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Characterization of the BPI-like gene from a subtracted cDNA library of large yellow croaker (*Pseudosciaena crocea*) and induced expression by formalin-inactivated *Vibrio alginolyticus* and *Nocardia seriolae* vaccine challenges

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Eukaryotic expression

Abstract One expressed sequence tag (EST 64LF004 clone), which is from the subtracted cDNA library of the head kidney of large yellow croaker (*Pseudosciaena crocea*) stimulated with peptidoglycan (PG) by suppression subtractive hybridization (SSH) method, was cloned using RACE–PCR. The full length cDNA, which possesses typical structural features of a signal peptide, a conserved LPS binding domain and two bactericidal permeability-increasing (BPI) motifs as in higher vertebrates, was identified as a novel homologue, namely of the large yellow croaker BPI-like molecule (Pc-BPI-L). Phylogenetic analysis showed this Pc-BPI-L of large yellow croaker as the most ancestral branch in bony fish clade. The recombinant Pc-BPI-L protein expressed in the Tn-5B1-4 insect cells was successfully produced and confirmed to have the predicted size of 52 kDa by Western blot analysis. At the message level, Pc-BPI-L mRNA was ubiquitously expressed in all tissues examined. Following formalin-inactivated *Vibrio alginolyticus* and *Nocardia seriolae* treatment, Pc-BPI-L message was differentially up-regulated in primary immune organs. These results indicate that Pc-BPI-L might be involved in the immune response to bacterial infection.

Introduction

The innate immune system acts as the first line of host defense against pathogens and plays a vital role in maintaining host–microbe homeostasis [1]. Bony fish have

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quick, powerful defense mechanisms to a wide range of pathogens [2,3]. The host immune system recognizes invading pathogens by their highly conserved pathogen-associated molecular patterns (PAMPs), which are unique to these pathogens and are normally not shared by host cells. Recognition is mediated by pattern recognition receptors (PRRs) and initiates the inflammatory processes [4–6]. Many components of PRRs are evolutionarily conserved from insects to humans [7]. The expressed immune-related genes in immune tissue after injection of PAMPs such as peptidoglycan (PG) or lipopolysaccharide (LPS) have been investigated in several fishes [8–11].

Bactericidal permeability-increasing protein (BPI) and LPS-binding protein (LBP) are highly conserved host-defense molecules and belong to a family of lipid binding proteins named the BPI-LBP-PLTP-CETP family [1,12]. They are two structurally and functionally related proteins [13,14] and bind to the Lipid A component of LPS from the outer envelope of Gram-negative bacteria via their N-terminal domain [1,12,15,16].

BPI was originally isolated from primary granules of rabbit and human polymorphonuclear leukocytes (PMN) [17,18], with in vitro antimicrobial activity attributed to an ''endogenous antimicrobial'' molecule in mammalian BPI [19,20]. Most work on BPI has been concentrated in mammals to date, with little information about the homolog of mammalian BPI in teleosts. In 2002 Inagawa et al. firstly identified and cloned LBP/BPI homolog from rainbow trout (*Oncorhynchus mykiss*) [21]. Since then, the presence of BPI /LBP was reported in three other teleosts, common carp, Atlantic cod and channel catfish [22–24].

Large yellow croaker (*Pseudosciaena crocea* Richardson, 1864) is an economically important mariculture species in China. In recent years, with the rapid development of marine-culture of large yellow croaker in the southern coastal region, serious epidemics with high mortalities in cultured juveniles emerged, resulting in severe economic losses. The pathogenic causes of these epidemics included *Nocardia seriolae*, *Vibrios* and *Iridovirus* [25–27]. Antibiotics and chemotherapeutics were usually used to control these diseases, which resulted in the development of a drug-resistant bacteria, environmental pollution and residues in fish [28].

In the present study, the bacterial permeability-increasing protein like gene of large yellow croaker (Pc-BPI-L) was sequenced and characterized using suppression subtractive hybridization (SSH) and RACE–PCR, and the expression profile in tissues sampled after induction with formalin-inactivated *Vibrio alginolyticus* (FI-Va) and *Nocardia seriolae* (FI-Ns) pathogens was also examined. BPI is an antimicrobial peptide and thus has therapeutic implications. In order to obtain a substantial amount of the Pc-BPI-L protein, it has been expressed as a recombinant in prokaryotic and eukaryotic expression systems.

Materials and methods

Fish challenge experiments and sample collection

Large yellow croakers, 50–80 g in body weight, were selected and cultured at the commercial mariculture farm at Xiangshan, Ningbo city, China. Fifty fish were anaesthetized with engenol (10 mg L^{-1}) and injected intraperitoneally (i.p.) with 0.2 ml 250 $\mu g\ ml^{-1}$ PG (Sigma, USA). Fifteen control individuals were injected with saline. Head kidneys were removed aseptically from three to five individuals after injection at 12, 24, 36, 48 and 72 h, respectively. Tissue samples were immediately frozen in liquid nitrogen and then stored at −80 °C until used for RNA extraction. The treatment of experimental animals complied with the Laboratory Animal Law.

Preparation of bacteria vaccine and fish challenges

Two formalin-inactivated bacteria vaccines were prepared as follows. Briefly, the strain of *V. alginolyticus* was inoculated in common sea water medium (yeast powder 1 g, beef extract powder 3 g, tryptone 5 g, agar 20 g, add 15‰ sea water up until 1000 ml, pH 7.3, 121 kPa autoclaving for 20 min), cultivated for 24 h at 28 °C [25]. The strain of *N. seriolae* was inoculated on tryptic soy agar (TSA) and plates were then incubated for 15 days at 28 °C [26]. Bacteria were washed with 10 mmol L^{-1} phosphate-buffered saline (PBS) (pH 7.2). The precipitate was washed three times and resuspended in PBS, and lastly inactivated for 48 h at 4 °C by the addition of 0.5% (v/v) formalin. The inactivated supernatant was centrifuged at 7000 rpm for 30 min at 4 °C to discard formalin; the pellet was resuspended in PBS and adjusted to 1×10^7 particles ml^{-1} and stored at 4 °C. The above operations were performed under sterile conditions.

Two groups of large yellow croaker, 120–150 g in body weight (15 fishes per group) were anesthetized with engenol (10 mg L^{-1}) and injected i.p. with 0.5 ml of FI-Va and FI-Ns bacterial vaccine consisting of 1.0×10^7 particles ml^{-1}, respectively. The third group was injected with saline as a control. The kidney, liver, heart, intestine, muscle, gills, spleen and head kidney were removed aseptically from five individuals at 48 h after induction and frozen at −80 °C until RNA extraction.

cDNA library construction and sequencing

Total RNA was extracted from the head kidney of the driver and the tester using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The quantity and quality of RNA were determined by UV spectrophotometer (absorbance at A_{260} and an $A_{260/280}$ ratio) and agarose gel electrophoresis, respectively. The cDNA was synthesized and amplified using a Clontech SMART PCR cDNA Synthesis Kit (BD Clontech, USA).

The SSH cDNA library was constructed using a PCR-Select™ cDNA subtraction kit (BD Clontech) according to the manufacturer's protocol. The cDNA fragments that remained 250–1000 bp were amplified and ligated to the pMD-19T vector (Takara, China), then DH5α *Escherichia coli* cells (Takara) were transformed with the recombinant DNA. Insert size was checked by PCR amplification followed by agarose gel electrophoresis. Approximately 200 randomly-selected positive clones were sequenced using the M13+/− primers. Bioinformatic analyses of EST sequences were conducted by using the Basic Local Alignment Search Tool (BLAST) [29].

Cloning of Pc-BPI-L cDNA by 5′-RACE and sequence analysis

The EST 64LF004 clone was selected based on the information gained from the BLAST-searches performed previously. To isolate the full-length cDNA of this EST clone, 5′-RACE PCR was performed using a Smart RACE cDNA amplification kit (BD Clontech). The gene specific primer was designed according to the known EST sequence, 5′-RACE primer: 5′-CAGCACCTTTGCCAAGTATCTCG-3′. The generated 5′-PCR product was purified and subcloned into the pMD19-T vector (Takara) for sequencing. Three clones were sequenced by the M13+/− universal primers in vector.

The open reading frame (ORF) was acquired with the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/). Domains/Motifs were predicted by the domain prediction program simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/) [30]. The potential cleavage site of signal peptide was analyzed by the SignalP program (http://www.cbs.dtu.dk/services/SignalP/) [31].

Alignment and phylogenetic analysis

The relevant sequences were retrieved from GenBank for multiple sequence alignments, and a phylogenetic tree was constructed using the neighbor-joining method [32,33] by the MEGA3.1 package [34], and the resulting trees were bootstrapped with 1000 replicates.

Tissue expression analysis by reverse transcription—PCR

The total RNAs (5 μg) were reverse-transcribed with a MMLV-RT—PCR Kit (Promega, USA). RT—PCR was used to analyze the level of Pc-BPI-L gene expression with the gene-specific primers (Pc-BPI-P1, 5′-CGAGATACTTGGCAAAGGT-3′, Pc-BPI-L-P2, 5′-GAAGGCTGCAATGGGTAAT-3′). PCR was carried out under the following conditions: 94 °C, 2 min; followed by 29 cycles of amplification (94 °C for 15 s, 56 °C for 15 s, 72 °C for 30 s) and a final extension of 1 min at 72 °C. As a positive control for RT—PCR, β-actin was amplified to determine the concentration of each template. The primers for β-actin were β-P1 (273 bp), 5′-GCGACCTCACAGACTACCTC-3′ and β-P2, 5′-GTAGGTG GTCTCGTGGAT-3′. The values were presented as the standard errors of the mean (SEM) of two independent experiments done in triplicates and differences were considered statistically significant when p values were less than 0.05.

Expression of BPI in prokaryotic expression vector

The complete ORF of Pc-BPI-L gene was amplified by PCR and subcloned into the vector pET28b and pET32c vector (Novagen, Germany), respectively. The recombinant plasmids containing the target gene were then transformed into *E. coli* BL21 and grown to $OD_{600} = 0.5$ at 30 °C, and then induced with 0.3—1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. The crude *E. coli* BL21 cell extractions were identified by SDS—PAGE and then subjected to Western blot as described below.

Expression of BPI protein in Tn-5B1-4 insect cells using Bac-to-Bac system

Recombinant vector construction

Plasmid pFastBac™-HTb, *E. coli* (DH10Bac) and Tn-5B1-4 cells of the cabbage looper moth, *Trichoplusia ni* were kindly provided by Professor Chuanxi Zhang (Institute of Applied Entomology, Zhejiang University, Hangzhou, China). The fetal bovine serum (FBS) (Gibco, USA), Grace's Insect Cell Culture Medium (Invitrogen, USA) and Lipofectin 2000 (Sigma, USA) were purchased. Anti-His monoclonal antibody and HRP-conjugated sheep anti-mouse IgG antibody were purchased from Novagen, Germany.

The complete ORF of Pc-BPI-L gene was amplified by PCR and subcloned into the pFastBac-HTb vector (Invitrogen, USA) to construct the recombinant donor plasmid pBacHT-BPI. The donor plasmid was then transformed into *E. coli* (DH10Bac) to form the recombinant Bacmid-BacHT-BPI.

Cell culture and transfection

The *Trichoplusia ni* cell line Tn-5B1-4 was cultured in Grace's Insect Cell Culture Medium with 10% fetal bovine serum (FBS). Bacmid-BacHT-BPI DNA was further transfected into Tn-5B1-4 cells using Lipofectin 2000 followed by three washes with ice-cold PBS.

Western blot

Total extracts from Tn-5B1-4 cells were subjected to SDS—PAGE with 4% stacking gel and 8% separating gel, and then transferred onto a polyvinylidene difluoride membrane (MagnaProbe, Osmonics) by an electrophoretic transfer system (Bio-Rad, USA). The membrane was then blocked with PBST (PBS pH 7.4, containing 0.1% Tween-20) containing 5% skim milk for 1 h at room temperature. After washing five times with PBST, the membrane was incubated with the His-Tag monoclonal antibody diluted in PBST (1/1000) for 2 h at room temperature and washed five times with PBST. HRP-conjugated sheep anti-mouse IgG antibody was diluted 1/1000 in PBST and incubated with the membrane for 2 h. The membrane was soaked in the working solution and prepared before the experiment for 5 min at 25 °C, terminated by ddH_2O, and then photographed.

Results

Construction of the subtracted cDNA library

One hundred and ninety-six randomly selected positive clones were sequenced and checked for homologues in the GenBank database using the BLASTX sequence comparison software on the NCBI website. Matches having an expected (E) value $\leq 10^{-4}$ were classified as significant. Partial ESTs were deposited to dbEST at the NCBI, GenBank accession numbers are from EB643249 to EB643371.

Characterization of Pc-BPI-L cDNA

A cDNA fragment of 581 bp from the EST 64LF004 clone with significant poly(A) signal and sequence homology to *Ictalurus punctatus* BPI protein was obtained. 5′-RACE

was conducted with the gene-specific primers to clone full-length cDNA. The sequence of the full-length cDNA has been deposited in GenBank (accession number DQ917778*)*. Sequence analysis indicated that the full-length cDNA of Pc-BPI-L gene is 1919 bp long, including 5′- and 3′-untranslated region (UTR) of 103 bp and 397 bp, respectively. There is a typical polyadenylation signal (AATAAA) located 23 bp upstream of the poly(A) tail and three mRNA instability motifs (ATTTA) in 3′-UTR. The isolated clone has an open reading frame (ORF) (104—1522) that encodes a 473 amino acid (aa) peptide (including stop codon) with a predicted size of 51.28 kDa. The deduced Pc-BPI-L amino acid sequence contains a putative 17-aa signal peptide, a cleavage site between 17 and 18 aa, followed by 456 aa identified as the putative mature protein (Fig. 1). SMART results showed two putative functional domains at positions 26—244 and 259—461 aa of Pc-BPI-L that are similar to the BPI/LBP/CETP N-terminal domain (5.02e-31) and BPI/LBP/CETP C-terminal domain (1.93e-32) found in human BPI/LBP/CETP. The BLASTP search showed that the overall amino acid sequence of large yellow croaker BPI-L shared 36%, 36%, 36%, 34%, 28% and 25% residue identity with the channel catfish, common carp, rainbow trout, Atlantic cod, cattle and human BPI/LBP, respectively. An alignment of the Pc-BPI-L amino acid sequence with other representatives of this family in teleosts is shown in Fig. 2.

Phylogenetic analysis of Pc-BPI-L

As shown in the phylogenetic tree (Fig 3), there are two large sister taxa that were clustered. One large clade consisted of four sister taxa: firstly, the mammals BPI and LBP proteins formed one clade, then bony fish BPI/LBP proteins and mammals BPI-like 2 respectively formed two separate clades, and the last clade came from the mammal CETP and PLTP proteins. Interestingly, the mammalian BPI-like 1, BPI-like 3 and PLUNC-like proteins assembled into another large clade. The Pc-BPI-L protein of large yellow croaker formed the branch at the base of the bony fish taxa with a high bootstrap value (to the number of 99). The next close branch was BPI/LBP of Atlantic cod, followed by a clade containing freshwater fish species: BPI of channel catfish and BPI/LBP of common carp and rainbow trout. This phylogenetic analysis showed that the Pc-BPI-L of large yellow croaker was the most ancestral split within the fish clade.

Expression pattern of Pc-BPI-L by RT—PCR

Pc-BPI-L gene expression in various tissues was analyzed by RT—PCR after treatment with two formalin-inactivated bacterial vaccines (Figs. 4 and 5). The Pc-BPI-L gene was ubiquitously expressed, with variable expression levels, in all untreated control tissues examined. After treatment with bacterial vaccines its expression was obviously up-regulated ($p < 0.05$) in the head kidney, spleen, intestine, kidney, gills and muscle (Figs. 4 and 5). In heart, head kidney and spleen tissues, the Pc-BPI-L expression was up-regulated ($p < 0.05$) in fish treated with FI-Ns more than those treated with FI-Va (Figs. 4 and 5). Furthermore, Pc-BPI-L expression was unchanged in the liver tissue of two experimental groups, FI-Va and FI-Ns. Expression of β-actin mRNA was nearly constant in all experimental samples, indicating that the total RNA concentrations were identical (Fig. 4).

Eukaryotic expression of BPI in insect cells and Western blot analysis

Pc-BPI-L of large yellow croaker was expressed using the Bac to Bac system in Tn-5B1-4 cells of the cabbage looper moth, *Trichoplusia ni*. The presence of the recombinant Pc-BPI-L product of the expected size, about 52 kDa, was confirmed by Western blot using the anti-His-Tag monoclonal antibody. The result indicated that the recombinant protein was expressed successfully in Tn-5B1-4 cells (Fig. 6A,B).

Expression of BPI in *E. coli*

The complete ORF of large yellow croaker Pc-BPI-L gene was cloned into the pET28b and pET32c expression vectors. After induction with 1 mM IPTG, a protein with an approximate molecular weight of 52 kDa, which correlated well with the size of deduced BPI amino acid sequence, failed to be observed in SDS—PAGE and Western blot.

Discussion

Impact of viral infections or immunostimulation on the immune-related genes in various fish has been demonstrated by the SSH technique and EST analysis [35]. These include *Oncorhyncus mykiss* injected with *Vibrio anguillarum* [2]; *P. crocea* injected with polyI:C [36]; *Sparus aurata* injected with *Nodavirus* [37]; *Paralichthys olivaceus* injected with PG [8]; *Cyprinus carpio* injected with PG, LPS and Con-A [9,10]; and *Epinephelus awoara* injected with LPS [11]. In this paper, a subtracted cDNA library from the head kidney of the large yellow croaker stimulated with PG has been constructed using SSH technology, and a novel Pc-BPI-L gene in large yellow croaker has been thus identified.

The large yellow croaker Pc-BPI-L gene contains three mRNA instability motifs (ATTTA) in the full-length cDNA. The instability motif is responsible for destabilizing mRNA by directing degradation and suppressing translation levels [38,39], and has been identified from a number of inflammatory mediators such as cytokines in fish [22,40,41]. Beamer et al. [42] have pointed out that the positive charge amino acids in the N-terminal domain can bind to the anionic portion of lipid A and may correlate with bactericidal activity. Our results showed that basic amino acid residues are clustered in the N-terminus of the Pc-BPI-L amino sequence (a conserved LPS-binding domain) similar to the mammalian BPI [15,43—47], suggesting that the Pc-BPI-L may bind LPS with high affinity and may also have bactericidal activity. Sequence analysis also suggested that the Pc-BPI-L gene is more like mammalian BPI than the LBP counterpart and therefore it may participate in host antimicrobial defense.

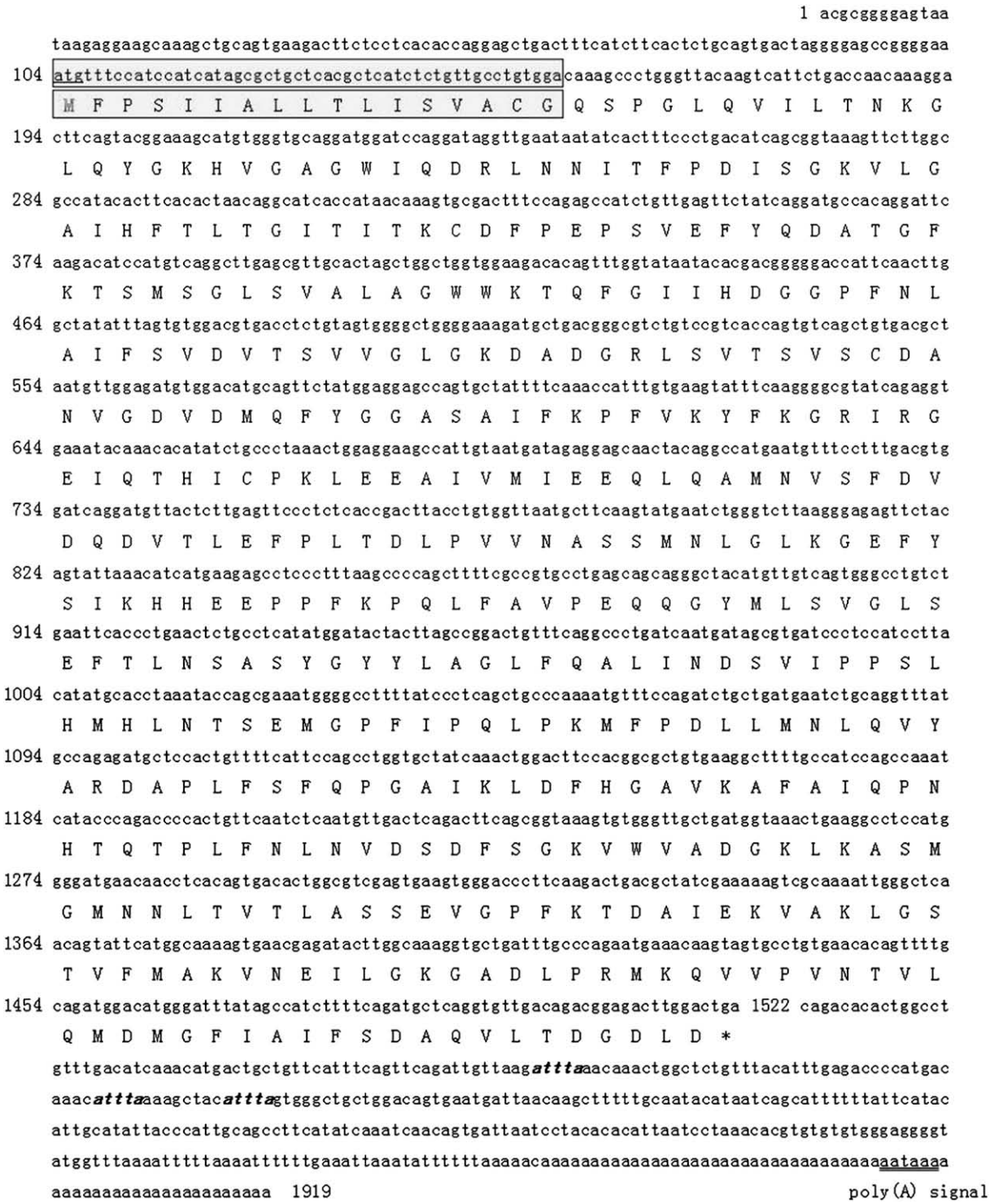

Figure 1 Nucleotide and deduced amino acid sequence of large yellow croaker Pc-BPI-L gene (GenBank accession no. DQ917778). Nucleotide and amino acid numbers, starting from the putative methionine initiation codon, are presented on the left side. Asterisk (*) indicates termination codon. The Pc-BPI-L putative signal peptide is shown as drop shadow. One typical polyadenylation signal AATAAA is shown as double underscore. Three mRNA instability motifs (ATTTA) in 3′-UTR are shown in boldface and italicized.

In this study, the ubiquitous expression of Pc-BPI-L gene was observed in all sampled control tissues, which were blood-filled and were not perfused free of blood prior to RNA isolation (Fig. 4), in agreement with the Atlantic cod expression that was detected in brain, head kidney, heart, posterior intestine, anterior intestine, liver, spleen, skin, gill and peripheral blood cells [23]. This indicated the possible presence of leukocytes as the main source of Pc-BPI-L

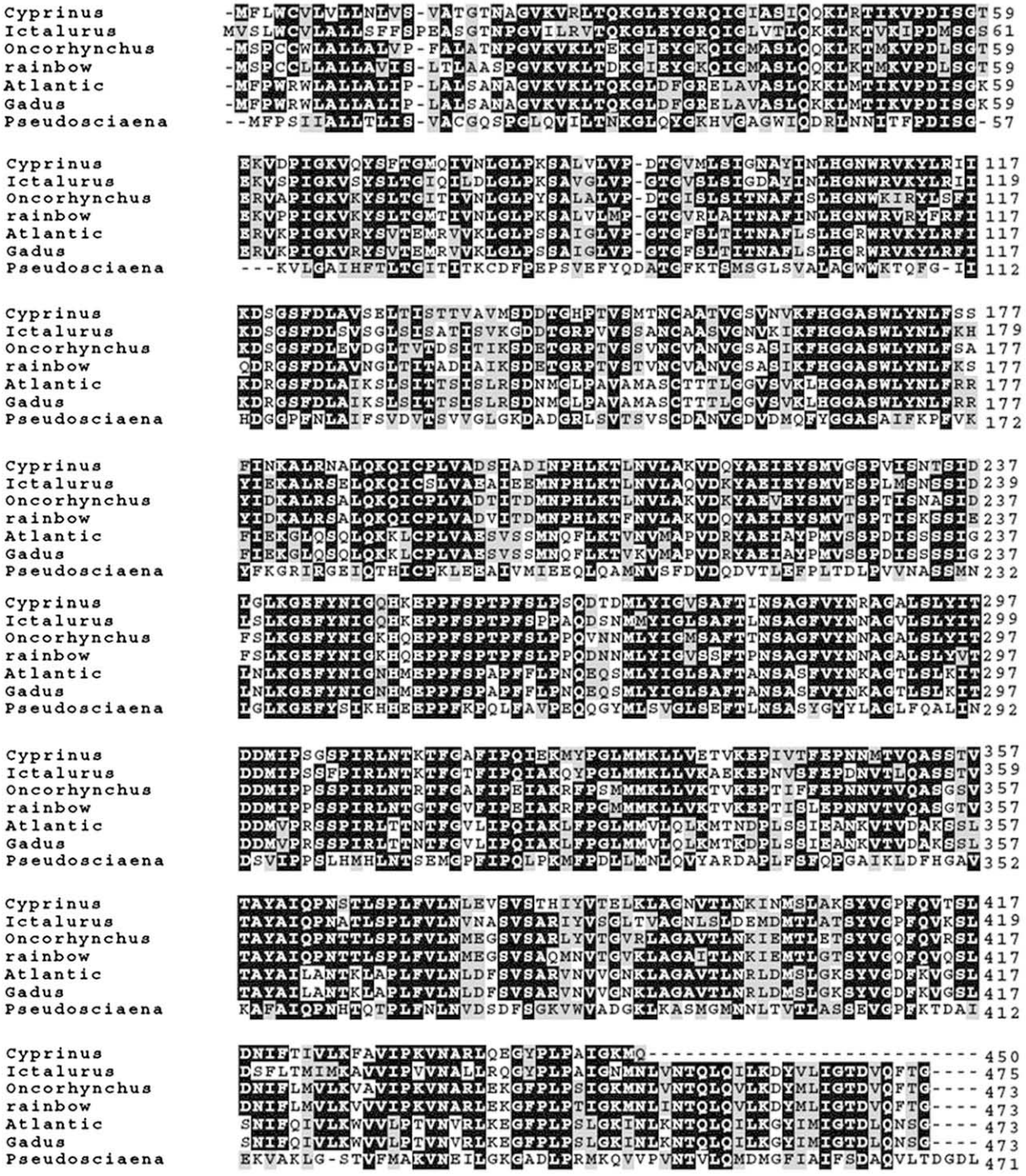

Figure 2 Alignment of the deduced amino acid sequence of large yellow croaker Pc-BPI-L with other bony fish LBP/BPI. Alignment was performed using the clustalw program. Identical residues shared among these LBP/BPI sequences are shaded. The accession numbers (DDBJ, EMBL and GeneBank) of sequences retrieved in this study are as follows: large yellow croaker Pc-BPI-L (DQ917778), rainbow trout LBP/BPI-1 (BAB91243), rainbow trout LBP/BPI-2 (BAB91244), Atlantic cod BPI/LBP a (AAM52335), Atlantic cod BPI/LBP b (AAM52336), carp BPI (BAC56095), channel catfish BPI (AAX20011).

just like the origin of BPIs of Atlantic cod and mammals [17,23,48,49]. Nevertheless, more attention is still needed on cell types expressing Pc-BPI-L and the Pc-BPI-L expression patterns in the large yellow croaker in order to elucidate the cellular origin of Pc-BPI-L.

According to Figs. 4 and 5, the obvious up-regulation ($p < 0.05$) of Pc-BPI-L mRNA in the head kidney, spleen, intestine, kidney, gill and muscle were observed after induction with two kinds of bacterial vaccines. In addition, it was found that the expression of the Pc-BPI-L gene in heart,

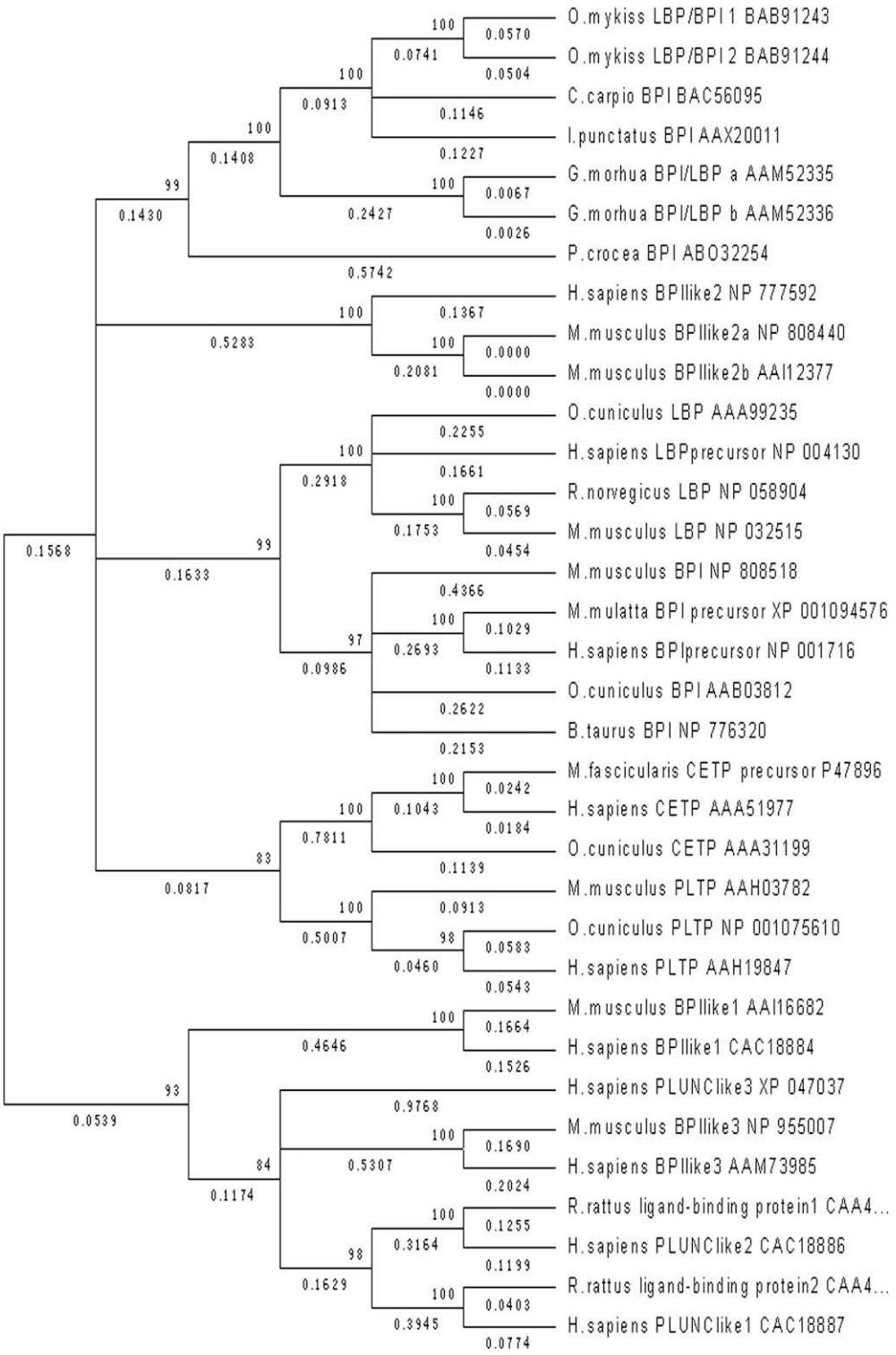

Figure 3 A phylogenetic tree based on the genetic distances of the deduced amino acid sequences between bony fish and mammalian LBP/BPI. All sequences were clustered and aligned with the following alignment parameters: Pairwise alignment parameters: gap opening penalty 10, gap extension penalty 0.10. Multiple alignment parameters: gap opening penalty 10, gap extension penalty 0.20 and delay divergent sequences 30%. The tree was constructed by the neighbor-joining method using the Mega 3.1 and TreeView, and the percentage of bootstrap values was set to 1000 replicates.

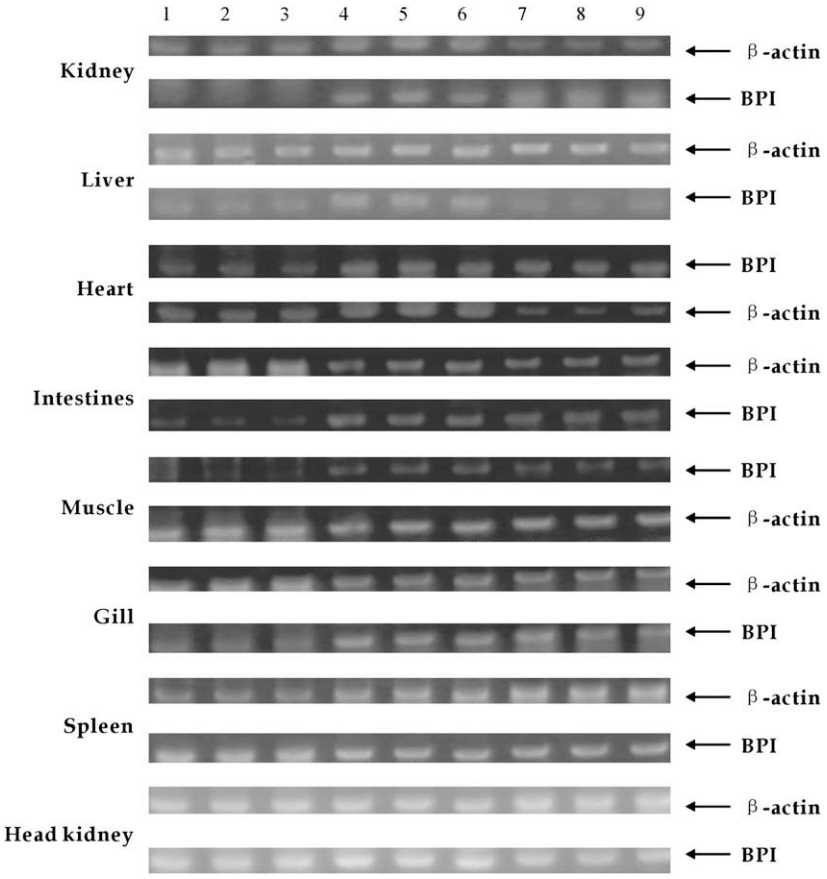

Figure 4 RT—PCR results of the Pc-BPI-L gene from normal tissues and tissues treated at 2 days after injection of FI-Va and FI-Ns. Normal group (lanes 1—3); FI-Va injection (lanes 4—6); FI-Ns injection (lanes 7—9). The RT—PCR products of the Pc-BPI-L gene are 256 bp. RT—PCR products (273 bp) of β-actin served as an internal control.

spleen and head kidney tissues of the group treated with FI-Va was significantly up-regulated ($p < 0.05$) compared to the group treated with FI-Ns. These facts, with the obvious up-regulation of the Pc-BPI-L in immune organs such as spleen, head kidney and kidney, indicated that Pc-BPI-L molecule of large yellow croaker appears to be a highly inducible molecule after bacterial vaccine challenges and might be involved in the first line of defense against bacterial pathogens, and plays a vital role in innate immune response.

It has been reported that *V. alginolyticus* is a marine Gram-negative bacterium [25] while *N. seriolae* is an aerobic, Gram-positive bacterium of the genus Nocardia which caused a systemic disease in fish [26]. Many pathological nodules were observed in the heart, spleen and kidney in the infected large yellow croaker caused by *N. seriolae* [26]. This pathological characteristic of the infection might provide a relevant explanation for why the level of Pc-BPI-L gene in heart, spleen and head kidney of the group challenged with FI-Ns is significantly up-regulated compared to individuals infected with FI-Va (Fig. 5). Further study of whether there exists a different immune response mechanism of Pc-BPI-L molecule to two kinds of bacterial infection or not may be an interesting direction to follow in the future.

The phylogenetic study showed that the Pc-BPI-L might have diverged from the common BPI-LBP-PLTP-CETP orthologue as in mammals and other teleosts, and both the Pc-BPI-L and BPI/LBP of Atlantic cod are located in the base corresponding to BPI/LBP of other freshwater fishes, which were supported by a high bootstrap value (to the number of 99). It seems that marine fish BPIs are more ancestral than freshwater fish BPIs. But given the limited available data at present, further work will need to focus on the information of a wide range of specimens from much more fish BPIs to test the phylogenetic evolution of this molecule family in teleosts in the future.

The recombinant Pc-BPI-L protein in the insect cells transfected with plasmid vector was successfully expressed, whereas the recombinant proteins in *E. coli* BL21 failed to produce detectable protein either by SDS—PAGE or Western blot analysis. Here it is inappropriate to say that Pc-BPI-L protein cannot be expressed in *E. coli*. We presumed that possible reasons for this result are: (1) the prokaryotic expression vectors (pET28b, Novagen, Germany) used in this study can express many foreign proteins but might not be suitable for Pc-BPI-L protein expression; (2) the expressed Pc-BPI-L protein is an antibacterial peptide that might inhibit further *E. coli* target protein expression because we have observed that large yellow croaker

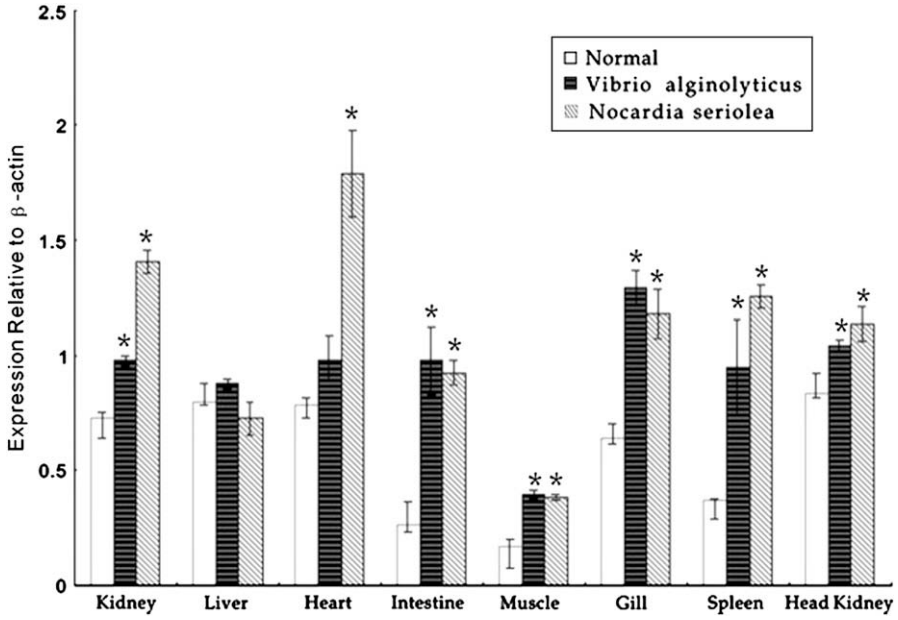

Figure 5 Tissue-specific expression profile of the Pc-BPI-L mRNA. The expression level of the Pc-BPI-L gene was performed by a comparison with β-actin level and tissue ratios were calculated from the mean results of three independent experiments. The data were analyzed by Student's *t*-test and error bars were presented as SEMs of three independent experiments done in triplicate. Differences were considered statistically significant when $p < 0.05$ and represented by an asterisk.

Dp-1 protein, a non-antibacterial peptide protein but a cell cycle-regulated component of the cellular transcription factor DRTF1/E2F, was successfully expressed in pET28b (data not shown).

In summary, all of the examined characteristics of the Pc-BPI-L gene in our present study, i.e., nucleotide, predicted amino acid sequence and expression pattern, demonstrate that we have identified a family member, Pc-BPI-L, of the BPI gene in the large yellow croaker. Following formalin-inactivated *Vibrio alginolyticus* and *Nocardia seriolae* treatment, Pc-BPI-L message was differentially up-regulated in primary immune organs. These results indicate that Pc-BPI-L might be involved in the immune response to bacterial infection.

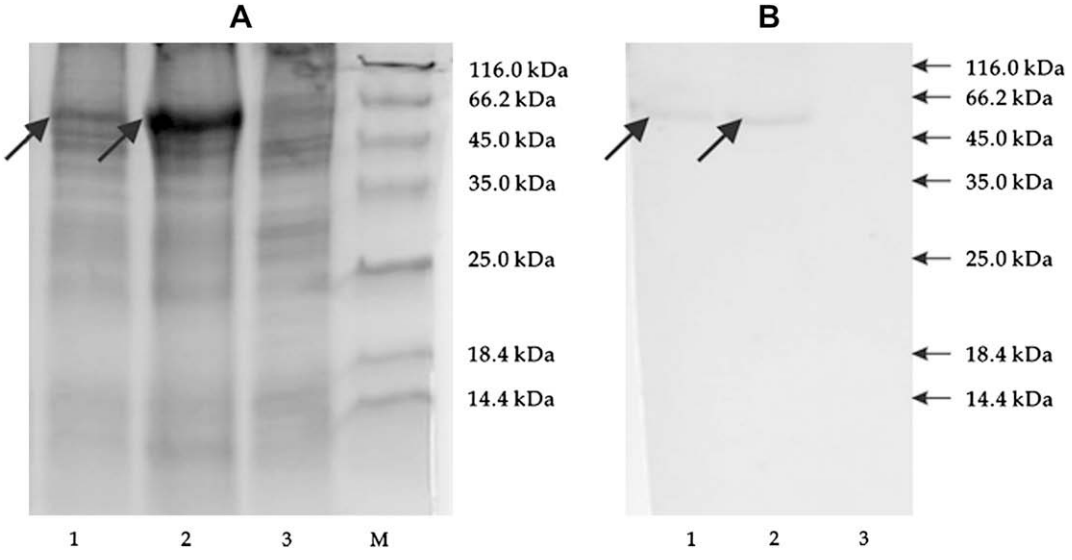

Figure 6 (A) Eukaryotic expression of the large yellow croaker BPI in insect cells and Western blotting analysis. Protein marker (14.4—116 kDa); lanes 1, 2: the cells infected with recombinant bacmid; lane 3: the cells infected with bacmid. (B) Western blot analysis. Lanes 1, 2: the cells infected with recombinant bacmid; lane 3: the cells infected with bacmid.

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