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A mammalian like interleukin-1 receptor-associated kinase 4 (IRAK-4), a TIR signaling mediator in intestinal innate immunity of black tiger shrimp (*Penaeus monodon*)

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ABSTRACT

Interleukin-1 receptor associated kinase-4 (IRAK-4) has been identified as a central signal transduction mediator of the Toll-like receptor (TLR) and Toll/interleukin-1 receptor (TIR) pathways in vertebrate innate immunity. An IRAK-4 homologue was cloned from the black tiger shrimp (*Penaeus monodon*) (*Pm*IRAK-4) and it shares domains and structures with other IRAK-4s. It was found to be mainly expressed in the hemocytes and midgut but also to a lower extent in several other tissues in shrimp. The *Pm*IRAK-4 responded to bacterial infection in the intestine by an enhancement of its expression level. These results indicate that *Pm*IRAK-4 may play a role at least in the intestinal innate immunity of *P. monodon*.

1. Introduction

Immunity sensors play an essential role to recognize pathogen components and then to generate a signal transduction which will lead to induction of host defense molecules. Toll-like receptor (TLR) and Toll-interleukin receptor (TIR) family members are such key immunity sensors, which induce an evolutionarily conserved intracellular signaling cascade in both vertebrates and invertebrates [1,2]. TLR/TIR signal transduction contains several interleukin-1 receptor-associated kinases (IRAKs) as downstream signaling mediators. All IRAK members have two typical domain structures, a N-terminal domain containing a death domain (DD) and a central kinase domain [3]. The kinase domain is required for the serine/threonin kinase activity during signal transduction. Among the four mammalian IRAKs (IRAK-1, IRAK-2, IRAK-M and IRAK-4), only IRAK-1 and IRAK-4 have been shown to have active kinase activity [4,5]. IRAK-1 and IRAK-4 are orthologs of the Pelle serine/threonin kinase molecules, in the *Drosophila* Toll signaling cascade, which is operating against Gram-positive bacteria and fungi [6,7]. Among IRAK family members, IRAK-4 shares the highest sequence homology with the *Drosophila* Pelle [5]. In contrast to *Drosophila* Pelle kinase, the mammalian IRAK-4 is also involved in response to Gram-negative bacteria infections [8,9].

IRAK-4 is present in several animal species from sponges to mammals and it is expressed in various tissues [4,10–12]. It is the central mediator in NF-κB activation and innate immunity signaling and its kinase activity is necessary for activation of IRAK-1 and perhaps other signal transducing substrates [7]. A bacterial defense function of this protein has been known for a long time, and for example IRAK-4 deficient mice have increased mortality upon a bacterial infection [13]. The IRAK-4 is a key kinase in signal transduction by the lipoprotein receptor Toll-like receptor (TLR) 2 and the lipopolysaccharide (LPS) receptor TLR 4 [14]. Both transcriptomic and proteomic techniques show that, TLR2 and TLR4 are detected in mammalian intestine epithelial cells (IECs) and they can also trigger pro-inflammatory responses [15–18] and the TLRs might be important to maintain the composition of the intestinal bacteria in the IECs [19].

Aquatic invertebrates live in an environment rich in a variety of microorganisms. In shrimp a natural entry of microbes is through oral routes by either feeding or drinking [20,21]. Hence, intestine is one of the target sites for bacterial invasion. The gastrointestinal (GI) track of shrimp can be separated into three main parts consisting of foregut, midgut and hindgut lined from head to tail, respectively [20]. The midgut, which is not covered by a cuticle layer like the other part of the GI track, is a favorable area for pathogen infection most likely just because of that reason [20,22]. Analysis of host-pathogen interactions has been performed by two different methods of infection, most of which by injection of pathogens into the hemocoel and a few by feeding (or immersion) methods [21]. The injection method always leads to the activation of an immune

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response but it bypasses the initial step of bacterial infection [21]. In contrast, the immersion method mimics a natural bacterial infection [23,24]. Both injection and immersion methods have been used to analyze the intestinal host-bacteria interaction in shrimp by us [20,25]. In the present study we identified an IRAK-4 homologous molecule (*Pm*IRAK-4) and its tissue mRNA expression in *Penaeus monodon*, the black tiger shrimp as well as the expression profile of *Pm*IRAK-4 in midgut of GI track after a bacterial injection through immersion.

2. Materials and methods

2.1. Cloning of full length PmIRAK-4 cDNA

Total RNA (at least 1 μg) was extracted from the intestine and then purified using a QuickPrep *micro* mRNA Purification Kit (GE Healthcare). The 3′ RACE PCR primers (forward: 5′-CTTGCCTGCTTAGGTGGGACTGAAC-3′) were designed from a partial sequence of IRAK-4 from black tiger shrimp EST library (Accession No. OV-N-N01-1169-W). 3′ RACE-PCR performed with SMART RACE cDNA Amplification Kit (BD Clontech). Thermal cycling was as follows: 25 cycles of 94 °C 30 s, 68 °C 30 s, and 72 °C 3 min. The 3′ RACE PCR products were cloned into pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* JM109.

2.2. PmIRAK-4 sequence analysis, homology modeling and phylogenetic analysis

The nucleotide sequence of *Pm*IRAK-4 was compared to others in Genbank using BlastX. Multiple sequence alignment was done by ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html). The deduced amino acid domain was predicted with SMART (http://smart.embl-heidelberg.de/). Homology models of *Pm*IRAK-4 were generated in Swiss-model by using the 3-D structure of human IRAK-4 kinase domain (PDB number for a dead domain of human IRAK-4: 3MOP and a kinase domain of human IRAK-4: 2OIB) [5,26]. Images were produced by using PyMol program (http://www.pymol.org/) and the PyMOL Molecular Graphics System (DeLano Scientific, PaloAlto, CA). The qualities of the final models were checked by What_Check programs [27]. A phylogenetic tree representing the relationship between *Pm*IRAK-4 and other proteins was analyzed by the maximum likelihood (ML) and Bayesian inference (BI) methods. A PhyML program (under the Whelan and Goldman (WAG) and gamma model with four categories) was used in ML analysis [28]. In case of BI method, we used MrBayes program [29] with CAT model (3000 cycles, first 1000 cycles removed as burn-in, and the analysis was repeated three times with identical results). Internal blanch support values was from analysis of 1000 ML bootstrap replicates.

2.3. Tissue distribution of PmIRAK-4 mRNA

RNA from various tissues, including epidermis, gills, heart, hemocytes, hepatopancrease, midgut, hindgut, lymphoid organ, muscle, abdominal ganglia, pleopod, stomach, antennal grand, testis, and ovary of juvenile shrimp, was extracted following the instruction of TriReagent® (Molecular Research Center) followed by treatment with RNase-Free DNase I. Complementary DNA was synthesized using ImPromII™ Reverse Transcription System Kit (Promega). *Pm*IRAK-4 gene specific primers (GSP-*Pm*IRAK4-F, 5′-GAGCCCCAGAGCAGGGACCA-3′; GSP-*Pm*IRAK4-R, 5′-CCAAGTAGACCACCCCAAAT-3′) were designed from full length cDNA of *Pm*IRAK-4. The elongation factor-1α (*EF-1α*) gene was used as internal control in all PCR experiments (*EF-1α* -F, 5′-ATGGTTGTCAACTTTGCCCC-3′; *EF-1α*-R, 5′-TTGACCTCCTTGATCACACC-3′). PCR conditions were as follows: 94 °C 2 min, followed by 30 cycles of 94 °C 20 s, 58 °C 20 s, and 72 °C 1 min for the *Pm*IRAK-4 gene and 25 cycles for *EF-1α* gene. The PCR products were analyzed on 1.2% agarose gel stained with ethidium bromide.

2.4. Bacterial challenge, sample collection and PmIRAK-4 transcription analysis

Vibrio harveyi and *Staphylococcus aureus* which are representative species of Gram-negative and Gram-positive bacteria were cultured separately in tryptone soya broth (TSB, Oxoid) supplemented with sterile 2% (W/V) NaCl at 28 and 37 °C, respectively for 18 h. The bacterial cells were washed twice with sterile 2% NaCl by centrifugation at 3500*g* for 10 min at 4 °C. The pellet was resuspended in sterile 2% NaCl and adjusted to OD_{600} = 2.0 (approximate concentration of 2×10^8 CFUs/ml). Then the bacterial solution was placed in a water bath at 60 °C for 1 h.

For injection, two groups of shrimp were injected with 100 μl of heat-killed *V. harveyi* or *S. aureus* into the muscle at the lateral side of the shrimp body (approximately 2×10^7 cells/shrimp). The control group was injected with 100 μl of 2% NaCl.

For bacterial feeding, shrimp received non-pathogenic *Vibrio* sp. isolated from GI track of shrimp via *Artemia*. Each shrimp was fed with *Artemia* containing live *Vibrio* sp. approximately 2.0×10^9 CFUs/day. Shrimp receiving only *Artemia* without bacteria served as the control.

Intestine of shrimp from all experiments was collected at 0, 2, 12 and 24 h post injection, while feeding method of samples were collected at 3, 5 and 7 days post feeding. The intestines were divided into two parts, midgut and hindgut (as showed in Fig. 1A) for RNA extraction. The transcript levels of *Pm*IRAK-4 were detected by quantitative RT-PCR using the QuantiTect SYBR green PCR kit (QIAGEN). The expression of *Pm*IRAK-4 was normalized to the expression of the mRNA encoding the *EF-1α* for each sample. PCR was performed with two oligonucleotide primers (q*Pm*IRAK4-F: 5′-GGAACACGAGAGTCCGTATTGG-3′ and -R: 5′-CCTCCTAAACGAACTAAACCGA-3′; for *Pm*IRAK-4 gene; q*EF-1α*-F: 5′-AGGCGTACTGGTAAGGAACTGGAA-3′ and -R: 5′-AGAGGAGCATACTGTTGGAAGGTCTC-3′ for *EF-1α* gene). The qPCR reactions contained 5 μl of 1:10 diluted cDNA template, 1× QuantiTect® SYBR Green PCR master mix (QIAGEN) and 5 μM forward and reverse primers in a 25-μl reaction volume. The following amplification profile was used: 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. All qPCR reactions were performed in duplicate. The intestines from at least three shrimps were used for each time point.

2.5. Statistic analysis

The relative expression levels of different time groups were examined by One-way ANOVA followed by Duncan's new multiple range test and Tukey test. Differences were considered statistically significant at $P < 0.05$. Results are expressed as the mean ± SE.

3. Results

3.1. Identification of PmIRAK-4

The 1757 nucleotide sequence of *P. monodon* IRAK-4 (*Pm*IRAK-4) begins with an 5′ untranslated region (UTR) of 158 nucleotides followed by a predicted open reading frame (ORF) extending from nucleotide 1 to a TAG stop codon at nucleotide 1518 and a 3′ UTR of 81 nucleotides (Fig. 1B). The ORF of *Pm*IRAK-4 encodes 505 amino acids, with a calculated molecular mass of 56.6 kDa. An N-terminal death-domain and a central kinase domain were predicted on a deduced *Pm*IRAK-4 protein sequence by SMART program

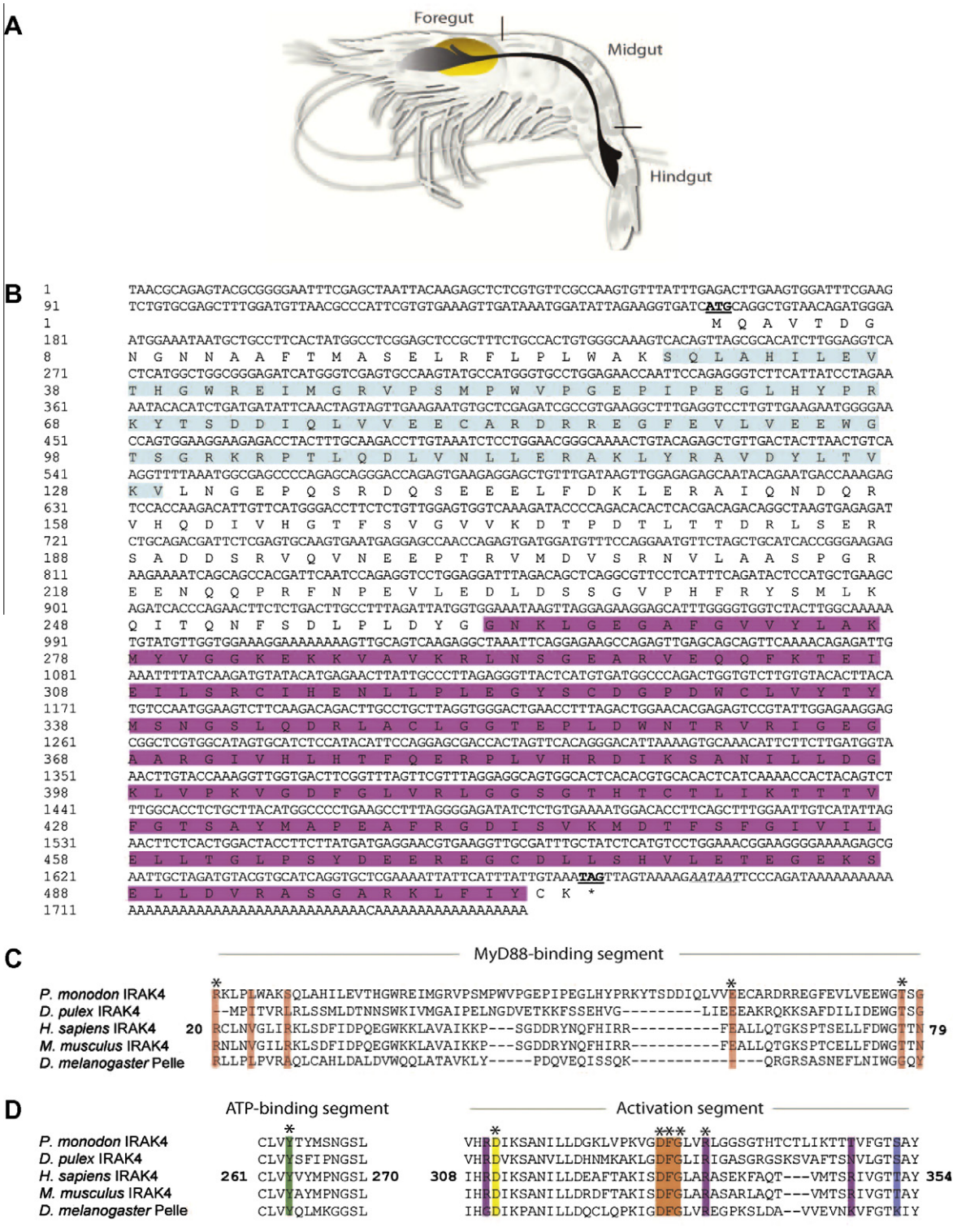

Fig. 1. The shrimp gastrointestinal tract and nucleotide and amino acid sequence of *Penaeus monodon* IRAK-4. (A) The gastrointestinal tract is divided into three different parts; consisting of the foregut, midgut and hindgut. The border of each part is indicated by a line. In addition, the whole shrimp digestive system also includes the hepatopancreas as represented with a yellow color. (B) Nucleotide sequence of *Pm*IRAK-4, bold and single underlined letters represent start and stop codons, while italic and underlined letters are the polyadenylation signal (AATAAA). The N-terminal death domain and central kinase domain of this protein are highlighted by blue and pink shades, respectively. (C) MyD88 binding site on the N-terminal death domain base on human IRAK-4 study, which consists of five residues as shown in red and the conserved residues are indicated by star. (D) Multi sequence alignment of IRAK-4 homologs in the kinase domain, including an ATP-binding- and activation segments shows a similarity of sequences between species. The ATP-binding segment has tyrosine as a gatekeeper (green shade). The activation segment also provides the conserved residues and motif; such as three arginine residues (violet), a catalytic base (yellow), DFG motif (orange) and a critical threonine in the P + 1 pocket (blue). The number in the picture refers to the human IRAK-4 sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

analysis (Fig. 1B). The N-terminal death domain of *Pm*IRAK-4 has three conserved residues, Arg (R), Glu (E) and Thr (T) similar to "MyD88 binding site" in the mammalian IRAK-4s (Fig. 1C). Actually, the kinase domain of IRAK-4 has two typical motifs including

an ATP-binding site (also known as "Hinge") and a serine/threonine protein kinase activation site. The kinase domain of the five kinases (Fig. 1D) has a tyrosine as a pivotal residue at the center of the ATP-binding site in this domain, commonly known as a "gatekeeper". Tyr335 of *Pm*IRAK-4 was predicted as a tyrosine gatekeeper. Additionally, multiple alignments reveal that several residues and DFG motif (DFGXXR) are conserved in the activation site of these IRAK-4 kinases (Fig. 1D).

The secondary structure of *Pm*IRAK-4 was predicted using the Swiss-model based on the crystal structure of human IRAK (Fig. 2A). The homology modeling analysis showed that the death domain of *Pm*IRAK-4 is composed of six α helices and two β sheets (Fig. 2A and B). In the central kinase domain, the ATP-binding site consists of five β -sheets and one α helix, while the activation site is made up of nine α helices and two β sheets (Fig. 2A and C). *Pm*IRAK-4 aligns well with the human IRAK-4 (Fig. 2B and C). Sequence conservation was detected in both the death domain and kinase domain of IRAK-4, especially the activation site.

3.2. Phylogenetic and tissue expression analysis of PmIRAK-4

A phylogenetic analysis clearly separated the IRAK-4 proteins of vertebrates and invertebrates in two different groups and *Pm*IRAK-4 clustered with other members of invertebrate IRAK-4s (Fig. 3A).

In juvenile shrimp, *Pm*IRAK-4 was abundantly expressed in the hemocytes and the midgut, moderately expressed in the gills, hepatopancrease, hindgut, lymphoid organ, pleopod, stomach, abdominal ganglia, antennal grand, testis, and ovary, whereas low expression was shown in the epidermis and heart (Fig. 3B).

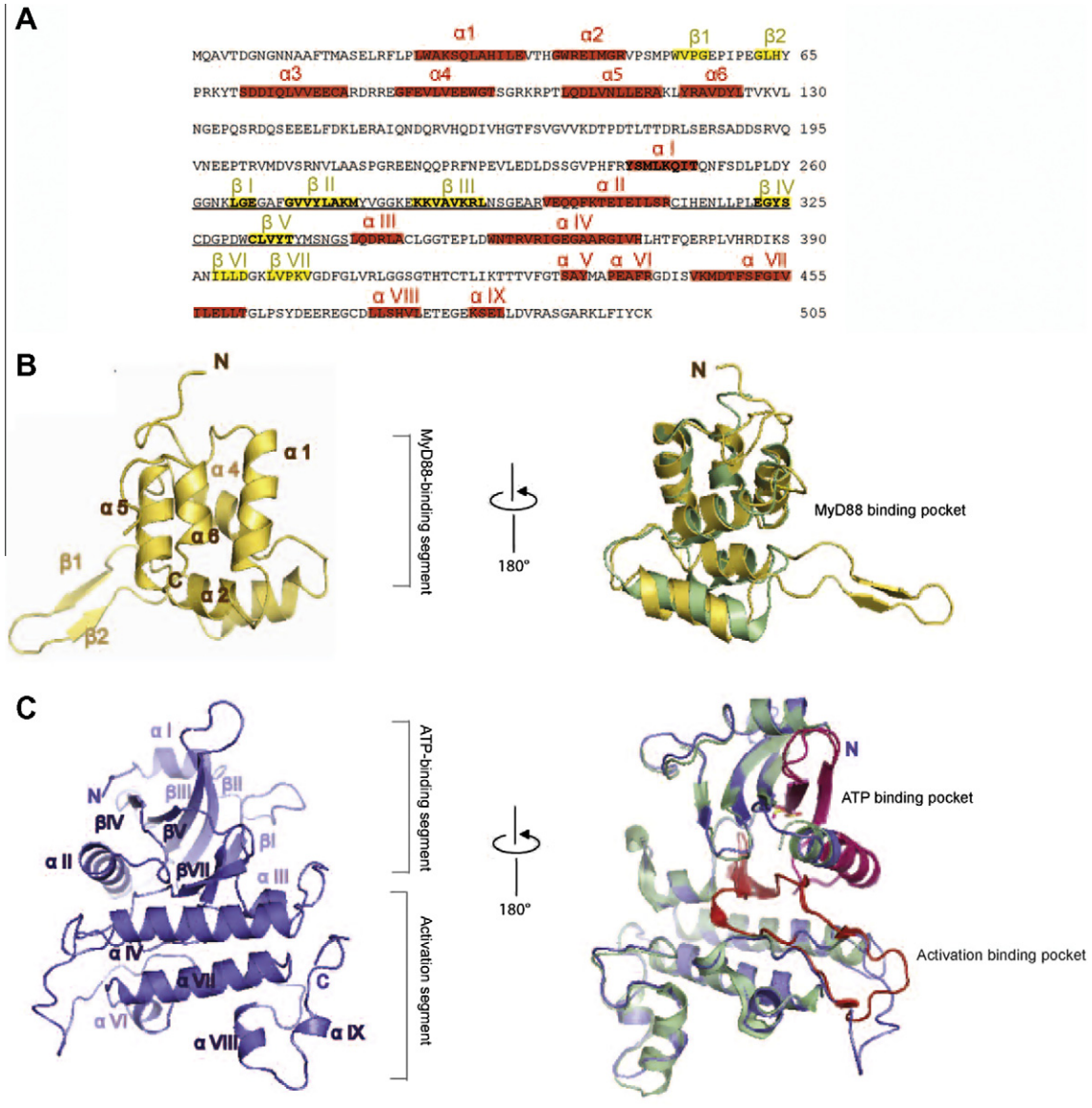

Fig. 2. Protein structure of the *Pm*IRAK-4. (A) The arrangement of secondary-structure elements in the *Pm*IRAK-4. Alpha helices and β-sheets are indicated by red and yellow shades, respectively. A death domain (DD) contains six α helices (α 1–6) and two β sheets (β 1–2), while a kinase domain (KD) consists of nine α helices (α I–IX) and seven β sheets (β I–-VII). The DD is composed of two regions, such as N-terminal ATP binding segment and activation segment. The residues in the ATP binding segment is represented by single underlined letters. (B and C) Stereo view of the the DD (B) and the KD (C) ribbon diagrams in two orientations related by a 180° rotation along a vertical axis. Each domain structure comparison of *Pm*IRAK-4 with human IRAK-4 is shown on the right side and the human IRAK-4 is shown in light green. In addition, the ATP binding pocket and the activation binding pocket are highlighted with pink and red, respectively. Figures were prepared using Pymol (http:// www.pymol.org). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

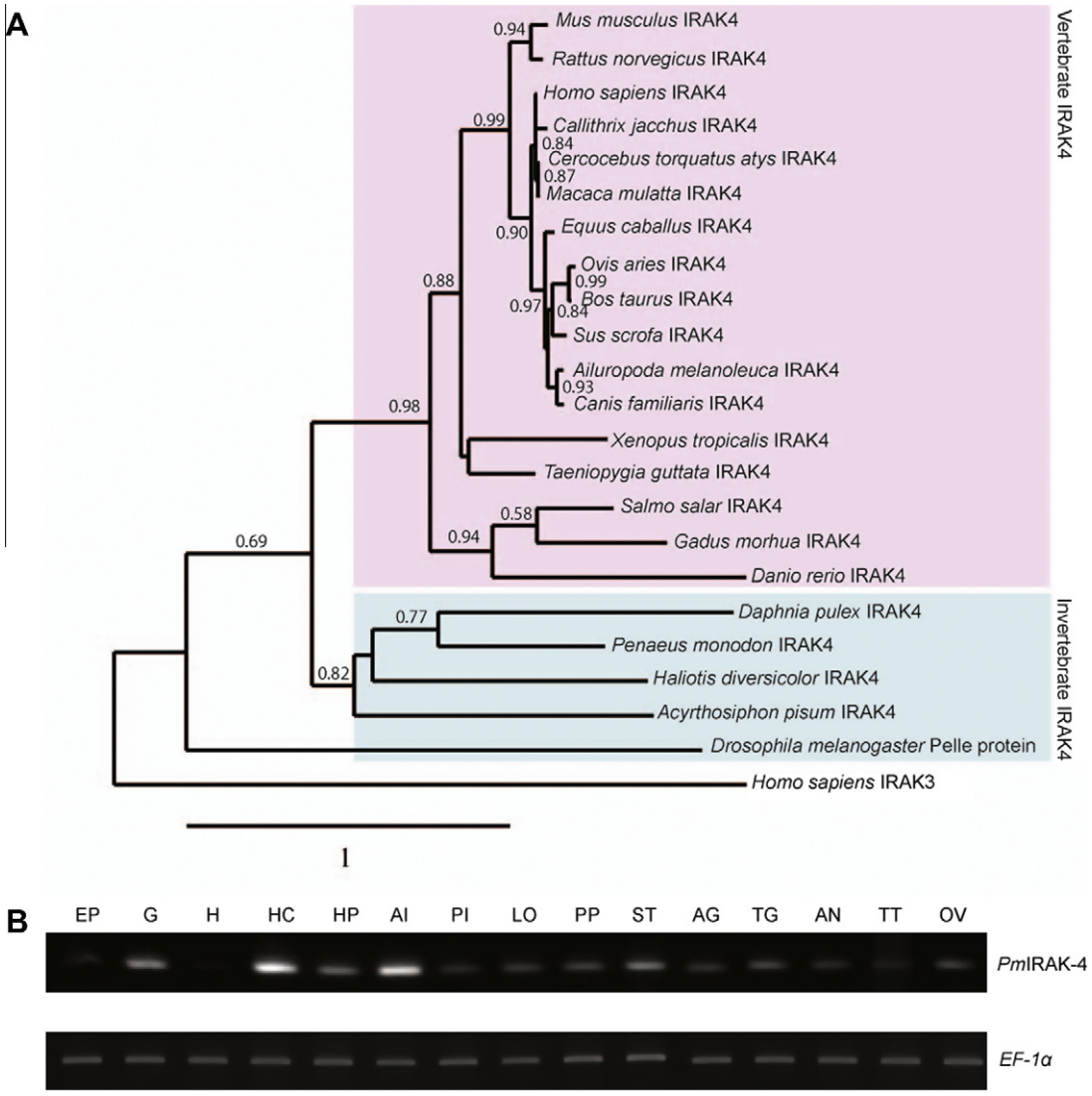

Fig. 3. Phylogenetic and tissue distribution analysis of the *Pm*IRAK-4 (A) A phylogram based on multiple alignments between *Pm*IRAK-4 and other IRAK-4 proteins; *D. pulex* (EFX85081), *D. melanogaster* (AAF56686), *A. pisum* (XP_001950616), *H. diversicolor* (ADC53123), *X.* (*Silurana*)*tropicalis* (NP_001116877), *B. taurus* (ACD50138), *C. familiaris* (XP_543727), *E. caballus* (XP_001488489), *C. torquatus atys* (ABY64981), *H. sapiens* (NP_001138728), *M. mulatta* (NP_001129573), *A. melanoleuca* (XP_002927878), *S. scrofa* (NP_001106163), *C. jacchus* (XP_002752400), *T. guttata* (XP_002194205), *O. aries* (NP_001135986), *R. norvegicus* (NP_001100261), *M. musculus* (NP_084202), *G. morhua* (ADG85742), *S. salar* (NP_001135238) and *D. rerio* (AAT37635). A *H. sapiens* IRAK3 was used as outgroup (AAH69388). (B) The expression of *Pm*IRAK-4 was studied in various tissues, such as EP = Epidermis, G = Gill, H = Heart, HC = Hemocyte, HP = Hepatopancrease, AI = Midgut, PI = Hindgut, LO = Lymphoid organ, PP = Pleopod, ST = Stomach, AG = Antennal grand, TT = Testis and OV = Ovary. An elongation factor-1α (*EF-1α*) gene was used as internal control.

3.3. Heat-killed Gram negative bacteria injection and live bacterial feeding induce PmIRAK-4 expression in intestine

To examine the expression pattern of the *Pm*IRAK-4 in shrimp intestine following a bacterial challenge, quantitative RT-PCR revealed that the *Pm*IRAK-4 mRNA expression was significantly induced by *V. harveyi* at 2 h to 24 h post injection in both midgut and hindgut compared to control (Fig. 4A and B). In hindgut, *S. aureus* also induced an expression level of *Pm*IRAK-4, although not as impressive as was the case with the *V. harveyi* injection (Fig. 4D). However, the *Pm*IRAK-4 transcript was not significantly changed after *S. aureus* injection in the midgut (Fig. 4C).

Furthermore to determine whether the expression profile of *Pm*IRAK-4 was affected in shrimp intestine during bacterial feeding, shrimp were fed with *Vibrio* sp. via *Artemia*. After feeding *Pm*IRAK-4 expression was significantly higher ($P < 0.05$) in both midgut and hindgut than those in the control at 7 days post feeding (Fig. 4E and F).

4. Discussions

The role of IRAK-4 protein or *Drosophila* IRAK-4 like kinase Pelle as mediators in innate immunity is well established [7,30].The IRAK-4 was identified as multi-domain protein, consisting of a conserved N-terminal death domain (DD) and a central kinase domain (KD) [3]. The TIR cascade transduces signals through the adaptor proteins MyD88 and IRAK, respectively. The MyD88 recruits IRAK-4 via their DD–DD interaction, which is important for its activation and downstream signaling ability [31]. Residues R12, V16, R20, E69, T76 and N78 of the IRAK-4 DD were used in a human IRAK-4-MyD88 interaction [32] and IRAK-4 KD is required for activating the TIR-mediated cytokine and chemokine productions [30,33]. The IRAK-4 KD contains two major sites, the N-terminal ATP-binding site and the C-terminal kinase activation site [5]. The binding pocket of IRAK-4 ATP-binding site is formed between Glu233 (E233) to a tyrosine gatekeeper (Tyr262) in human and these two residues are conserved through multiple

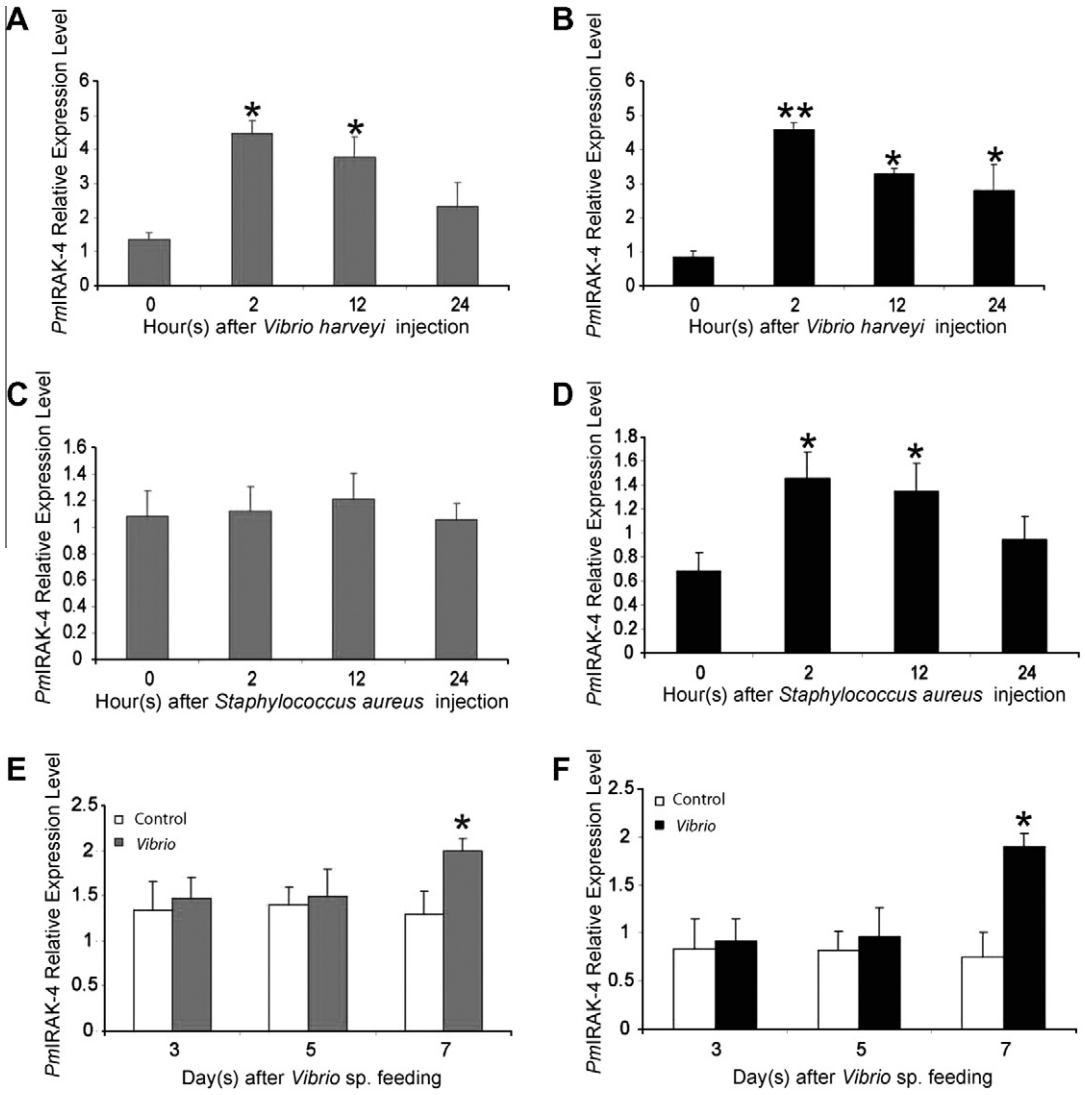

Fig. 4. Expression of *Pm*IRAK-4 in response to bacterial injection and feeding. (A–D) *Pm*IRAK-4 expression was significantly higher than the control from 2 h post-injection with heat-killed *Vibrio harveyi* in midgut (A) and hindgut (B), whereas *S. aureus* induced a transcription of *Pm*IRAK-4 only in hindgut (D) and did not have any effect in midgut (C). In midgut (E) and hindgut (F), the *Pm*IRAK-4 was significantly up-regulated and higher than control from 7 days post-immersion with *Vibrio* sp. The asterisk indicates that the expression levels are significantly different ($*P < 0.05$, $**P < 0.01$).

structure-base sequence alignment [5]. The tyrosine gatekeeper is unique for IRAK kinases, including IRAK-4 [5]. Also, the kinase activation site has several conserved components such as three arginine residues clustering a binding loop, a catalytic base, DFG motif and a critical threonine in the P + 1 pocket [5]. The *Pm*IRAK-4 shares the domain organization and most structural compositions as the other IRAK-4s. Combining multiple sequence alignment and 3D structure analysis may allow us to speculate that the *Pm*IRAK-4 might have the same biological activities as human IRAK-4.

The TIR-signaling transduction is one of the most ancient conserved pathways of innate immunity in animals [2,9,17]. Hence the IRAK-4 is a key molecule in the TIR cascade [7,12]. Furthermore the IRAK-4 expression has been observed in a variety of tissues, suggesting that it might be involved in many physiological processes [34]. According to the tissue distribution analysis in our present study, the *PmIRAK-4* was expressed higher in the midgut than in the hindgut of normal juvenile shrimps. The main function of the invertebrate hindgut is storage of fecal matter and also host defense against microbes, whereas midgut plays an important role for uptake of digested compounds [35]. The observed difference in expression between midgut and hindgut might be explained by the fact that this part has no cuticle as hindgut and since the cuticle can protect against pathogen invasion the expression in midgut is higher [36]. Interestingly, the *PmIRAK-4* transcripts in both midgut and hindgut were up-regulated approximately to the same level by heat-killed *V. harveyi* injection. In contrast only *Pm*IRAK-4 was increased in expression by *S. aureus* injection in the hindgut and this may suggest that the hindgut is more responsive to bacteria than the midgut which probably reflects the fact that most bacteria penetrates into the body cavity through the midgut (Jiravanichpaisal, unpublished). In non-pathogenic *Vibrio* sp. feeding trial the *PmIRAK-4* transcripts in both midgut and hindgut were significantly up-regulated at day 7. This can be explained so that this bacterium is already present as part of the normal flora in shrimp intestine where cells always interact with this bacterium and the *PmIRAK-4* transcripts is therefore constitutively expressed in both tissues. Until these tissues are continuously stimulated by this bacterium in high numbers this will eventually affect the *PmIRAK-4* transcripts. Bacterial infection also increased the expression level of IRAK-4 or *Drosophila* Pelle in *D. melanogaster*, *M. arenaria*, *H. diversicolor* and *D. rerio* [11,12,37,38]. In contrast, no-significant

up-regulation of an IRAK-4 homologue was detected in sponge (*S. domuncula*) [39]. Therefore, it is possible that IRAK-4 may have a direct involvement in the immune response against bacterial infections in several organisms [4,40] and maybe also in intestine in shrimp.

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