Fish and Shellfish Immunology 89 (2019) 623–631

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Identification and function of penaeidin 3 and penaeidin 5 in *Fenneropenaeus merguiensis*

Bin Wu[a], Chaohua Zhang[b], Xiaoming Qin[b], Lili Shi[c,*], Mouming Zhao[a,**]

[a] *School of Food Science and Engineering, South China University of Technology, Guangzhou 510641, PR China*
[b] *College of Food Science and Technology, Guangdong Ocean University, Zhanjiang 524088, PR China*
[c] *College of Fisheries, Guangdong Ocean University, Zhanjiang 524088, PR China*

ARTICLE INFO

Keywords:
Fenneropenaeus merguiensis
Penaeidins
Antimicrobial peptides
Vibrio parahaemolyticus
Micrococcus lysodeikticus

ABSTRACT

Antimicrobial peptides (AMPs) participate in immune defenses of invertebrate, vertebrate and plant species. As a kind of AMPs, penaeidins play important roles in innate immunity of shrimp. In this study, two penaeidin homologues termed FmPEN3 and FmPEN5 were cloned and identified from *Fenneropenaeus merguiensis* for the first time. The complete open reading frames (ORFs) of FmPEN3 and FmPEN5 were 216 bp and 240 bp, encoding 71 and 79 amino acids, respectively. Both FmPEN3 and FmPEN5 contain an N-terminal proline-rich domain (PRD) and a C-terminal cysteine-rich domain (CRD). The genome structure of FmPEN3 and FmPEN5 genes both consist of 2 exons and 1 intron. qPCR analysis showed that FmPEN3 was constitutively expressed but FmPEN5 transcripts were found only in hemocytes, gills, epidermis, nerve and pyloric cecum. The FmPEN3 and FmPEN5 expression were responsive to *Vibrio parahaemolyticus* and *Micrococcus lysodeikticus* infection and their transcription levels were downregulated by RNAi silencing of the transcription factors FmDorsal and FmRelish. In addition, recombinant proteins of FmPEN3 (rFmPEN3) and FmPEN5 (rFmPEN5) were successfully expressed in *E. coli*. The antibacterial assays revealed that rFmPEN3 and rFmPEN5 could inhibit the growth of *M. lysodeikticus* but only rFmPEN5 could inhibit the growth of *V. parahaemolyticus in vitro*. In summary, the results presented in this study indicated the functions of FmPEN3 and FmPEN5 played in anti-bacterial immunity of *F. merguiensis*, providing some insights into the function of AMPs in shrimp.

1. Introduction

Antimicrobial peptides (AMPs), the important humoral immune effectors of the innate immunity system, are widely distributed in invertebrates, vertebrates and plants species [1–3]. In addition to play a role as endogenous antibiotics, AMPs participate in wound healing, the inflammatory response and the regulation of adaptive immunity [4]. Many kinds of AMPs have been identified and characterized in shrimp, including penaeidins, crustins, anti-lipopolysaccharide factors (ALFs) [5] and stylicins [6]. Moreover, some precursor proteins, such as hemocyanin and histones, could be converted to the active AMPs by proteolysis [7,8]. According to the net charge, AMPs in shrimp can be classified into cationic and anionic classes, respectively. In shrimp, cationic AMPs are commonly found. However, few anionic AMPs have been reported until now, including only stylicins, hemocyanin derived peptides and some ALFs [1,5].

Penaeidins, which are considered as a unique AMP family specifically identified in penaeid shrimp, play important roles in the host defense system [9,10]. The penaeidins are highly cationic AMPs, having the major characteristics of an N-terminal proline-rich domain (PRD) and a C-terminal cysteine-rich domain (CRD). In CRD, six cysteine residues form three disulfide bridges [11]. Penaeidins have been identified in many species of penaeid shrimp, including *Litopenaeus vannamei*, *Fenneropenaeus chinensi* and *Penaeus monodon*. Based on the amino acid sequence similarities, penaeidins can be divided into 5 classes (penaeidin1-5) [12]. Recently, a penaeidin called MjPen-II in *Marsupenaeus japonicus* was identified as a separate classe because of the additional N-terminal serine-rich region (SRR) [13]. Peneaidins have broad antimicrobial spectrum activities, displaying strong activity against Gram-positive bacteria and fungi, and some peneaidins exhibit moderate activity against Gram-negative strains [14]. There is more than one subgroup of peneaidins in a species of penaeid shrimp. For example, Penaeidin 2, 3 and 4 were expressed in *L. vannamei* [15], and penaeidins 3 and 5 were reported in Chinese white shrimp *F. chinensis*

---

[*] Corresponding author.
[**] Corresponding author.
E-mail addresses: 13590054296@163.com (L. Shi), femmzhao@scut.edu.cn (M. Zhao).

https://doi.org/10.1016/j.fsi.2019.04.032
Received 9 November 2018; Received in revised form 31 March 2019; Accepted 10 April 2019

[14,16]. The diversity of sequences resulted in the various functions among different subgroups of penaeidins in shrimp [12,17].

The banana prawn, *Fenneropenaeus merguiensis*, is one of the most valuable penaeidae family of marine crustaceans [18]. Worldwide efforts have being made to develop secure captive breeding schemes to mitigate the risks from relying on wild broodstock. In Indonesia, Vietnam and Australia, the large-scale aquaculture production of *F. merguiensis* has been carried out [19]. However, little was known about *F. merguiensis* yet, including the breeding, physiology and immunity, and so on. Even though a huge number of AMPs have been described in various shrimp species, only a few AMPs have been reported in *F. merguiensis*, such as Fm-SOP derived peptides [20]. Until now, there was no penaeidin identified in *F. merguiensis*.

In this study, two kinds of penaeidin homologues were obtained from *F. merguiensis*, their tissue distribution and expression profile against *Vibrio parahemolyticus* and *Micrococcus lysodeikticus* were performed, and effects of Dorsal/Relish silencing on the transcription of two penaeidin homologues were analyzed. In addition, the antibacterial activities of recombinant penaeidins were identified *in vitro*.

2. Materials and methods

2.1. Biological materials

F. merguiensis shrimp (8–10 g) were purchased from the commercial farm in Zhanjiang city, Guangdong province, China. They were acclimated in a recirculating tank system for over 7 days before experiments with water salinity and temperature maintained at 30‰ salinity at 24–26 °C, respectively. The shrimp were fed with a commercial shrimp pellet diet twice daily. The bacteria *V. parahemolyticus*, *M. lysodeikticus* were maintained in our laboratory.

2.2. Total RNA isolation, cDNA synthesis, and genomic DNA preparation

Total RNA was prepared from shrimp tissue samples using a commercial spin column purification kit (Sangon, China). RNA was quantified by NanoVue Plus Spectrophotometer (GE, USA) at 260 and 280 nm. Only RNAs with absorbance ratios (A_{260}:A_{280}) greater than 1.8 were used for further experiments. The total RNA was reverse-transcribed into first strand cDNA using a PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) for gene cloning. For real-time quantitative polymerase chain reaction (qPCR) analysis, the cDNA samples were prepared using the PrimeScript™ RT reagent kit (TaKaRa, Japan). The cDNA template for the rapid amplification of the cDNA ends (RACE) PCR was prepared using the SMARTer™ RACE cDNA amplification kit (Clontech, Japan). Genomic DNA was extracted from muscle tissues using the Universal Genomic DNA Extraction Kit Ver.5.0 (TaKaRa, Japan) according to the manufacturer's protocol.

2.3. Cloning of cDNA and genome of penaeidins from F. merguiensis

Partial EST sequences, coding for penaeidin 3 and penaeidin 5 proteins, were retrieved from the *F. merguiensis* transcriptome data [21]. Based on the sequences, specific primers were designed to clone the cDNA fragments of penaeidin 3 and penaeidin 5 genes (Table 1). The full-length sequences of penaeidins were amplified with the RACE method using the SMARTer™ RACE cDNA Amplification kit (Clontech, Japan) following the manufacturer's instructions. The genomic DNA sequences of penaeidins were obtained by PCR using the genomic DNA as the template, using the specific primers listed in Table 1. The specific products were purified by Gel Extraction Kit (Omega, USA), then cloned into the PMD-20 vector (TaKaRa, Japan) and sequenced. All the sequences obtained in this study have been deposited in the National Center for Biotechnology information databases (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

2.4. Bioinformatics analysis

The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to analyze the nucleotide sequences and search for protein sequences from other species in the NCBI. Signal peptides were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). SMART program (http://smart.embl-heidelberg.de) was used to analyze the protein domain topology. Multiple sequence alignments were performed by ClustalW2 (http://www.ebi.ac.uk/tools/clustalw2). The neighbor-joining (NJ) phylogenic trees were constructed using the MEGA 6.0 software, and bootstrapping procedure with a minimum of 1000 bootstraps.

2.5. Immune challenge and gene expression analysis

Eleven kinds of tissues, namely hemocytes, muscle, hepatopancreas, heart, pyloric cecum, eyestalk, gill, epidermis, stomach, nerve and intestine, were obtained from healthy *F. merguiensis* for RNA extraction and cDNA synthesis as mentioned in section 2.2. Primers qFmPEN3-F/R and qFmPEN5-F/R (Table 1) were used to detect the relative mRNA expression of FmPEN3 and FmPEN5 in different tissues by qPCR analysis, respectively. qPCR assays were performed at a volume of 10 μL comprised of 1 μL of 1:10 cDNA diluted with ddH_2O, 5 μL of 2 × SYBR Green Master Mix (Takara, Japan), and 250 nM of each primer. FmPEN3 and FmPEN5 expression were measured using a LightCycler (Roche) with the following program: one cycle at 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 57 °C for 30 s, and 78 °C for 5 s. The elongation factor-1 gene (EF-1) of *F. merguiensis* was used as internal control [22]. Expression levels of FmPEN3 and FmPEN5 were calculated using the Livak ($2^{-\Delta\Delta Ct}$) method [23] after normalization to EF-1 gene. Three replicate qPCR analyses were performed per sample.

For the immune challenge experiments, the solution of *V. parahaemolyticus* and *M. lysodeikticus* were prepared as previous described [24]. Healthy *F. merguiensis* was intramuscularly injected with 50 μL of *V. parahaemolyticus* (5.0×10^5 CFU/g) or *M. lysodeikticus* (2.5×10^6 CFU/g) at the third abdominal segment. The negative control group received an injection of 50 μL PBS only. Both hemocytes and gills of the immunity-challenged *F. merguiensis* were sampled at 0, 4, 8, 12, 24, 48, and 72 h post-injection, and three shrimps from each group were randomly selected. The relative mRNA expression of FmPEN3 and FmPEN5 genes were detected by qPCR using the same program described above.

2.6. Detection of expression of FmPEN3 and FmPEN5 genes in FmDorsal/FmRelish-knockdown F. merguiensis

Double-stranded RNA (dsRNA) was generated by *in vitro* transcription using T7 RiboMAX™ Express RNAi System (Promega, USA) as described in our previous studies [24]. The open reading frame (ORF) of the Dorsal homolog (FmDorsal) and Relish homolog (FmRelish) were obtained from the *F. merguiensis* transcriptome data and the primers containing a 5′ T7 RNA polymerase binding site (Table 1) were used to synthesize FmDorsal and FmRelish dsRNA. As the same way, the dsRNA targeting to green fluorescent protein (GFP) gene was also generated as a control. The lengths of FmDorsal, FmRelish and GFP dsRNA are 486 bp, 477 bp and 554 bp, respectively. The dsRNA-mediated RNA interference (RNAi) experiment was carried according to the reference [25]. Briefly, the experimental shrimp were treated with injection of FmDorsal/FmRelish dsRNA (1 μg/g shrimp), while the control group was injected with equivalent GFP dsRNA. At 36 h after the dsRNA injection, hemocytes and gills of each group (9 shrimps) were collected for total RNA extraction and the synthesis of the first strand cDNA as the template. qPCR was performed to detect the knockdown efficiency. Meanwhile, the expression levels of FmPEN3 and FmPEN5 genes in hemocytes and gills of FmDorsal/FmRelish-knockdown shrimps at 36 h were also detected by qPCR. EF-1 of *F. merguiensis* was used here as an

Table 1
PCR primers used in this study.

Primers	Primer sequences (5’ -3’)
For cDNA cloning	
FmPEN5-F	ATGCGTCTCGCGGTCTG
FmPEN5-R	TTATCCTTTTAATGCAGAACAACAG
FmPEN3-F	ATGCGTCTCGCGGTCTGC
FmPEN3-R	TCAGCCTCTGTATCCTCTCCCA
FmPEN5-5′RACE	CAGTTTAAAATCAAGGTTGCAGTCT
FmPEN5-3′RACE	ACGTCCTGTACCCAGACCATTC
FmPEN3-5′RACE	TCTGTATCCTCTCCCAGTCGTG
FmPEN3-3′RACE	CTGGGAGAGGATACAGAGGCTG
For Genomic DNA cloning	
GFmPEN5-F	ATGCGTCTCGCGGTCTG
GFmPEN5-R	TTATCCTTTTAATGCAGAACAACAG
GFmPEN3-F	ATGCGTCTCGCGGTCTGC
GFmPEN3-R	TCAGCCTCTGTATCCTCTCCCA
For qPCR	
qFmPEN5-F	GGGTACAAGGCTGGTTACACAC
qFmPEN5-R	GCAACAAGCAACAGCCTCACT
qFmPEN3-F	CACCCTATGGGGGAAGCAG
qFmPEN3-R	TCAGCCTCTGTATCCTCTCCCA
qFmDorsal-F	CAGTTGGTGCTGTTGCTGTGAG
qFmDorsal-R	AATACATTTTTGAGAATCCAAGTCCA
qFmRelish-F	GAGACATCCAAGTTTCCCACCT
qFmRelish-R	TGTTGGCATTGAATCTTCCTGA
FmEF-1F	TGTTCCAGCGAGACAAGCCC
FmEF-1R	TGTAATCGGCATGGCCTGGG
For dsRNASynthesis	
FmDorsal-F	ATGGAATGATAGAATGGGAAGC
FmDorsal-T7-F	GGATCCTAATACGACTCACTATAGGATGGAATGATAGAATGGGAAGC
FmDorsal-R	CCAGGAGAGAGGGGAGAGC
FmDorsal-T7-R	GGATCCTAATACGACTCACTATAGGCCAGGAGAGAGGGGAGAGC
FmRelish-F	TGAACGACCAACGGACAGA
FmRelish-T7-F	GGATCCTAATACGACTCACTATAGGTGAACGACCAACGGACAGA
FmRelish-R	GGTAAACTCAGTGTTCGGGG
FmRelish-T7-R	GGATCCTAATACGACTCACTATAGGGGTAAACTCAGTGTTCGGGG
GFP-F	CGACGTAAACGGCCACAAGTT
GFP-T7-F	GGATCCTAATACGACTCACTATAGGCGACGTAAACGGCCACAAGTT
GFP-R	ATGGGGGTGTTCTGCTGGTAG
GFP-T7-R	GGATCCTAATACGACTCACTATAGGATGGGGGTGTTCTGCTGGTAG
For Protein Expression	
pFmPEN5-F	GGggtaccCAAGGGTACAAGGCTGGTTACA
pFmPEN5-R	CGgaattcTTATCCTTTTAATGCAGAACAACAG
pFmPEN3-F	GGggtaccCAAGGGTACCATGGTGGTCA
pFmPEN3-R	CGgaattcTCAGCCTCTGTATCCTCTCCC

internal control. Primer sequences were listed in Table 1.

2.7. Recombinant expression and purification of FmPEN3 and FmPEN5

The open reading frame (ORF) of FmPEN3 and FmPEN5 without the signal peptide coding sequence was amplified using specific primers pFmPEN3-F/R and pFmPEN5-F/R (Table 1) with protective based and restriction sites (*Kpn* I and *EcoR* I), respectively. The purified PCR fragments of FmPEN3, FmPEN5 and pET-32a vector were digested with *Kpn* I and *EcoR* I and ligated at 16 °C overnight. The recombinant plasmids were then transformed into competent *Escherichia coli* strain Origami (DE3) (Invitrogen, USA) cells for recombinant expression under the induction condition (0.1 mM isopropyl-β-D-1-thiogalactopyranoside, IPTG). Bacterial cells were harvested and sonicated, and then the supernatant fractions were purified with Ni–NTA agarose affinity chromatography (GE, USA). The purification of recombinant proteins was analyzed by SDS-PAGE. Concentration of the purified protein was determined using a BCA protein assay kit (ComWin Biotech, China). Meanwhile, the transformants with parent vector expressed a unique product representing rTrx. rTrx protein was also purified to use as the control protein in the subsequent assays.

2.8. SDS-PAGE and Western blot analysis

Standard 15% SDS-PAGE was used to analyze and follow the expressed FmPEN3 and FmPEN5 upon expression and purification. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250. The proteins separated by the SDS-PAGE were electro-transferred to a pre-wet nitrocellulose (NC) membrane in electroblotting buffer. A standard operation of Western blot was carried out to identify the expression of rFmPEN3 and rFmPEN5, and mouse anti-6His antibody (TransGen Biotech, China) was used as the primary antibody.

2.9. Antibacterial assay for FmPEN3 and FmPEN5

V. parahaemolyticus and *M. lysodeikticus* were selected to examine the antibacterial activity of the purified recombinant proteins (rFmPEN5 and rFmPEN3) by liquid growth inhibition assays as previous described [26]. The antimicrobial activity was determined by minimal inhibitory concentration 50 (MIC_{50}), which is the minimum concentration at which 50% of a microorganism was inhibited. Briefly, serial of 2-fold dilutions of peptides ranging from 0 to 50 μM were made in the dialysis buffer. 20 μL of each concentration was added to corresponding well in a 96-well microtiter plate, and each well was inoculated with 180 μL of a suspension of mid-log bacteria (5×10^5 CFU/mL) in Poor Broth medium containing 0.5% NaCl. Cultures were grown for 16 h at 30 °C, and bacterial growth was evaluated by the culture absorbance at 600 nm measured by the ELX800 Universal Microplate Reader (Bio-Tek). Bacteria incubated with Trx-His-tag were used as control.

Fig. 1. Nucleotide and deduced amino acid sequences of FmPEN3 (A) and FmPEN5 (B) from *Fenneropenaeus merguiensis*. The nucleotide (lower row) and deduced amino acid (upper row) sequences are shown and numbered on the left. The predicted signal peptide sequences are shaded yellow and the polyadenylation signal sequence (aataaa) shaded gray. The six conserved cysteine residues are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

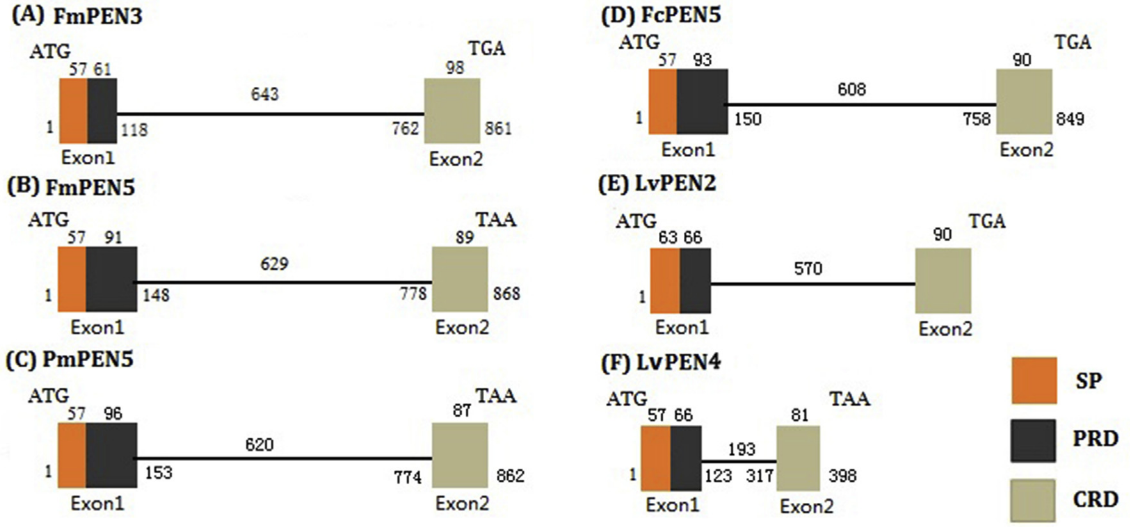

Fig. 2. Genomic structure of FmPEN3 and FmPEN5. (A) FmPEN3; (B) FmPEN5; (C) PmPEN5; (D) FcPEN5; (E) LvPEN2; (F) LvPEN4. FmPEN3, *Fenneropenaeus merguiensis* penaeidin 3 (Accession No.MK092338); FmPEN5, *F.merguiensis* penaeidin 5 (Accession No. MK092339); PmPEN5, *Penaeus monodon* penaeidin 5 [29]; FcPEN5, *Fenneropenaeus chinensi* penaeidin 5 [14]; LvPEN2, *Litopenaeus vannamei* penaeidin 2 [31]; LvPEN4, *L.vannamei* penaeidin 5 [31].

2.10. Statistical analysis

Student's *t*-test performed using OriginPro 8.0 (OriginLab, China) was used to compare means between samples. Differences were considered significant at $p < 0.05$ and highly significant at $p < 0.01$.

3. Results

3.1. Cloning and sequence analysis of FmPEN3 and FmPEN5 from *F. merguiensis*

The full-length cDNA of two FmPEN genes from *F. merguiensis* were obtained with RACE. The length of FmPEN3 (GenBank accession No. MK038876) is 698 bp, with an ORF of 216 bp encoding a protein of 71 amino acids (Fig. 1A). The length of FmPEN5 is 691 bp, with an open reading frame (ORF) of 240 bp encoding a protein of 79 amino acids (GenBank accession No. MK038877) (Fig. 1B). The deduced amino acid sequence of FmPEN5 displays 56% identity with FmPEN3, and deduced mature peptides of the two FmPENs possess the same signal peptide of 19 amino acids. Based on the deduced mature peptides, the calculated molecular masses of FmPEN3 and FmPEN5 were 5.8 kDa and 6.4 kDa, and the theoretical isoelectric points were 9.76 and 9.69, respectively. Like other penaeidins, both FmPEN3 and FmPEN5 possess the unique characteristic of the penaeidin family, which are containing a highly conserved signal peptide, a PRD, a CRD and six conserved cysteine residues at the C-terminus [1,11]. The genomic sequences of two FmPEN genes were also obtained. The entire coding region of FmPEN3 and FmPEN5 genes both consist of 2 exons and 1 intron, and the length of intron was 643 bp and 629 bp, respectively (Fig. 2). Analysis of genome sequences showed that the exon-intron boundaries in FmPEN3 and FmPEN5 conform to the consensus GT/AG rule for splicing [27]. Meanwhile, there was no similarity within the intron sequences between these two genes.

3.2. Multiple sequence alignment and phylogenetic analysis

Multiple alignment sequence analysis reveals that FmPEN3 and FmPEN5 are similar to penaeidins from other shrimps (Fig. 3). FmPEN3 and FmPEN5 is 41–67% similarity to the penaeidin of other shrimps. FmPEN3 shares higher similarity to the penaeidin 3b of *P.monodon* (ACQ66007, 64%), penaeidin 3-1 (AAP33450, 64%) and penaeidin 3-2 of *F. chinensis* (ABC33920, 63%). FmPEN5 possess higher similarity to the penaeidin 5-1 (AAZ79334, 67%) and penaeidin 5-2 (AAZ80041, 67%) of *F. chinensis*. To compare a full-length alignment of FmPEN3 and FmPEN5 with penaeidins of other penaeid shrimps, a phylogeny tree was constructed based on the Neighbor-joining method utilizing MEGA 6.0 software. As shown in Fig. 4, the penaeidin family was divided into four classes, (1) penaeidin 2 from *L. vannamei* and *Litopenaeus stylirostris;* (2) penaeidin 3 from *L. vannamei*, *L. stylirostris, F. chinensis* and *P.*

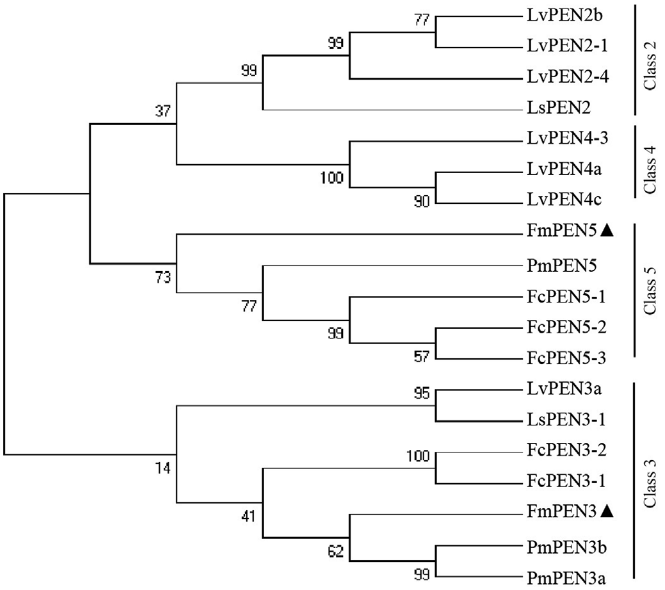

Fig. 4. Phylogenetic tree of FmPEN3 and FmPEN5. The deduced amino acid sequence of FmPEN3 and FmPEN5 are aligned with other known penaeidins by the ClustalW program and the tree is constructed using MEGA 6.0 software with neighbor-joining method. The bootstrap sampling is performed with 1000 replicates. FmPEN3 and FmPEN5 were marked by ▲. LvPEN2b, *Litopenaeus vannamei* penaeidin 2b (Accession No. Q963C4); LvPEN2-1, *L.vannamei* penaeidin 2-1 (Accession No. ABA54999); LvPEN2-4, *L.vannamei* penaeidin 2-4 (Accession No. ABA63166); LsPEN2, *Litopenaeus stylirostris* penaeidin 2 (Accession No. AAQ62565); LvPEN4-3, *L.vannamei* penaeidin 4-3 (Accession No. ABA63168); LvPEN4a, *L.vannamei* penaeidin 4a (Accession No. AAK77540); LvPEN4c, *L.vannamei* penaeidin 4c (Accession No. AAK77542); PmPEN5, *Penaeus monodon* penaeidin 5 (Accession No. ACQ66008); FcPEN5-1, *Fenneropenaeus chinensis* penaeidin 5-1 (Accession No. AAZ79334); FcPEN5-2, *F. chinensis* penaeidin 5-2 (Accession No. AAZ80041); FcPEN5-3, *F. chinensis* penaeidin 5-3 (Accession No. ABC33919); LvPEN3a, *L.vannamei* penaeidin 3a (Accession No. AAK77532); LsPEN3-1, *L. stylirostris* penaeidin3-1 (Accession No. AAQ62566); FcPEN3-1, *F. chinensis* penaeidin 3-1 (Accession No. AAP33450); FcPEN3-2, *F. chinensis* penaeidin 3-2 (Accession No. ABC33920); PmPEN3a, *P. monodon* penaeidin 5 (Accession No. AAQ05769); PmPEN3b, *P. monodon* penaeidin 3b (Accession No. ACQ66007).

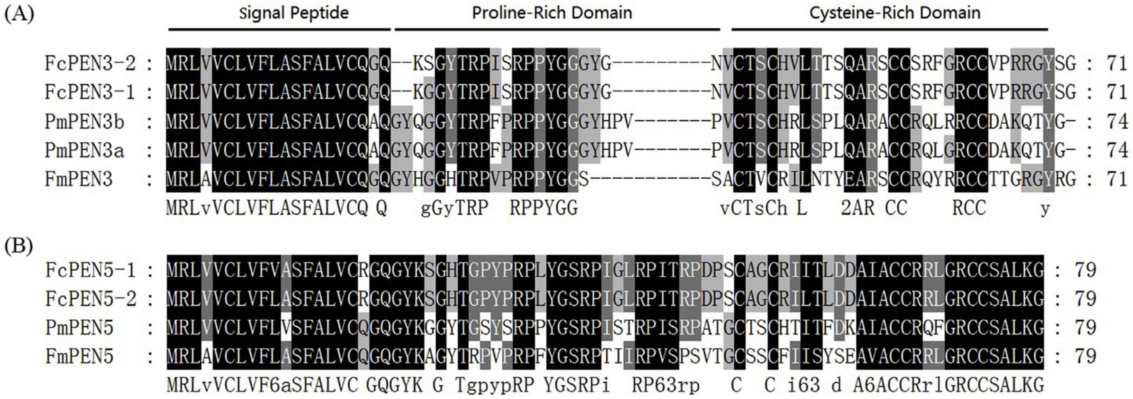

Fig. 3. Multiple alignment of the deduced amino acid sequences. (A) FmPEN3 and (B) FmPEN5. The black shade represented 100% identity, dark gray represented 80% identity, light gray represented 60% identity. FcPEN5-1, *Fenneropenaeus chinensis* penaeidin 5-1 (Accession No. AAZ79334); FcPEN5-2, *F. chinensis* penaeidin 5-2 (Accession No. AAZ80041); PmPEN5, *Penaeus monodon* penaeidin 5 (Accession No. ACQ66008); FcPEN3-1, *Fenneropenaeus chinensis* penaeidin 3-1 (Accession No. AAP33450); FcPEN3-2, *F. chinensis* penaeidin 3-2 (Accession No. ABC33920); PmPEN3a, *P. monodon* penaeidin 3a (Accession No. AAQ05769); PmPEN3b, *P. monodon* penaeidin 3b (Accession No. ACQ66007).

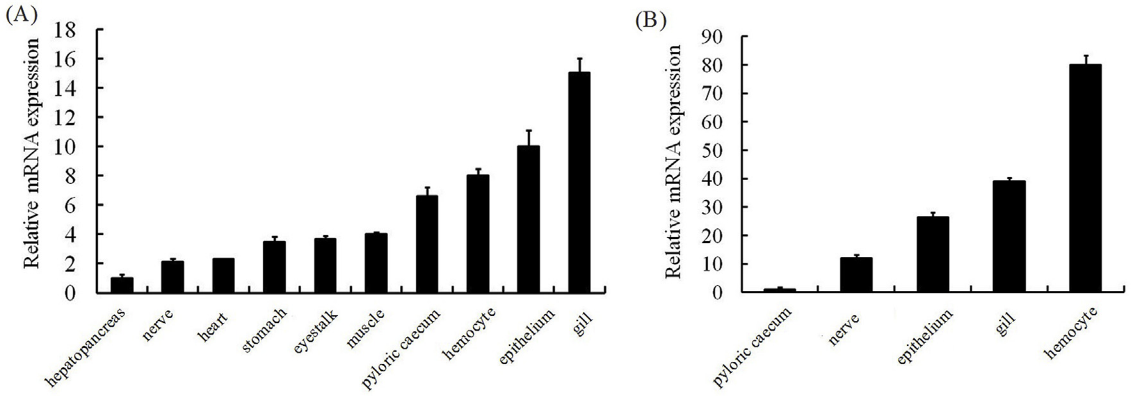

Fig. 5. Tissue distribution of FmPEN3 (A) and FmPEN5 (B) in healthy *F. merguiensis*. Ten animals were used for tissue sampling. FmEF-1 was used as the internal control to normalize the cDNA template used for qPCR analysis. The expressions of FmPEN3 in hemocytes and FmPEN5 in pyloric caecum were set as 1.0, respectively. The results were based on three independent experiments and expressed as mean values ± SD.

monodon; (3) penaeidin 4 from *L. vannamei*; (4) penaeidin 5 from *P monodon* and *F. chinensis*. FmPEN3 and FmPEN5 are close to the class penaeidin 3 and penaeidin 5, which can be denominated as Fmpenaeidin 3 and Fmpenaeidin 5, respectively.

3.3. *Tissues distribution of FmPEN3 and FmPEN5 in healthy F. merguiensis*

qPCR analysis was employed to determine the tissue specificity of FmPEN3 and FmPEN5 mRNA expression, with EF-1 gene transcript fragment being used as the internal reference control. Different tissue distributions were detected in the two penaeidins in *F. merguiensis* (Fig. 5). FmPEN3 transcripts were detected in the all other tissues except for intestine, with the highest level in gills and lowest level in hepatopancreas. The expression level of FmPEN3 in gills is nearly 15 times of that in hepatopancreas. In addition, FmPEN3 was also highly expressed in hemocytes (Fig. 5A). Differently, FmPEN5 transcripts were not found in all the detected tissues. FmPEN5 were expressed only in hemocytes, gills, epidermis, nerve and pyloric cecum, with the highest level in hemocytes and lowest level in pyloric cecum (Fig. 5B).

3.4. *Expression analysis of FmPEN3 and FmPEN5 in hemocytes and gills of immune-challenged F. merguiensis*

Considering the high expression of FmPEN3 and FmPEN5 in hemocytes and gills, hemocytes and gills were chosen to analyze the time-course expression changes of the two genes in *F. merguiensis* after *V. parahaemolyticus* and *M. lysodeikticus* infection.

In the hemocytes, the transcription of FmPEN3 had no changes at 4 h and 8 h compared with the control group after *V. parahaemolyticus* infection. Subsequently, it increased significantly to the highest level at 12 h and then gradually declined at 24–72 h (Fig. 6A). By comparison, the expression profile of FmPEN5 differed from that of FmPEN3. As shown in Fig. 6B, the mRNA expression of FmPEN5 gradually upregulated from 4 to 12 h and reached the highest level at 12 h. Afterwards, it was dropped rapidly to the original level at 24 h. In response to *M. lysodeikticus* challenge, the expression profiles of FmPEN3 and FmPEN5 were similar to that of *V. parahaemolyticus* injection group (Fig. 6A and B).

In the gills, with the *V. parahaemolyticus* infection, the FmPEN3 expression sharply increased and reached to the peak at 4 h by 2.78-fold of the control, then moderately decreased to about 2.39-fold at 8 h followed by reduced into the lower level than the control at 24 h, 48 h and 72 h (Fig. 7A), while the expression level of FmPEN5 increased from 12 h and reached a peak of 3.16-fold at 48 h followed by a gradual decrease to 1.77-fold at 72 h (Fig. 7B). By contrast, in response to *M. lysodeikticus* challenge, the FmPEN3 expression increased sharply at 12 h to about 2.6-fold followed by decreased to 1.72-fold at 24 h, then increased again to 3.19-fold at 48 h and decreased to baseline at 72 h (Fig. 7A). At the same time, the FmPEN5 expression increased from 4 h and reached to the peak at 12 h followed by a decrease to baseline at 48 h-72 h (Fig. 7B).

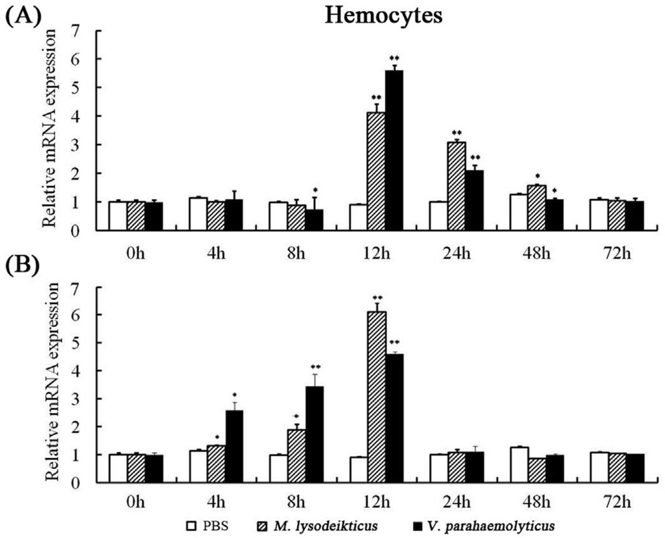

Fig. 6. Expression profiles of FmPEN3 (A) and FmPEN5 (B) in hemocytes from *V. parahemolyticus* and *M. lysodeikticus* challenged shrimps. The relative expression of FmPENs after *V. parahemolyticus* and *M. lysodeikticus* challenges was compared to control group (PBS). The expression of FmPENs in the untreated *L. vannamei* (0 h) was set as 1.0. qPCR was performed in triplicate for each sample. Expression values were normalized to those of FmEF-1 using the Livak ($2^{-\Delta\Delta Ct}$) method and the data were provided as the means ± SD of triplicate assays. The statistical significance was calculated using Student's t-test (* indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with control).

3.5. *Effects of Dorsal/Relish silencing on the expression of FmPEN3 and FmPEN5*

Dose dependent experiment of dsDorsal/Relish and dsGFP was done (Data not shown). The effects of dsDorsal and dsRelish on their mRNA expression were determined using qPCR. With the injection dose of 1ug/g shrimp, the relative expression levels of FmDorsal (Fig. 8A) and FmRelish (Fig. 8B) in hemocytes of *F. merguiensis* were significantly knocked down by dsRNA injection at 36 h ($p < 0.01$), with the levels 0.21 and 0.19-fold of the GFP dsRNA injection group, respectively. The similar results were obtained in gills, the expression of FmDorsal and FmRelish were also down-regulated to 0.13 and 0.39-fold of the GFP

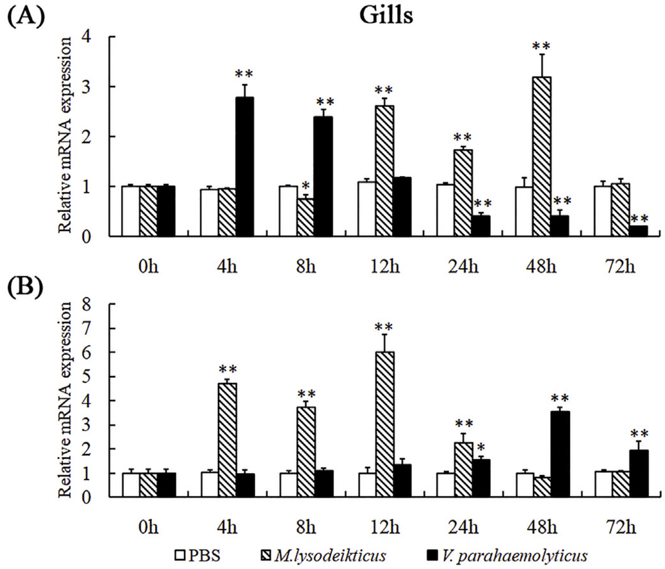

Fig. 7. Expression profiles of FmPEN3 (A) and FmPEN5 (B) in gills from *V. parahemolyticus* and *M. lysodeikticus* challenged shrimps. The relative expression of FmPENs after *V. parahemolyticus* and *M. lysodeikticus* challenges was compared to control group (PBS). The expression of FmPENs in the untreated *L. vannamei* (0 h) was set as 1.0. qPCR was performed in triplicate for each sample. Expression values were normalized to those of FmEF-1 using the Livak ($2^{-\Delta\Delta Ct}$) method and the data were provided as the means ± SD of triplicate assays. The statistical significance was calculated using Student's t-test (* indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with control).

control group, respectively. We also examined the effects of silencing of dsDorsal and dsRelish on the expression of FmPEN3 and FmPEN5. Regardless of in hemocytes and gills, both transcription of FmPEN3 and FmPEN5 in Dorsal/Relish silencing groups were significantly suppressed compared with those in GFP groups (Fig. 8C and D).

3.6. Recombinant expression of FmPEN3 and FmPEN5 and Western blot analysis

Recombinant rFmPEN3 and rFmPEN5 were successfully expressed in *E. coli* and part of recombinant proteins existed in cells as the soluble proteins (Fig. 9). The bacteria were harvested by centrifugation after induction with IPTG at 0.1 mM at 20 °C overnight. After being purified by Ni-NTA agarose column and analyzed by 15% SDS-PAGE, a distinct band of each protein was shown with molecular mass of about 25 kDa (rFmPEN3) and 26 kDa (rFmPEN5), which accorded with the estimated molecular mass of each fusion protein that contained an extra Trx-His tag of about 19.2 kDa of the expression vector (Fig. 9A and B). Moreover, rTrx was produced and purified from empty pET-32a vector as a control. Western blot analysis exhibited specific bands towards rFmPEN5, rFmPEN3 and rTrx, which were consistent with the predicted molecular weight (Fig. 9C).

3.7. Antibacterial activity of FmPEN3 and FmPEN5

To detect the antibacterial activity of FmPEN3 and FmPEN5 *in vitro*, the bacterial growth inhibition assay was performed. As shown in Table 2, there was almost no antibacterial activity of FmPEN3 in inhibiting the growth of *V. parahaemolyticus* (> 50 μM). However, FmPEN3 revealed high antibacterial activity in inhibiting the growth of *M. lysodeikticus* (6.25 μM). Differently, FmPEN5 had moderate inhibitory activity against *V. parahaemolyticus* (Gram-negative bacteria) and *M. lysodeikticus* (Gram-positive bacteria), with the MIC$_{50}$ values at 50 μM and 12.5 μM, respectively.

4. Discussion

AMPs are paramount substances of the immune defense reactions developed by living organisms to fight infection by microorganisms [1,9]. Penaeidins are members of AMP family specifically existing in penaeid shrimp and play an important role in the host defense system of shrimp [13,28]. To date, about 40 penaeidins have been identified in different penaeid shrimps such as *L. vannamei*, *P. monodon*, *F. chinensis*

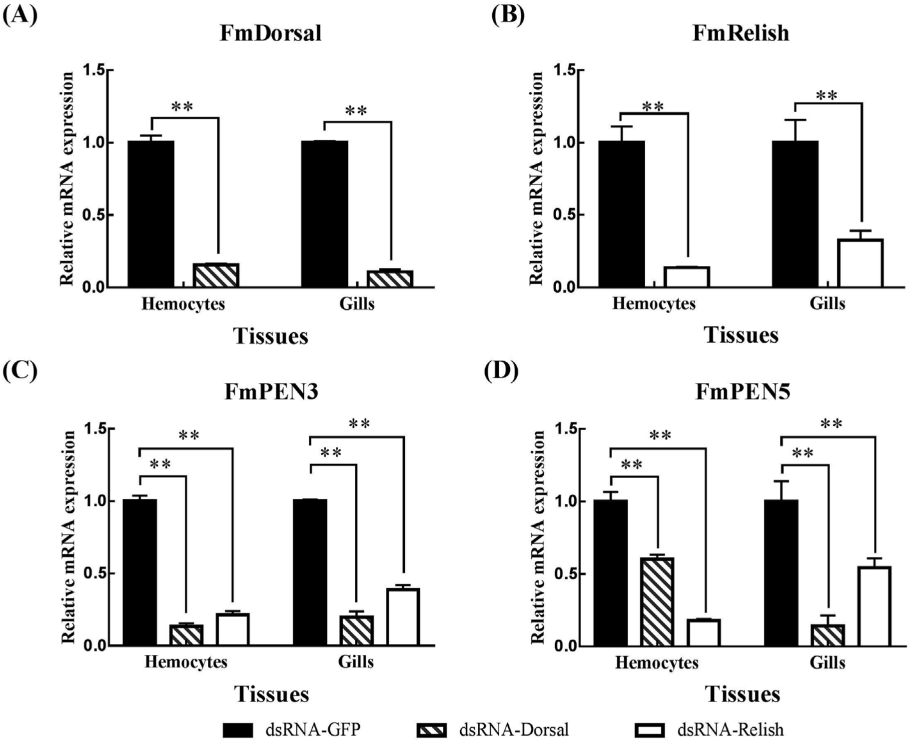

Fig. 8. Effects of Dorsal/Relish silencing on the transcription of FmPEN3 and FmPEN5. (A) Expression of FmDorsal in hemocytes and gills at 36 h of dsGFP and dsFmDorsal injection; (B) Expression of FmRelish in hemocytes and gills at 36 h of dsGFP and dsFmRelish injection; (C) Transcription level of FmPEN3 in hemocytes and gills at 36 h of dsGFP, dsFmDorsal and dsFmRelish injection; (D) Transcription level of FmPEN5 in hemocytes and gills at 36 h of dsGFP, dsFmDorsal and dsFmRelish injection. The statistical significance was calculated using Student's t-test (* indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with control).

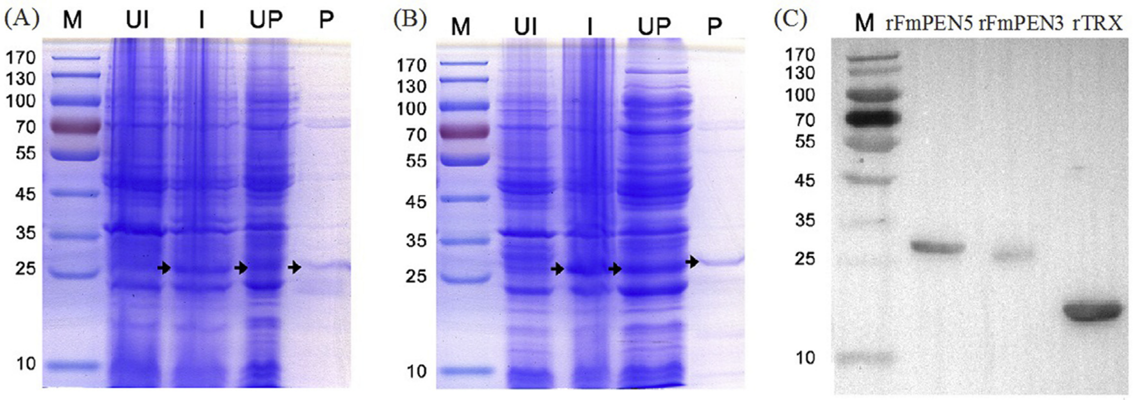

Fig. 9. Recombinant expression of FmPEN3 and FmPEN5. (A) Recombinant expression of FmPEN3; (B) Recombinant expression of FmPEN5; (C) Western blot analysis of rFmPEN3 and rFmPEN5.

Table 2
The antibacterial activities and minimal growth inhibition concentrations (MIC_{50}) of the recombinant proteins rFmPEN3 and rFmPEN5.

Microorganism	MIC_{50} (uM)	
	rFmPEN3	rFmPEN5
Gram-positive bacteria		
Vibrio Parahemolyticus	> 50	50
Gram-positive bacteria		
Micrococcus lysodeikticus	6.25	12.5

and so on [13]. However, no penaeidin has been reported in *F. merguiensis* until now. In present study, two penaeidins termed FmPEN3 and FmPEN5, were identified from the penaeid shrimp *F. merguiensis* for the first time.

FmPEN3 and FmPEN5 genes were obtained by RACE-PCR technology. FmPEN3 encoded a mature peptide of 71 aa with a signal peptide of 19 aa. Like other penaeidin3, FmPEN3 also contained conserved Gly5 and His/Arg 37 in penaeidin 3 family. However, the position 35 was Val rather than the specific residue Ser [5,29]. FmPEN5 encoded a mature peptide of 79 aa with a signal peptide of 19 aa, which is the same as that of FmPEN3. In present, penaeidin5 family has only been reported from *F. chinensis* and *P. monodon* [14,29]. Phylogeny tree analysis showed that FmPEN5 closed to penaeidin5 family, and FmPEN5 contains Ser18, which is the specific residue in penaeidin5 family [5]. However, another specific residue of penaeidin 5 family at the position 43 was replaced by Glu instead of Lys/Asp. These results indicated that FmPEN3 and FmPEN5 were the new member of penaeidin3 and penaeidin5 family, respectively.

FmPEN3 and FmPEN5 included the conserved N-terminal PRD and CRD with six conserved cysteine residues at the C-terminus, which was the characteristic feature of the penaeidin family [11]. The six cysteine rich motifs in the C-terminal domain were essential for the antimicrobial and chitin-binding activity [9,30]. Gene structures of FmPEN3 and FmPEN5 were similar to that of penaeidin5 from *F. chinensis* [14] and *P. monodon* [29], penaeidin2 and penaeidin4 from *L. vannamei* [31]. The entire coding sequence of these penaeidin genes were interrupted by only one intron, which separated the PRD and CRD (Fig. 2). However, the lengths of introns in these genes were various. On the whole, the gene structures of different kinds of penaeidins, or the same kind of penaeidin from distinct species were similar to some extent.

Penaeidin mRNA has been shown to be strongly expressed in many penaeid shrimp including *F. chinensis* [14], *P. monodon* [29] and *L. vannamei* [31]. Expression analysis showed that abundances of FmPEN3 and FmPEN5 expressions were present in the hemocytes, which was consistent with the fact that the expression of other penaeidins occurs primarily in hemocytes and in highly vascular tissues [12]. However, the expression profiles of FmPEN3 and FmPEN5 in different tissues were greatly different except for the high level in hemocytes. FmPEN3 was expressed constitutively in *F. merguiensis*. Differently, FmPEN5 transcripts were found only in few tissues including hemocytes, gill, epidermis, nerve and pyloric cecum. That suggested that transcription of FmPEN3 and FmPEN5 may be controlled by distinct regulatory elements [11]. On the other hand, the differences in tissue distribution indicated that the two penaeidins may have diverse functions as immunity effectors in response to various physiological stresses or pathogenic infection [32].

In the immune challenged experiments *V. parahaemolyticus* and *M. lysodeikticus* injected, time course analysis of FmPEN3 and FmPEN5 revealed that both the two genes responded to the pathogenic exposure, suggesting FmPEN3 and FmPEN5 involved in the anti-bacterial host defense. Upon *V. parahaemolyticus* challenge, expression of FmPEN3 and FmPEN5 could be induced in hemocytes and gills, but the time-order expression of FmPEN3 and FmPEN5 was different in different tissues. For example, FmPEN3 expression in gills rapidly increased and reached the peak at 4 h, but it had no changes at the beginning followed by an increase at 12 h in hemocytes, while the expression profiles of FmPEN5 in gills and hemocytes were contrary to that of FmPEN3. In addition, the different expression profiles of FmPEN3 and FmPEN5 were obtained after treated with *M. lysodeikticus*, these results indicated that FmPEN3 and FmPEN5 may have different regulation in immune response in different tissues. Moreover, the expression patterns of FmPEN3 and FmPEN5 were different in the same tissues treated by bacteria injection, suggesting different members belong to the same kind of AMPs may have discrepant expression patterns in the same tissues when suffer pathogens infection. Similar situation was already reported in *M. japonicus*, that the expressions of MjCrustinI-2 and MjCrustinI-3 in gills were rapidly upregulated, but the expression of MjCrustinI-5 increased after microorganism challenged at 24 h [33].

In shrimp, it has been shown that Dosal and Relish are the main Rel/NF-κB transcription factors, which are involved in the Toll and immune deficiency (IMD) signal transduction pathways of innate immune reactions. Dorsal is activated in the Toll pathway for antifungal and antibacterial responses, while Relish is required for the IMD pathway to activate the gene expression of AMPs [34]. Up to now, some Dosal and Relish genes from penaeid shrimp such as *L. vannamei* [31], *P. monodon* [29] and *F. chinensis* [35] are cloned and proved to relate to the transcription of penaeidins. For example, previous research reported that both FcDorsal and FcRelish from *F. chinensis* were involved in the immune response of shrimp to bacteria challenge and dsRNA injection experiments further confirmed that FcDorsal and FcRelish regulated the transcription of Penaeidin 5 [35,36]. In the present study, similar results were observed that FmDorsal and FmRelish activated positively the transcription of FmPEN3 and FmPEN5, suggesting the transcription of FmPEN3 and FmPEN5 may be regulated by both Toll and IMD signal pathways in *F. merguiensis*.

To further characterize the antibacterial activity *in vitro* of FmPEN3 and FmPEN5, the recombinant proteins rFmPEN3 and rFmPEN5 were recombinantly expressed in *E.coli*. Using the *E. coli* Origami (DE3) as the host strain, the soluble fraction of recombinant proteins was increased by changing the temperature and the concentration of IPTG. Research showed that penaeidins possessed antimicrobial activity predominantly directed against Gram-positive bacteria and filamentous fungi [1]. In this study, the antibacterial assays revealed that rFmPEN3 and rFmPEN5 could inhibit the growth of *M. lysodeikticus* (Gram-positive bacteria) *in vitro*, which was similar to penaeidin5 from *F. chinensis* (FcPEN5) and penaeidin4-1 from *Litopenaeus setiferus* (Litset Pen4-1), although the activity against *M. lysodeikticus* were lower than rFcPEN5 and rLitset Pen4-1 [12,14]. In addition to the activity against Gram-positive bacteria and filamentous fungi, some penaeidins such as FcPEN5, penaeidin-II from *M. japonicus* (MjPen-II) [13] exhibited the antibacterial activity against Gram-negative bacteria. More interestingly, some penaeidins such as penaeidin 5 from *P. monodon*even have antiviral activity [29]. In our study, rFmPEN5 exhibited moderate antibacterial activity against *V. parahaemolyticus* but rFmPEN3 didn't inhibit the growth of *V. parahaemolyticus in vitro*. The diverse activities against *V. parahaemolyticus* between rFmPEN3 and rFmPEN5 indicated the different antibacterial ability of FmPEN3 and FmPEN5.

In conclusion, penaeidin 3 and penaeidin 5 were characterized from banana prawn *F. merguiensis.* Although the two penaeidin genes could be regulated by FmDorsal and FmRelish, their expression profiles and antibacterial activities against bacteria varied from each other. All the results indicated that the two penaeidins possess the functional diversity in *F. merguiensis,* which will be helpful to better understand the function of AMPs in shrimp and be beneficial to improve and perfect the knowledge of the innate immunity in shrimp.

Acknowledgements

This study was supported by Science and technology project of Guangdong Province, China (2013B020312006); The PhD Start-up Fund of Natural Science Foundation of Guangdong Province, China (2016A030310334); Science and technology project Zhanjiang City, Guangdong Province, China (2014C01019). We are also grateful to anonymous reviewers for their constructive suggestions on an earlier version of this manuscript.

References

[1] A. Tassanakajon, V. Rimphanitchayakit, S. Visetnan, P. Amparyup, K. Somboonwiwat, W. Charoensapsri, S. Tang, Shrimp humoral responses against pathogens: antimicrobial peptides and melanization, Dev. Comp. Immunol. 80 (2018) 81–93.
[2] I. Dolezilkova, M. Mackova, T. Macek, Antimicrobial Peptides: the relationship of their structure and antibacterial activity, Chem. Listy 105 (2011) 346–355.
[3] C.Z. Li, S.P. Weng, J.G. He, WSSV-host interaction: host response and immune evasion, Fish Shellfish Immunol. 84 (2018) 558–571.
[4] W.S. Fredrick, S. Ravichandran, Hemolymph proteins in marine crustaceans, Asian Pac. J. Trop. Biomed. 2 (2012) 496–502.
[5] A. Tassanakajon, P. Amparyup, K. Somboonwiwat, P. Supungul, Cationic antimicrobial peptides in penaeid shrimp, Mar. Biotechnol. 12 (2010) 487–505.
[6] J.L. Rolland, M. Abdelouahab, J. Dupont, F. Lefevre, E. Bachere, B. Romestand, Stylicins, a new family of antimicrobial peptides from the Pacific blue shrimp *Litopenaeus stylirostris*, Mol. Immunol. 47 (2010) 1269–1277.
[7] C.P. Li, F. Wang, J.J. Aweya, D.F. Yao, Z. Zheng, H. Huang, S.K. Li, Y.L. Zhang, Trypsin of *Litopenaeus vannamei* is required for the generation of hemocyanin-derived peptides, Dev. Comp. Immunol. 79 (2018) 95–104.
[8] S.A. Patat, R.B. Carnegie, C. Kingsbury, P.S. Gross, R. Chapman, K.L. Schey, Antimicrobial activity of histones from hemocytes of the pacific white shrimp, Eur. J. Biochem. 271 (2004) 4825–4833.
[9] E. Bachère, D. Destoumieux, P. Bulet, Penaeidins, antimicrobial peptides of shrimp: a comparison with other effectors of innate immunity, Aquaculture 191 (2000) 71–88.
[10] Y. Gueguen, J. Garnier, L. Robert, M.P. Lefranc, I. Mougenot, J. de Lorgeril, M. Janech, P.S. Gross, G.W. Warr, B. Cuthbertson, M.A. Barracco, P. Bulet, A. Aumelas, Y.S. Yang, D. Bo, J.H. Xiang, A. Tassanakajon, D. Piquemal, E. Bachere, PenBase, the shrimp antimicrobial peptide penaeidin database: sequence-based classification and recommended nomenclature, Dev. Comp. Immunol. 30 (2006) 283–288.
[11] B. Vaseeharan, S. Shanthi, J.C. Chen, M. Espineira, Molecular cloning, sequence analysis and expression of Fein-Penaeidin from the haemocytes of Indian white shrimp *Fenneropenaeus indicus*, Results Immunol. 2 (2012) 35–43.
[12] B.J. Cuthbertson, L.J. Deterding, J.G. Williams, K.B. Tomer, K. Etienne, P.J. Blackshear, E.E. Buellesbach, P.S. Grosse, Diversity in penaeidin antimicrobial peptide form and function, Dev. Comp. Immunol. 32 (2008) 167–181.
[13] M.Y. An, J. Gao, X.F. Zhao, J.X. Wang, A new subfamily of penaeidin with an additional serine-rich region from kuruma shrimp (*Marsupenaeus japonicus*) contributes to antimicrobial and phagocytic activities, Dev. Comp. Immunol. 59 (2016) 186–198.
[14] C.J. Kang, J.F. Xue, N. Liu, X.F. Zhao, J.X. Wang, Characterization and expression of a new subfamily member of penaeidin antimicrobial peptides (penaeidin5) from *Fenneropenaeus chinensis*, Mol. Immunol. 44 (2007) 1535–1543.
[15] P.S. Gross, T.C. Bartlett, C.L. Browdy, R.W. Chapman, G.W. Warr, Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*, Dev. Comp. Immunol. 25 (2001) 565–577.
[16] C.H. Kang, J.X. Wang, X.F. Zhao, X.M. Yang, H.L. Shao, J.H. Xiang, Molecular cloning and expression analysis of Ch-penaeidin, an antimicrobial peptide from Chinese shrimp, *Fenneropenaeus chinensis*, Fish Shellfish Immunol. 16 (2004) 513–525.
[17] B.J. Cuthbertson, E.F. Shepard, R.W. Chapman, P.S. Gross, Diversity of the penaeidin antimicrobial peptides in two shrimp species, Immunogenetics 54 (2002) 442–445.
[18] D. Powell, W. Knibb, C. Remilton, A. Elizur, De-novo transcriptome analysis of the banana shrimp (*Fenneropenaeus merguiensis*) and identification of genes associated with reproduction and development, Mar. Genom. 22 (2015) 71–78.
[19] N. Nguyen Hong, J. Quinn, D. Powell, A. Elizur, T. Ngo Phu, J. Nocillado, R. Lamont, C. Remilton, W. Knibb, Heritability for body colour and its genetic association with morphometric traits in Banana shrimp (*Fenneropenaeus merguiensis*), BMC Genet. 15 (2014).
[20] S. Anuchan, P. Deachamag, N. Siammai, S. Phongpaichit, W. Chotigeat, Antimicrobial activity of engineered shrimp ovarian peritrophin fragments from *Fenneropenaeus merguiensis*, Protein Pept. Lett. 22 (2015) 73–80.
[21] W. Wang, S.P. Yang, C.G. Wang, L.L. Shi, H. Guo, S.M. Chan, Gill transcriptomes reveal involvement of cytoskeleton remodeling and immune defense in ammonia stress response in the banana shrimp *Fenneropenaeus merguiensis*, Fish Shellfish Immunol. 71 (2017) 319–328.
[22] R.Q. Zhuo, T.T. Zhou, S.P. Yang, S.F. Chan, Characterization of a molt-related myostatin gene (FmMstn) from the banana shrimp *Fenneropenaeus merguiensis*, Gen. Comp. Endocrinol. 248 (2017) 55–68.
[23] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$Method, Methods 25 (2001) 402–408.
[24] L.L. Shi, S.F. Chan, C.Z. Li, S. Zhang, Identification and characterization of a laccase from *Litopenaeus vannamei* involved in anti-bacterial host defense, Fish Shellfish Immunol. 66 (2017) 1–10.
[25] S. Wang, H.Y. Li, Z. Qian, X. Song, Z.J. Zhang, H.L. Zuo, X.P. Xu, S.P. Weng, J.G. He, C.Z. Li, Identification and functional characterization of the TAB2 gene from *Litopenaeus vannamei*, Fish Shellfish Immunol. 46 (2015) 206–216.
[26] C. Sun, X.J. Du, W.T. Xu, H.W. Zhang, X.F. Zhao, J.X. Wang, Molecular cloning and characterization of three crustins from the Chinese white shrimp, *Fenneropenaeus chinensis*, Fish Shellfish Immunol. 28 (2010) 517–524.
[27] P.A. Sharp, C.B. Burge, Classification of introns: U2-Type or U12-Type, Cell 91 (1997) 875–879.
[28] D. Destoumieux, P. Bulet, D. Loew, A. Van Dorsselaer, J. Rodriguez, E. Bachere, Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda), J. Biol. Chem. 272 (1997) 28398–28406.
[29] N. Woramongkolchai, P. Supungul, A. Tassanakajon, The possible role of penaeidin5 from the black tiger shrimp, Penaeus monodon, in protection against viral infection, Dev. Comp. Immunol. 35 (2011) 530–536.
[30] D. Destoumieux, M. Munoz, C. Cosseau, J. Rodriguez, P. Bulet, M. Comps, E. Bachere, Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge, J. Cell Sci. 113 (Pt 3) (2000) 461–469.
[31] N.A. O'Leary, P.S. Gross, Genomic structure and transcriptional regulation of the penaeidin gene family from *Litopenaeus vannamei*, Gene 371 (2006) 75–83.
[32] M. Li, C.X. Ma, H.Y. Li, J.X. Peng, D.G. Zeng, X.H. Chen, C.Z. Li, Molecular cloning, expression, promoter analysis and functional characterization of a new Crustin from *Litopenaeus vannamei*, Fish Shellfish Immunol. 73 (2018) 42–49.
[33] H.S. Jiang, W.M. Jia, X.F. Zhao, J.X. Wang, Four crustins involved in antibacterial responses in *Marsupenaeus japonicus*, Fish Shellfish Immunol. 43 (2015) 387–395.
[34] F.H. Li, J.H. Xiang, Signaling pathways regulating innate immune responses in shrimp, Fish Shellfish Immunol. 34 (2013) 973–980.
[35] F.H. Li, D.D. Wang, S.H. Li, Y. Hui, J.K. Zhang, B. Wang, J.Q. Zhang, J.H. Xiang, A Dorsal homolog (FcDorsal) in the Chinese shrimp *Fenneropenaeus chinensis* is responsive to both bacteria and WSSV challenge, Dev. Comp. Immunol. 34 (2010) 0-883.
[36] F.H. Li, Y. Hui, D.D. Wang, T.A. Jose Priya, S.H. Li, B. Wang, J.Q. Zhang, J.H. Xiang, Identification of a novel relish homolog in Chinese shrimp *Fenneropenaeus chinensis* and its function in regulating the transcription of antimicrobial peptides, Dev. Comp. Immunol. 33 (2009) 1093–1101.