PAGE. The bound protein was detected by western blotting using mouse antiserum against AmphiITLN-like. The band intensity was estimated using Bandscan software (BioMarin Pharmaceutical Inc. UK).

2.9. Bacteria agglutination

E. coli, S. aureus, or Streptococcus faecalis were labeled with 50 μl FITC (10 mg/ml in DMSO) per ml of bacteria suspension at 37 °C for 1 h as described by Yu et al. [3]. The labeled bacteria (10^7) were incubated overnight with recombinant protein at different concentrations (0–100 μg/ml) in TBS at 37 °C, with or without the presence of 10 mM CaCl_2 and 10 mM EDTA. TBS buffer alone was used as a negative control and Fcεε4, an E. coli expressed shrimp protein with agglutinin activity, was used as a positive control [24]. Agglutination was examined under fluorescence microscopy at 400 × and degree of agglutination is scored in the bright field at 100 × by averaging independent readings of at least two persons as described by Lee et al. [6].

2.10. The quantitative binding of AmphiITLN-like to LPS or PGN

An ELISA assay was performed to quantitatively measure the binding of AmphiITLN-like to LPS (Sigma, extracted from E. coli) and PGN (Sigma, extracted from Bacillus subtilis). High absorption capacity polystyrene microtiter plates (Techno Plastic Products AG) were coated with a total of 5 μg of LPS or PGN (PGN was ultrasonically solubilized) per well in 100 μl TBS, or equal amount of TBS alone as negative controls. After an overnight incubation at 4 °C, the coated wells were washed with Tween-TBS (0.05% Tween-20 in TBS) and then blocked with 200 μl/well of blocking solution (5% (w/v) BSA in Tween-TBS). A range of concentrations of purified AmphiITLN-like protein (0–30 μg/ml) in blocking solution, in the presence or absence of 10 nM calcium, were added to the wells and the mixture was incubated for 2 h at 37 °C. Bound AmphiITLN-like was visualized with diluted mouse antiserum (1:2000) for 1 h at 37 °C. After four washes, the plates were incubated with 100 μl peroxidase-conjugated goat anti-mouse IgG (1/2000 diluted) at 37 °C for 1 h. The plates were washed as above and developed with 0.01% 3’, 3’, 5’, 5’-tetramethylbenzidine (Amresco). The reaction was stopped with 50 μl/well 2 M H_2SO_4 and the absorbance was read at 450 nm. Experiments were performed in at least 3 independent repeats and data were subject for statistical analyses.

The apparent dissociation constant (Kd) was calculated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA), using the following model equation: A = A_{max} [L]/(K_{d} + [L]), where A is the absorbance at 450 nm and [L] is the ligand concentration [25].

2.11. Bacteriostatic activity of AmphiITLN-like

S. aureus or E. coli (10^7) were added to 20 ml warm Luria-Bertani solid medium in Petri dishes. After the medium solidified, holes of 0.5 cm diameter were punched into the surface and 100 μl ampicillin (1 μg/μl), recombinant AmphiITLN-like protein (1 μg/μl), or TBS was added to each punch. After incubation at 37 °C for 12 h, a transparent ring around pores signified antibacterial activity.

3. Results

3.1. Cloning, sequence comparison, and phylogenetic analysis of AmphiITLN-like

Using D. rerio intelectin sequences, we carried out a blastp search for amphioxus intelectin homologs in the JGI database. One sequence, jgi/Braf1/171469, was identified with the highest homology. Primers for RT-PCR amplification from adult amphioxus were designed and the resulting cDNA clone was named AmphiITLN-like (Gene bank accession number HQ588911), which had an open reading frame of 909 bp, encoding 302 amino acids (Fig. 1).

A conserved fibrinogen-related domain (FR6d) commonly present in vertebrate intelectins was identified in the N-terminal 1/3 of AmphiITLN-like (aa 25-91, E-value 2.70e-04) by the SMART program. The C-terminal 2/3 sequence, termed the Intelectin domain by Lin et al. [11], is highly conserved and unique within the intelectin gene family. Potential N-glycosylation sites were not identified by NetNGlyc 1.0.

A blast search identified intelectin homologs in several representative organisms (Figs. 1 and 2), including 2 homologs in human (H. sapiens, Pan troglodytes, I. punctatus (blue catfish or channel catfish), and Lethenteron japonicum; 2 in the BABL/c and 6 in the 129SV strain of M. musculus, respectively; 1 homolog in Anolis carolinensis, C. idella, Salmo salar, and Oncorhynchus mykiss; 7 in D. rerio and 6 in Ciona intestinalis, but not in lower species such as Drosophila melanogaster or Caenorhabditis elegans.

The amino acid sequence alignment showed that AmphiITLN-like is highly conserved with other intelectins, with overall 47.2%, 48.4%, 44.5%, and 41.6% identity to intelectin 1 of H. sapiens, M. musculus, X. laevis, and D. rerio, respectively, and even higher identity of 65.2%, 65.2%, 60.6%, and 65.2% for the FR6d and lower identities of 49.3%, 49.8%, 48.8%, 50.7%, and 49.5% for the Intelectin domain. Interestingly, eight cysteine residues are conserved among all vertebrate intelectins. However, invertebrate intelectins, such as AmphiITLN-like and Ciona ITLN-1b, lacked the 4th and 6th cysteines, while...
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AmphiITLN-like contained two non-conserved cysteines as boxed in Fig. 1. To examine the evolutionary relationship of AmphiITLN-like, a NJ tree was constructed with MEGA4.0 using intelectin homologous from representative organisms (listed in Fig. 2). The ascidian (urochorda) and amphioxus (cephalochordata) intelectins were located at the base, suggesting that they are likely the ancestors of the intelectin gene family. The vertebrate intelectins could be split into two main branches: one branch contained advanced vertebrate and the other contained fish plus lamprey, suggesting that they evolved independently. In most species, except for human and Chimpanzee, intelectins from the same species clustered together, suggesting their expansion occurred only recently after species divergence. Interestingly, animals that live in water, including}

**Fig. 1.** Multiple alignments of amino acid sequences between AmphiITLN-like and intelectins from some representative species. Identical residues are shaded in black, and residues shared by >50% of the sequences are shaded in gray. The FReD domain is underlined in black and the intelectin domain is underlined in gray. The eight conserved cysteines are numbered 1–8 above the sequences and the two non-conserved cysteines are boxed. Sequences are shown as species names followed by gene names. The GenBank accession numbers are as follows: Human ITLN1 (NP_060095), Human ITLN2 (NP_543154), Mouse ITLN1 (NP_034714), Mouse ITLN2 (NP_001007553), X. laevis ITLN1 (NP_001082570), X. laevis ITLN2 (NP_001085762), Zebrafish ITLN1 (NP_001153013), Zebrafish ITLN2 (NP_001153013), Zebrafish ITLN3 (NP_001153056), Lamprey ITLN1 (BAD98810), and Ciona (XP_002121647).

**Fig. 2.** Neighbor-joining phylogenetic tree of intelectins. Numbers near the node indicate bootstrap confidence values derived from 1000 replicates. The scale bar shows the number of substitutions per site. Zebrafish and Ciona sequences are shown as species names followed by Ensembl Genome Browser number and others are shown as species names followed by gene names. GenBank accession numbers for Human, X. laevis, and Lamprey intelectins are the same as in Fig. 1 and those of other sequences are as follows: Mouse ITLN1 (ADJ67489), Mouse ITLN2 (ADJ67487), Mouse ITLN3 (ADJ67488), Mouse ITLN4 (ADJ67486), Mouse ITLN5 (ADJ67484), Mouse ITLN6 (ADJ67483), Chimpanzee ITLN1 (XP_513928), Chimpanzee ITLN2 (XP_513929), Green anole ITLN-2-like (XP_003229481), Rainbow trout putative ITLN (AAG30025), Grass carp (AAY43357), Atlantic salmon ITLN-1a (NP_001135002), Blue catfish ITLN1 (ABW07848), Blue catfish ITLN2 (ABW07849), Channel catfish ITLN1 (ABW07846), Channel catfish ITLN2 (ABW07847), and Lamprey serum lectin (BAB32787). AmphiITLN-like is signified by the triangle.
ascidian, amphioxus and fish, have many copies of intelectins while most animals that live on land have only two copies.

### 3.2. Tissue distribution of AmphiITLN-like transcripts in adult

To demonstrate the expression profile of AmphiITLN-like transcripts in adult animal, in situ hybridization was conducted. AmphiITLN-like transcripts were most abundant in epithelial cells of the gut (Fig. 3A,C), hepatic caecum, and endstyle (Fig. 3A–D). Strong signals were also observed in the skin, the muscle septum, and the exothermal cells of the gill, but not in the muscle and notochord. Since the digestive tract and outer skin layer are known as the primary immune tissue/organs in amphioxus [26], this distribution of AmphiITLN-like implied its role in the immune response.

### 3.3. Up-regulation of AmphiITLN-like in response to bacterial challenge

To explore the response of AmphiITLN-like to bacteria challenge, quantitative real-time PCR was employed to determine the expression level of AmphiITLN-like. In all cases, the dissociation curves for both AmphiITLN-like and β-actin exhibited a single peak, indicating the amplification was specific (data not shown). AmphiITLN-like expression was up-regulated following S. aureus challenge and peaked (7.12 fold, \( p < 0.01 \)) at 8 h. However, the level of expression dropped sharply to a lower-than-normal level at 12 h before increasing again by 24 h (1.55 fold higher). In contrast, E. coli challenge only evoked a modest increase in AmphiITLN-like expression, peaking (3.16-fold, \( p < 0.05 \)) after 12 h (Fig. 4) and returning to baseline levels by 24 h.

![Fig. 3. Expression pattern of AmphiITLN-like transcripts in adult amphioxus. Micrographs of whole vertical sections (A, B) and enlarged inboxes (C, gut and D, hepatic caecum) of adult amphioxus showing the presence of AmphiITLN-like transcripts. (E) Negative control by DIG-tagged sense strand probe. Scale bars represent 100 μm.](image-url)
3.4. Expression and purification of recombinant AmphitILN-like protein

To further explore its function, the recombinant AmphitILN-like protein was expressed in E. coli system. The majority of the recombinant protein existed as inclusion bodies. Purified and refolded protein migrated as a single 30-kDa band on SDS-PAGE, matching the expected size (Fig. 5A). Western blotting using anti-His antibody confirmed that it was produced by the pET29b-AmphitILN-like vector (Fig. 5B).

Mouse antiserum was successfully obtained with the recombinant protein and was able to recognize AmphitILN-like in the western blot analysis (Fig. 5B).

3.5. Bacterial binding of AmphitILN-like

To explore the potential immunological functions of AmphitILN-like, we incubated recombinant AmphitILN-like protein with bacteria to measure its binding activity. As shown in Fig. 6A, recombinant AmphitILN-like could still bind to S. aureus after being washed by TBS buffer five times. Then we investigated the binding of AmphitILN-like to other bacteria and the potential role of calcium in this process. In the absence of calcium, 26 ± 1.45, 36 ± 2.3, 30 ± 1.45 percent of AmphitILN-like were bound to E. coli, S. aureus and S. faecalis, respectively, estimated by band intensity compared with the equal amount of unincubated AmphitILN-like (Fig. 6B). While in the presence of calcium, 37 ± 2.0, 57 ± 2.4, 36 ± 0.8 percent of proteins were bound to these bacteria, respectively, all with apparent statistic differences (P < 0.01, P < 0.01 and P < 0.05, respectively).

3.6. Ca²⁺-dependent agglutination activity of AmphitILN-like

To determine whether bacterial binding could induce the aggregation of bacteria, we performed the bacterial agglutinating assay in vitro. FITC-labeled Gram-negative E. coli or Gram-positive S. aureus and S. faecalis were incubated with recombinant protein at various concentrations and conditions. As shown in Fig. 7, recombinant AmphitILN-like agglutinated both Gram-negative and Gram-positive bacteria, but apparently to different degrees.

3.7. Differential binding of AmphitILN-like to PGN and LPS

Binding of AmphitILN-like to bacteria is likely dependent on the interaction with bacteria cell wall components, particularly carbohydrates. Therefore, we further employed ELISA analysis to quantify the recognition of AmphitILN-like to LPS or PGN, the major components of Gram-negative and Gram-positive bacteria cell wall, respectively.

The binding curves for AmphitILN-like were similar for both LPS and PGN, and both became saturated (Fig. 8). Nonlinear regression analysis suggested the binding actions fit a one-site binding model (R² > 0.98). The apparent dissociation constants (Kd, value for the

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binding affinity) and the maximum binding (Bmax, value for the binding rate) parameters were calculated (Table 2). The Bmax values of AmphiITLN-like binding to LPS and PGN were comparable without Ca2⁺; but in the presence of Ca2⁺, the Bmax values of LPS binding is smaller than that of PGN binding, (0.6109 ± 0.02474 vs 0.7645 ± 0.01948, p < 0.05), indicating a faster reaction rate. In all cases, the Kd values for PGN binding were lower than those for LPS binding, indicating that AmphiITLN-like had higher affinity for PGN than LPS (LPS vs PGN: p < 0.05; LPS + Ca2⁺ vs PGN + Ca2⁺: p < 0.05). Statistic analysis of Kd values also revealed that addition of Ca2⁺ did increase affinities for both PGN and LPS (LPS vs LPS + Ca2⁺: p < 0.05; PGN vs PGN + Ca2⁺: p < 0.05).

### 3.8. Bacteriostatic activity of AmphiITLN-like

To test direct bacteriostatic activity of AmphiITLN-like, inhibition of bacteria growth was tested. Ampicillin of 1 μg/ml could surely inhibit both S. aureus or E. coli growth while both TBS and recombinant AmphiITLN-like protein of up to 1 μg/ml did not display any apparent inhibition effect.

**Table 1**

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Agglutination was examined under a fluorescence microscope and the degree of agglutination was scored as: ++++, total agglutination of bacteria; ++, <10% of bacteria not agglutinated; +, 50% of bacteria not agglutinated; –, some agglutination detectable; –, no agglutination detectable.

4. Discussion

A number of studies have documented the presence and role of intelectin in vertebrates. In contrast, little is known about the role of intelectin in invertebrates. We cloned successfully one sequence of intelectin and evaluated its expression and function in the cephalochordate *B. belcheri tsingtauense*. The deduced 302 amino acid sequence of AmphiITLN-like contained a FReD domain in the N-terminus and a putative intelectin domain in the C-terminus, which is similar to the arrangement in vertebrate intelectins. All vertebrate intelectins we examined contained eight conserved
cysteines which could form inner-chain disulfide bonds to stabilize the multimer structure [27]. However, both amphioxus and ascidians lack the 4th and 6th cysteines, suggesting that these two cysteines have evolved in vertebrates later, possibly to further stabilize the intelectin structure. On the other hand, AmphiITLN-like contained two non-conserved cysteines. It is thought that intelectin multimers can only be formed by non-conserved cysteines through inter-chain disulfide bonds. Further investigation is needed to explore whether these non-conserved cysteines could help to form multimers in native protein.

Our phylogenetic analysis revealed that intelectin gene families duplicated following the divergence of species and suggested that these events may be relatively recent and ongoing. An interesting observation is that there are many intelectins in ascidian, amphioxus and fish, whereas only two in human, chimpanzee and frog. Expansion of a gene family usually represents an evolutionary mechanism to adapt to a particular selection pressure [28]. Dupli-
cated genes (paralogs) can evolve distinct functions, possibly explaining the diverse functions of intelectin among different species.

The specific distribution of AmphiITLN-like in the intestine was similar to that of intelectin in humans, mice, and catfish, and it is believed that there may be a similar innate immune function in the intestine. The fact that AmphiITLN-like was also present in the intestine, which plays a major role in the primitive immune system of amphioxus [26], suggests that the functions of intelectin in the intestine may be conserved among species.

Significant up-regulation of AmphiITLN-like occurred 8 h after bacterial infection, and was as rapid as for acute phase proteins, suggesting that intelectin may play a role in the acute phase response [29]. Similarly, this phenomenon has been observed in channel catfish following E. ictaluri infection [4,30], and in zebrafish following S. aureus infection [31]. Furthermore, the differential response to E. coli and S. aureus challenges suggest that AmphiITLN-like may be particularly sensitive to S. aureus.

Generally, animal defense lectins can function as phagocytosis receptors, soluble agglutinins, and opsonins against bacterial (or parasitic) invasion [4]. The agglutination of lectins allows the host to consolidate the targets and then initiate the secondary immune defense [17]. Our bacterial binding, agglutinating, and polysaccharide-binding assays revealed that AmphiITLN-like recognizes PGN or LPS, the chief cell wall components of bacteria, and then induces direct bacterial binding and agglutinating. However, previous studies have shown that human and ascidian intelectins are able to enhance phagocytosis of macrophages but not agglutinating [12], although the recombinant XL35 expressed in E. coli can aggregate trypsinized and glutaraldehyde-fixed rabbit erythrocytes [27]. To the best of our knowledge, our observations may be the first evidence that recombinant intelectin can recognize PGN or LPS and aggregate a pathogen.

All experimental evidence was consistent with a role for AmphiITLN-like as an acute phase reactant that is induced by bacterial challenge, and then binds to and agglutinates bacteria via its affinity for PGN and LPS. The fact that AmphiITLN-like preferably agglutinates S. aureus over E. coli is likely due to different binding capacity to PGN and LPS, the major and characteristic PAMPs of gram-positive and -negative bacteria, respectively.

Another interesting observation was that calcium is essential for bacterial agglutination, but not for bacterial binding and poly-
saccharide binding. However, calcium did increase the affinity and binding rate of AmphiITLN-like to polysaccharides and enhanced the interaction between AmphiITLN-like and the bacteria. This phenomenon can be explained assuming that calcium is not crucial for carbohydrate recognition but for the formation of intelectin complexes, resulting in aggregation of AmphiITLN-like bound bacteria. Indeed, such mechanism is found in many other lectins, such as immulectin in insects and Regliy in mice [32]. However, classical Ca2+-binding motifs have not yet been identified in AmphiITLN-like, thus, an unknown calcium binding motifs or ligand binding mechanism should be explored further.

The FcRd domain is highly conserved in intelectins and has been characterized as the specific binding site to ClCNaC in ficolin [33]. Recombinant human intelectin1 has the affinity to α-pentoses and β-galactofuranosyl, but no corresponding carbohydrate recognition domain (CRD) was identified. The mechanism of carbohydrate recognition is not fully understood for intelectin and should be further evaluated.

Intelectin acts as a receptor for lactoferrin (LF) in human infants [34]. LF is an iron-binding glycoprotein with antimicrobial activity [35]. Receptor-mediated interaction with LF or its peptide fragments, which enter into intestinal epithelial cells, may play an important role in the initial step for subsequent cellular processes, such as iron metabolism or antibiotic activity [36].

Intelectin is also named omentin and can exhibit decreased expression in the omental adipose tissue of individuals with Crohn’s disease, a kind of transmural intestinal inflammation. In mouse lung epithelial cells, intelectin is required to activate IL-13 inducing monocyte chemotactic protein, an essential chemokine involved in Th2 inflammation and participating in allergen-induced airway inflammation [37]. Taken together, these functions suggest that intelectin is involved in the inflammatory response in vertebrates.

In summary, we demonstrated that AmphiITLN-like is primarily localized in the digestive tract and that expression of AmphiITLN-like increases rapidly after bacterial challenge. Recombinant AmphiITLN-like binds with higher affinity to PGN than LPS and can act as agglutinin. Our results provide insight into the evolution of the intelectin gene family. Further studies focusing on the mechanisms controlling lectin-activity and iron adsorption of intelectin could be performed to improve our knowledge of intelectin in host immune defense.

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References


