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Molecular and antimicrobial characterization of a group G anti-lipopolysaccharide factor (ALF) from *Penaeus monodon*

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ABSTRACT

Anti-lipopolysaccharide factors (ALFs) are important host-defense molecules of crustaceans. They all contain a lipopolysaccharide-binding domain (LBD) and some ALFs exhibit strong antimicrobial activity. In this research, a Group G ALF from *Penaeus monodon* (ALF*Pm*11) was studied. It is an anionic peptide specifically having a cationic and highly amphipathic LBD, with five positively charged residues separated by aromatic residues. It was abundantly expressed in the hepatopancreas of *P. monodon* normally but the expression level in other tissues was relatively low or undetectable. However, in the shrimps challenged by *Vibrio*, expression of ALF*Pm*11 could be detected in all tissues. Chemically synthesized ALF*Pm*11-LBD displayed high inhibitory activity (minimum inhibition concentration ≤ 4 μM) against various bacteria, *e.g. Exiguobacterium* sp. L33, *Bacillus* sp. T2, and *Acinetobacter* sp. L32. It also displayed apparent activity in the agar well diffusion assay. Furthermore, it could efficiently induce agglutination of both Gram-positive and Gram-negative bacteria and cause significant membrane permeabilization of the bacteria. As a comparative study, ALF*Pm*11-LBD showed a better or equal antimicrobial function to ALF*Pm*3-LBD which was reported to possess strong antimicrobial activity against Gram-positive, Gram-negative bacteria and fungi. Thus, this research found a new effective ALF in *P. monodon* and demonstrated its antimicrobial mechanism, suggesting its potential applications in the future.

1. Introduction

Anti-lipopolysaccharide factor is one type of antimicrobial peptide (AMP) widely distributed in crustaceans [1,2]. It was first discovered in horseshoe crabs and as it could bind to the lipopolysaccharide (LPS) to inhibit the coagulation cascade, it was called anti-lipopolysaccharide factor (ALF) [3,4]. Recently, various ALFs were discovered in shrimps [5]. These ALFs play very important roles in the anti-*Vibrio* [6,7] or anti-virus [8] immunity responses of shrimps, and some of them showed high antimicrobial [9,10] or anti-inflammatory [11] activities. The ALF contained a signal peptide and a mature peptide. The 3D structure of the mature peptide showed that it was a wedge-shaped molecule consisting of three α-helices packed against a four-stranded β-sheet [12,13]. Two conserved cysteines in the mature peptide part formed a disulfide linkage, which was demonstrated to be essential for the stability of 3D structure [13]. This β-hairpin loop flanked by two cysteines was considered to play an important role in LPS binding activity, thus called LPS-binding domain (LBD) [14]. The striking charge distribution and high amphipathicity were considered to contribute to the membrane insertion ability of ALF [12].

Previously, the shrimp ALFs were classified into five groups [15–18]. However, in a recent study, more ALFs were mined from public databases and seven ALF groups were generated [19]. There are eleven ALF isoforms discovered from *Penaeus monodon* and they were classified into seven groups including Group A (ALF*Pm*1-2), Group B (ALF*Pm*3-5), Group C (ALF*Pm*6-7) [20], Group D (ALF*Pm*8), Group E (ALF*Pm*9), Group F (ALF*Pm*10) and Group G (ALF*Pm*11)[19]. Among them, one of the members in Group B ALF, ALF*Pm*3, was intensively studied [21–25]. The recombinant ALF*Pm*3 was reported to inhibit the growth of broad spectrum of Gram-positive/negative bacteria and fungi at very low concentration, especially that it killed three types of *Vibrio* efficiently [21]. ALF*Pm*3 also played an important role in the defense

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against white spot syndrome virus (WSSV) infection in *P. monodon* [22], by binding to the virus envelope protein WSSV189[23]. ALF*Pm*3 was found to be able to bind to both Gram-positive and Gram-negative bacterial cells and their major cell wall components, lipopolysaccharide (LPS) and lipoteichoic acid (LTA), with high affinity [24]. Moreover, the β-hairpin part of ALF*Pm*3 alone exhibited high inhibitory activity against various microorganisms [25]. One member of the Group C ALF, ALF*Pm*6, was described to be involved in the anti-*Vibrio* and anti-virus responses in *P. monodon* [26,27]. The LBD of ALF*Pm*6 displayed agglutination activity and killed *Escherichia coli* 363 and Gram-positive bacteria, such as *Bacillus megaterium*, *Aerococcus viridans* and *Micrococcus luteus*, at the concentration of 25–50 μM [28]. The Group D ALFs were negatively charged (Isoelectric point (p*I*) = 5.58–6.10), first described in *Litopenaeus stylirostris*. However, compared with Group B ALFs, their LPS binding and antimicrobial activities were limited [25]. The Group E ALF was first reported in *Marsupenaeus japonicas* and it can inhibit the growth of Gram-negative bacteria at the concentration of 30 μM[17]. The β-hairpin of the Group F ALF from *Litopenaeus vannamei* possesses no antimicrobial activity while *Litvan*ALF-G showed efficient inhibitory activity against Gram-positives and *Fusarium oxysporum* [19]. So far, Group D-G ALFs have not been characterized in *P. monodon*.

The antimicrobial mechanism of some ALFs was studied using various methods. Binding ability of ALFs to bacteria or bacterial components were studied [17,24,29]. ALF*Pm*3 was found to permeabilize both the outer and inner membrane of *Vibrio harveyi* 639[30]. With electron microscopy, severe membrane damage, bleb and cytoplasm leakage were clearly observed when the bacteria were incubated with either ALF*Pm*3 or recombinant m*Fc*ALF2 or LBDv [10,30,31]. In this research, the Group G ALF, ALF*Pm*11, was studied in detail. Its gene sequence was analyzed with bioinformatic tools and the expression pattern in different tissues was checked. The bactericidal activity of its LBD region was tested, in comparison with the LBD of ALF*Pm*3. Moreover, the inhibition mechanism of ALF*Pm*11 was described.

2. Materials and methods

2.1. Animals and bacteria

The black tiger shrimps (about 16 cm in length) were collected from a prawn breeding base (Dapeng, Shenzhen, China) and were cultured at 25 ± 1 °C in 50 L glass boxes, each containing 10 L of filtered seawater.

Vibrio parahaemolyticus strain OS4 was a gift from Prof. Li Deng. It was isolated from a diseased Red-spotted grouper *Epinephelus akaara* and was pathogenic to black seabream *Acanthopagrus schlegelii* [32]. *V. parahaemolyticus* OS4 was cultured with Luria-Bertani (LB) broth containing 3% sodium chloride (NaCl) shaken at the speed of 200 rpm at 30 °C.

Bacillus sp. T2 was collected by our lab from Lake Taihu (Jiangsu Province, China). *Acinetobacter* sp. L32 and *Exiguobacterium* sp. L33 were collected by our lab from Wenshan Lake in Shenzhen University. *Vibrio* sp. W28 was isolated from the seawater of Da mei sha (Shenzhen, China). *Staphylococcus aureus* was a gift kindly provided by Prof. Li Deng [33]. *Escherichia coli* JM107 was a lab collection. *Aeromonas hydrophila* CGMCC 1.2017 was purchased from China General Microbiological Culture Collection Center (CGMCC). *Vibrio* sp. W28 was grown in a modified LB medium (Tryptone 5 g, yeast extract 1 g, and NaCl 30 g per liter, pH 7.6) shaken at the speed of 200 rpm at 37 °C. *A. hydrophila* CGMCC 1.2017 was cultured with LB medium at 30 °C in a shaker at the speed of 200 rpm. The rest strains were also cultured with LB medium shaken at 200 rpm, but at 37 °C.

2.2. Antimicrobial peptide discovery and bioinformatic analyses

The nucleotide sequence of the new ALF was obtained by transcriptome sequencing of the hepatopancreas of *Penaeus monodon* (unpublished data). The unigene was defined as ALF by Blast against NT, NR, KOG, KEGG and SwissProt database.

The open reading frame and amino acid sequence of ALF*Pm*11 were deduced by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptide was predicted with SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). DNAman version 6 was used for generating gene structure information. Sequence similarity of ALF*Pm*11 with other ALFs was analyzed by Basic Local Alignment Search Tool (BLASTP). Multi-sequences alignment was performed with Clustalw (https://www.genome.jp/tools-bin/clustalw) and the results were generated with the online software ESPript 3.0 [34]. The physicochemical properties were predicted using Protparam (http://web.expasy.org/protparam/).

2.3. Vibrio challenge, tissue collection and total RNA extraction

The shrimps were cultured with the method described above for one day before the experiment and then divided into two groups. To stimulate the shrimps, *V. parahaemolyticus* OS4 was cultured to logarithmic phase as described above. The bacteria were harvested and washed twice with sterilized PBS. For the challenged group, 100 μL bacteria (1×10^7 CFU/mL) were injected into the ventral blood sinus of shrimps and the shrimps were challenged for 4 h. Shrimps in the control group were challenged with same volume of PBS.

Stomach, heart, gills, intestine, hemocytes, and hepatopancreas were collected from at least three individuals in each group. To collect the hemocytes, at least 600 μL hemolymph were syringed from pericardial sinus of shrimps and mixed with 100 μL anticoagulant (0.1 M sodium citrate, 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.6) immediately. The cells were collected and washed with 1 mL anticoagulant by centrifuging at 830 g, for 10 min at 4 °C, and then suspended with 600 μL lysis buffer for total RNA extraction. Total RNAs were extracted using RNeasy Mini Kit (Qiagen, USA), according to the manufacturer's instruction. The first strand of cDNA was synthesized using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan).

2.4. Confirmation of the ALFPm11 gene

To amplify the ORF of ALF*Pm*11, specific primers were designed by Primer Premier 5 software (Table 1, ALF*Pm*11conF and ALF*Pm*11conR). The reverse-transcription PCR (RT-PCR) was performed in a reaction of 30 μL containing 1.5 μL cDNA of hepatopancreas as template, 1 × premix Taq (Takara, Japan) and 0.4 μM primers, at the conditions of 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 60 s; 72 °C for 5 min. The PCR product was connected to a T-vector and transformed into *Escherichia coli* TOP10, following the manufacturer's instruction (pMD™18-T Vector Cloning Kit, Takara, Japan). Positive clones were sequenced (Igebio, Guangzhou, China).

2.5. Tissue distribution of ALFPm11

The relative expression level of ALF*Pm*11 in different tissues of PBS or *Vibrio* challenged *P. monodon* was tested by semi-quantitative reverse transcription PCR. EF-1α was used as an internal reference. The primers to quantify ALF*Pm*11 (ALF*Pm*11 F and ALF*Pm*11 R) and EF-1α (EF-1α F and EF-1α R) were designed by Primer Premier 5 software and were

Table 1
Primers used in the present study.

Name	Primer sequence (5′-3′)
ALF*Pm*11conF	TCATGACGAATCAGAGAACATC
ALF*Pm*11conR	TAAATCTCGCACATAAATCCC
ALF*Pm*11 F	TCGAGCTTCTGGGTCACTA
ALF*Pm*11 R	GCTTTCTGGACGAAGTTGA
EF-1α F	GGACAGCACCGAGCCCAAG
EF-1α R	TGCTTCTTCCACCAGCCCATA

Fig. 1. Nucleotide and deduced amino acid sequence of ALF*Pm*11. The initiation and termination codons are bolded; the signal peptide is underlined; the LBD region is on a gray background, the two cysteines are in red and positively charged residues are in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

listed in Table 1. The PCR reaction consisting of 1.5–2.4 μL cDNA, 1 × premix Taq (Takara, Japan) and 0.4 μM primers in 30 μL volume was performed with 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 15 s; 72 °C for 5 min. The PCR product was checked by electrophoresis on a 2% agarose gel.

2.6. Artificial synthesis of the LBD of ALFPm11 and ALFPm3

The LBD region of ALF*Pm*3 (E**C**KFTVKPYLKRFQVYYKGRMW**C**P--NH_2) and ALF*Pm*11 (Y**C**SYSTRPYFLRWQLKFKTKIW**C**P-NH_2) flanked with one amino acid residue in each end was chemically synthesized by DGpeptides Co. Ltd., (Hangzhou, China). The peptide was amidated in the C terminus and a disulfide bond was formed between the two cysteines. The purity of the peptide was 95%.

2.7. Antimicrobial activity of the LBD of ALFPm11 and ALFPm3

To test the minimum inhibition concentration (MIC), the indicator strains *Bacillus* sp. T2, *Acinetobacter* sp. L32, *Exiguobacterium* sp. L33, *Vibrio* sp. W28, *Staphylococcus aureus*, *Escherichia coli* JM107, *Aeromonas hydrophila* CGMCC 1.2017 and *Vibrio parahaemolyticus* OS4 were cultured with the method described above until OD 600 reached 0.4 and diluted 1000 times with relevant medium. Synthesized LBD of ALF*Pm*11 and ALF*Pm*3 were dissolved with PBS and 20 μL peptide was mixed with 80 μL diluted strains in a 96 well plate. The final concentration of the peptide was from 1 μM to 128 μM with the fold of two. PBS was added in the negative control. The plate was incubated at 37 °C or 30 °C (depending on the type of strain) for 18 h and OD_{600} was checked at 0 h and 18 h. The lowest concentration at which no growth of indicator was observed was considered as the MIC of the peptide. The experiment was repeated three times.

For the agar well diffusion assay, *Bacillus* sp. T2 and *Acinetobacter* sp. L32 were grown to logarithmic phase and 10 μL bacteria were mixed with 15 mL LB with 1.5% agar and poured into plates. Identical sizes of wells (7.5 mm diameter) were made. About 640 μM peptides dissolved in 40 μL PBS were added. Same volume of PBS or ampicillin (the concentration was about 670 μM) was used as negative or positive control. The plates were incubated at room temperature for 1 h for peptide diffusion and then at 37 °C for 17 h.

2.8. Bacterial agglutination assay

Gram-positive bacteria (*Exiguobacterium* sp. L33) and Gram-negative bacteria (*Acinetobacter* sp. L32, *Vibrio* sp. W28 and *A. hydrophila*) were cultured to logarithmic phase (OD_{600} = 0.4–0.5) and collected by centrifugation at 6000*g* for 2 min. The medium was removed and same volume of PBS was added to resuspend the bacteria. The bacteria were incubated with 128 μM peptides, 400 μg/mL Bull Serum Albumin (BSA) or PBS at room temperature for 1 h. Agglutination was then observed under a Leica DMi1 Microscope (Leica, Germany).

2.9. Membrane permeabilization activity

The membrane permeability assay was performed as described by Tang Ya-Li et al. [35] with some modifications. The logarithmic phase culture of *Bacillus* sp. T2 was suspended in PBS as described above and then diluted 10 times (about 5×10^7 CFU/mL) for use. The bacteria were incubated with 16 μM or 64 μM peptide at room temperature. BSA (200 μg/mL) or PBS were used as control. Samples were collected at 0, 10, 20, 30, 40, 60, 120, 180 and 240 min, and the bacteria cells were removed immediately by passing through a 0.22 μm filter. The total nucleotide leakage was measured by recording the absorbance at 260 nm (A260) of the filtrate. The experiment contained three duplicates. GraphPad Prism 5 was used for the statistical analysis and table generation.

3. Results

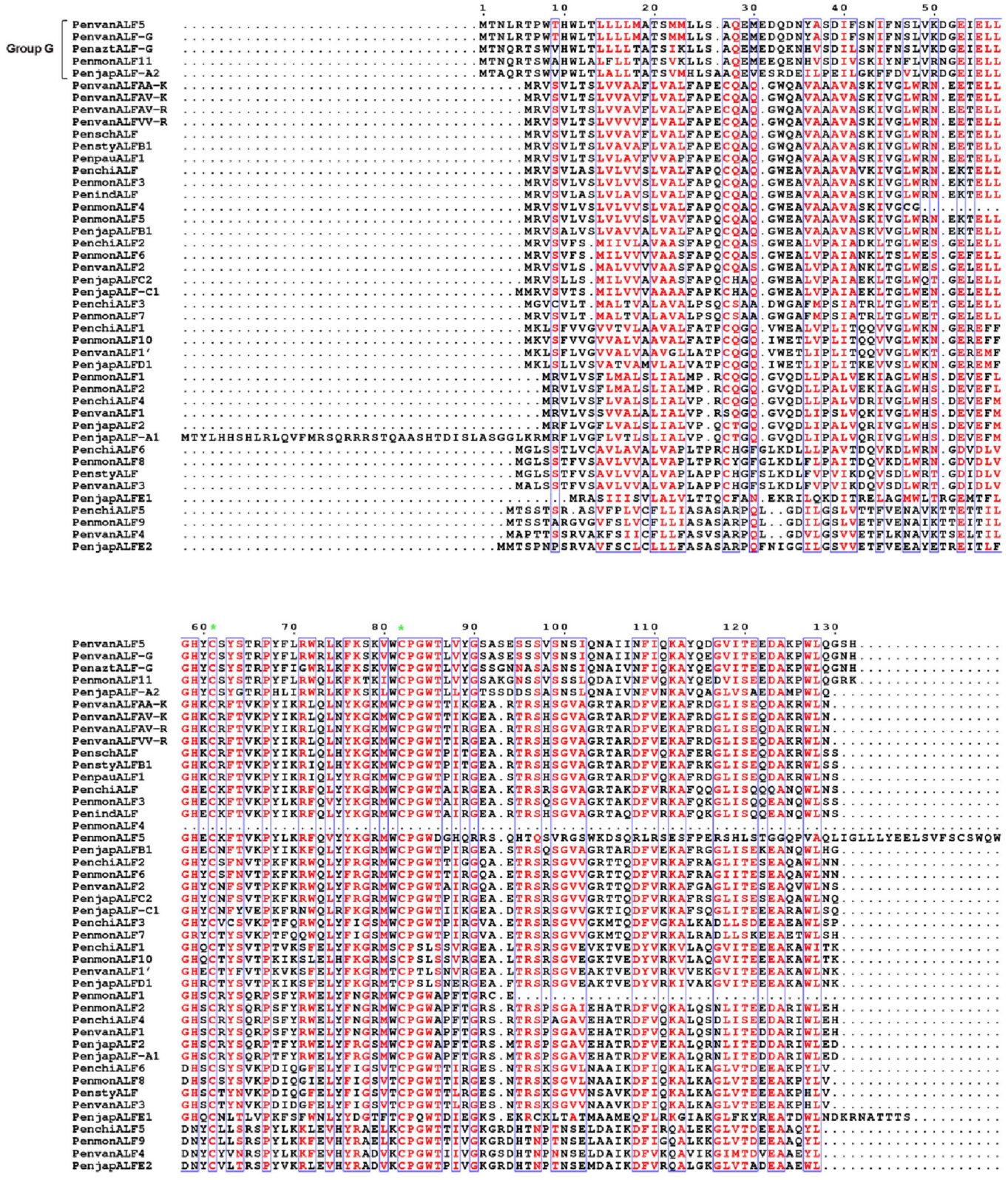
3.1. General information of ALFPm11

The anti-lipopolysaccharide factor described in this research was found from the trancriptome data of hepatopancreas of *P. monodon* (SRA accession: PRJNA473435) and the ORF region was confirmed by RT-PCR. According to the report of Gabriel Machado Matos et al. (2018)[19], it was named ALF*Pm*11 and classified into Group G ALF. The ORF of ALF*Pm*11 was 399 bp, encoding a 27 residues signal peptide and a 105 residues mature peptide. The LPS-binding domain (LBD) was from residue 61 to 82, flanked by two cysteines (Fig. 1). The molecular weight of the mature peptide is 12.2 kDa with a theoretical p*I* of 6.13.

Notably, the LBD region of ALF*Pm*11 contained 5 positively charged residues and the theoretical p*I* was 9.90, which was similar with some Group A and Group E ALFs, that having low p*I* value (5.46–6.13) in the mature peptide but very cationic (p*I* > 9) in the LBD region (Table S1).

3.2. Sequence similarity analysis of ALFPm11

The precursor of ALF*Pm*11 showed 78%, 75%, and 63% identity in amino acid sequences to ALF-G from *P. aztecus*, ALF5 from *P. vannamei*, and ALF-A2 from *P. japonicas,* respectively. These four ALFs were



(caption on next page)

Fig. 2. Amino acid sequences alignment of ALF*Pm*11 with other ALFs from shrimps. Similar residues were in red. The two cysteines were marked with green asterisks. The GenBank IDs of ALFs sorted by species are as follows: *Penaeus monodon* (*Penmon*ALF1: ABP73290.1; *Penmon*ALF2: ABP73291.1; *Penmon*ALF3:AEW91477.1; *Penmon*ALF4: ABP73293.1; *Penmon*ALF5: CF415871.1; *Penmon*ALF6: AER45468.1; *Penmon*ALF7: ANP92039.2; *Penmon*ALF8: GEME01010116; *Penmon*ALF9: GEEP01059175; *Penmon*ALF10: GEME01077326); *Penaeus chinensis* (*Penchi*ALF: AHN13886.1; *Penchi*ALF1: AFU61124.1; *Penchi*ALF2: AFU61125.1; *Penchi*ALF3: AFU61126.1; *Penchi*ALF4: AFU61127.1; *Penchi*ALF5: AFU61128.1; *Penchi*ALF6: AFU61129.1); *Penaeus indicus* (*Penind*ALF: ADE27980.1); *Penaeus japonicas* (*Penjap*ALF2: BAH22585.1; *Penjap*ALF-A1: ANA91278.1; *Penjap*ALFB1: ASR74829.1; *Penjap*ALF-C1: AB210110; *Penjap*ALFC2: AME17862.1; *Penjap*ALFD1: AME17863.1; *Penjap*ALFE1:ASR74830.1; *Penjap*ALFE2: ASR74831.1); *Penaeus paulensis* (*Penpau*ALF1: ABQ96193.1); *Penaeus schmitti* (*Pensch*ALF: ABJ90465.1); *Penaeus stylirostris* (*Pensty*ALF: AAY33769.1; *Pensty*ALFB1: AGH32549.1); *Penaeus vannamei* (*Penvan*ALF1: AVP74301.1; *Penvan*ALF1': AHG99284.1; *Penvan*ALF2: AVP74302.1; *Penvan*ALF3: AVP74303.1; *Penvan*ALF4: AVP74304.1; *Penvan*ALF5: AVP74305.1; *Penvan*ALFAA-K: ABB22833.1; *Penvan*ALFAV-K: ACT21197.1; *Penvan*ALFAV-R: ABB22832.1; *Penvan*ALFVV-R: ABB22831.1; *Penvan*ALF-G: GETZ01049665) ; *Penaeus aztecus* (*Penazt*ALF-G: GEUA01069818). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

grouped to Group G ALF (Fig. S1). However, it showed ≤45% identity to other group of ALFs. Amino acid sequence alignment of ALF*Pm*11 with other ALFs from shrimps showed that the residues in and beside the LBD region were highly conserved (Fig. 2). The hydrophobic and positively charged residues were alternate in the LBD of Group G ALF, which maintained the amphipathicity of the peptide.

3.3. Tissue specific expression pattern of ALFPm11

In the PBS challenged shrimps, expression of ALF*Pm*11 was detected in stomach, gills and hepatopancreas of *P. monodon*, not in intestine, heart and hemocytes (Fig. 3). Interestingly, it was abundantly expressed in hepatopancreas. When the shrimps were challenged by *V. parahaemolyticus* OS4 for 4 h, expression of ALF*Pm*11 in intestine, heart and hemocytes could be detected and higher expression level was shown in stomach and gills. However, relatively lower expression level was detected in hepatopancreas post challenging, and this was in accordance with the transcriptome sequencing result.

3.4. Antimicrobial activities assay

The LBD region of ALF*Pm*3 was reported to possess strong antimicrobial activity against Gram-positive, Gram-negative bacteria and fungi [18]. In this study, both the ALF*Pm*3-LBD and ALF*Pm*11-LBD were chemically synthesized and their bactericidal activity were tested and compared. The results (Table 2) showed that ALF*Pm*11-LBD was highly effective against all tested Gram-positive bacteria, including *S. aureus* and two aquatic bacteria. Moreover, it could also efficiently inhibit the growth of one Gram-negative aquatic bacterium, *Acinetobacter* sp. L32. However, it could not kill the two aquacultural pathogens, *V. parahaemolyticus* and *A. hydrophila* efficiently. Notably, ALF*Pm*11-LBD displayed higher or equal inhibition activity to ALF*Pm*3-LBD against most tested bacteria except *Exiguobacterium* sp. L33. We found that ALF*Pm*11-LBD was not completely dissolvable when the concentration was over 32 μM, and this might affect the activity.

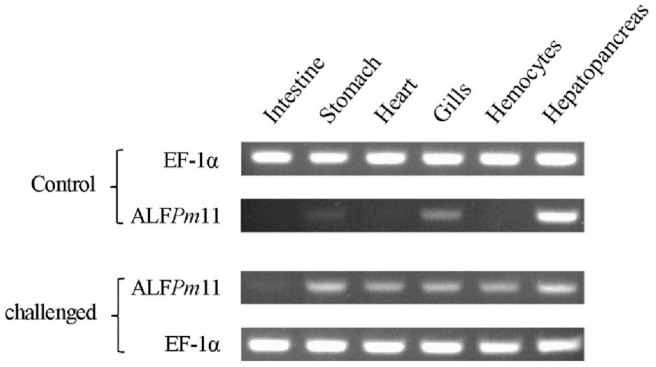


Fig. 3. Relative expression level of ALF*Pm*11 in six different tissues of *P. monodon* post challenge detected by semi-quantitative RT-PCR. In the challenged group, shrimps were challenged with 1×10^6 CFU *V. parahaemolyticus* OS4 for 4 h. In the control group, shrimps were injected with PBS. EF-1α was used as an internal reference. The experiment was repeated thrice.

Table 2
Minimum inhibition concentration (MIC) of ALF*Pm*3-LBD and ALF*Pm*11-LBD.

Indicator strains	MIC (μM)	
	ALF*Pm*3-LBD	ALF*Pm*11-LBD
Gram-positive bacteria		
Staphylococcus aureus	64	16
Exiguobacterium sp. L33	2	4
Bacillus sp. T2	2	2
Gram-negative bacteria		
Acinetobacter sp. L32	16	4
Vibrio sp. W28	64	64
Vibrio parahaemolyticus OS4	> 128	> 128
Escherichia coli JM107	> 128	128
Aeromonas hydrophila CGMCC 1.2017	> 128	> 128

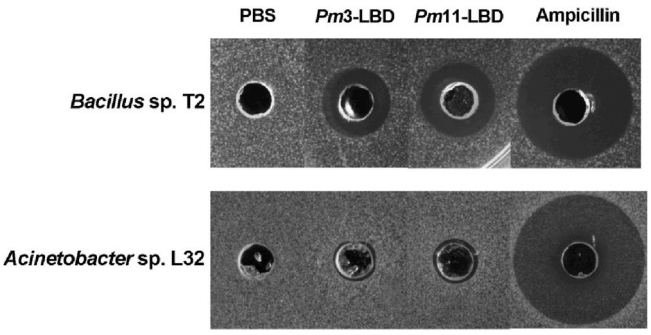


Fig. 4. Antimicrobial activity of ALF*Pm*3-LBD and ALF*Pm*11-LBD on plate. *Bacillus* sp. T2 and *Acinetobacter* sp. L32 were used as indicator strains. About 40 μL PBS was added as a negative control. Ampicillin was used as a positive control. The concentration of the peptides and ampicillin were 640 μM and 670 μM, respectively, in 40 μL volume.

Antimicrobial activity of the peptides on plate was tested by agar well diffusion assay. The result (Fig. 4) showed that both peptides displayed significant activity against *Bacillus* sp. T2 but the halos were much smaller than the positive control. A slightly larger halo was shown when ALF*Pm*11-LBD was added but the halo caused by ALF*Pm*3-LBD was more transparent. When *Acinetobacter* sp. L32 was the indicator strain, a small but apparent halo was observed for both peptides and the activity of ALF*Pm*11-LBD was a bit higher, which was in accordance with the MIC test. For ampicillin, a very large halo was found but only the bacteria around the well were totally inhibited.

3.5. Agglutination activity

As shown in Fig. 5, compared to the control, adding BSA did not make the bacteria agglutinate, but when the peptide was added, agglutination of bacteria could be observed. Specifically, both ALF*Pm*3-LBD and ALF*Pm*11-LBD caused significant agglutination of *Exiguobacterium* sp. L33 and *Vibrio* sp. W28. ALF*Pm*11-LBD displayed higher agglutination activity than ALF*Pm*3-LBD against *Acinetobacter*

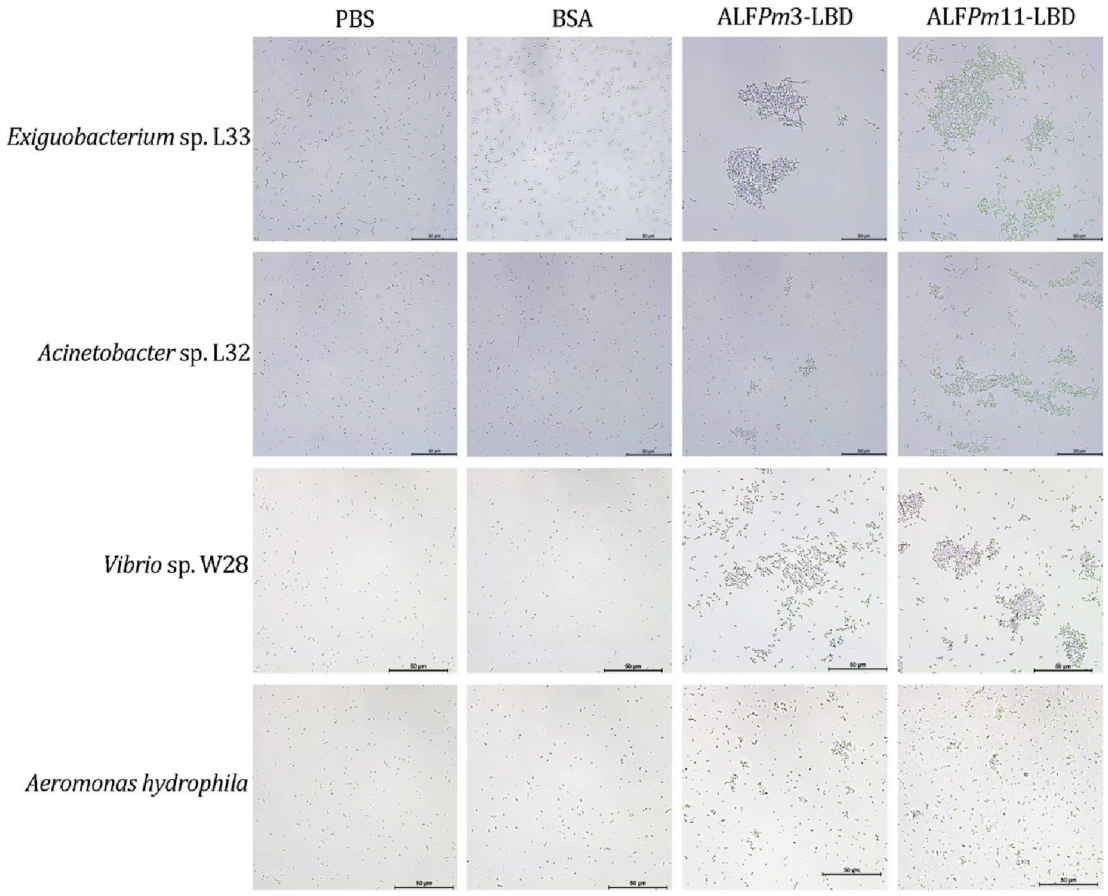


Fig. 5. Agglutination of *Exiguobacterium* sp. L33, *Acinetobacter* sp. L32, *Vibrio* sp. W28 and *Aeromonas hydrophila* induced by ALF*Pm*3-LBD and ALF*Pm*11-LBD. About 10^8 CFU/mL bacteria were incubated with 128 μM peptides for 1 h and observed under optical microscope. PBS and 400 μg/mL BSA were used as control. Scale bar is 50 μm.

sp. L32. However, both peptides showed unapparent agglutination activity when *A. hydrophila* was tested. The result was accordant to the inhibition activity, as the sensitive bacteria agglutinated seriously.

3.6. Membrane permeabilization activity

To test whether ALF*Pm*11-LBD could permeabilize bacterial membrane, release of total nucleotides was tested. The result (Fig. 6) showed that total amount of nucleotides in the supernatant started to increase when *Bacillus* sp. T2 was incubated with ALF*Pm*11-LBD for 20 min and reached maximum at 60 min or 240 min depending on the concentration of peptide was 64 μM or 16 μM. Adding BSA did not induce the bacteria to release more nucleotide compared to the control. This suggested that ALF*Pm*11-LBD could damage the membrane of bacteria, cause nucleotide leakage and ultimately kill the bacteria.

4. Discussion

ALFs have become a huge family of AMPs [36]. Although their amino acid sequences were diverse, all ALFs formed a similar 3D structure [13]. They all contained a β-hairpin structure, which was the main part of ALF that display LPS binding activity [14]. According to the sequence and charge, all shrimp ALFs were classified into seven groups [19]. The Group G ALF is a new group of ALFs and only five

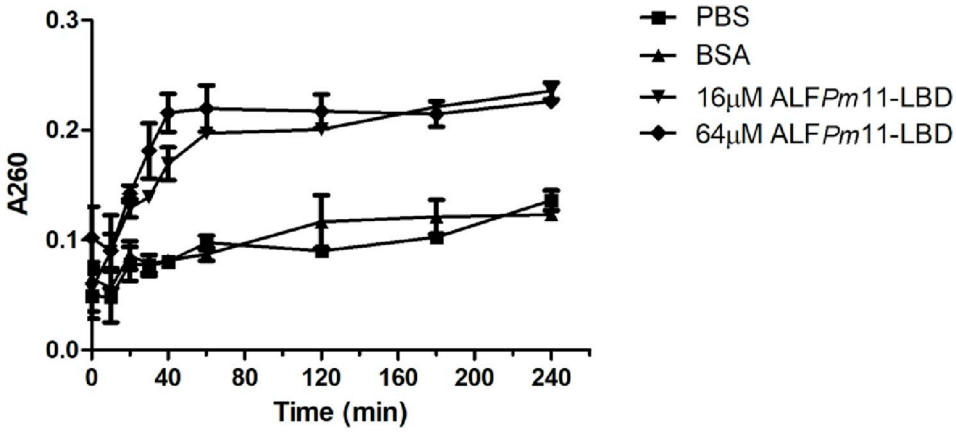


Fig. 6. Total nucleotide leakage of *Bacillus* sp. T2 treated with ALF*Pm*11-LBD. Total nucleotide was detected by recording the absorbance at 260 nm. The 10 times diluted bacteria suspended in PBS were incubated with PBS, 200 μg/mL BSA or 16/64 μM ALF*Pm*11-LBD in PBS buffer for different time. The experiment was conducted with three repeats and data was shown as mean value ± SD.

Fig. 7. Sequence information of ALF*Pm*3-LBD and ALF*Pm*11-LBD synthesized in this study. Cationic residues were in blue; aromatic residues were in green. Three unique lysines were in orange. The two β-strands were marked referred to the structure of ALF*Pm*3 (PDB ID: 2JOB). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

members have been discovered (Fig. S1). As the Group G ALF showed a p*I* value of 5.02–6.12, they were considered as anionic ALFs [19]. However, they had a very positively charged LBD region (Table S1).

The LBD region of ALF*Pm*3 and ALF*Pm*11 were shown in Fig. 7. Compared to ALF*Pm*3-LBD, the LBD of ALF*Pm*11 contained two fewer cationic residues in the β2 strand but one more cationic residue in the β3 strand. They both contained six aromatic amino acids, which were distributed alternately with lysines or arginines. As described by Adolf Hoess [12] and Christine Ried [14], when forming β-hairpin structure, the positively charged residues of the LBD will be on one side and hydrophobic residues will be on the other side, which make the molecule possess high amphipathicity.

Tissue distribution of ALF*Pm*11 showed that it was conventionally expressed in hepatopancreas, stomach and gills of *P. monodon*, which indicated that ALF*Pm*11 might play an important role for the shrimp to defend microbials from water and food. In the research of Gabriel M. Matos et al. [19], the *Litvan* ALF-G was found to distribute in hemocytes and gills in a low amount. But in this research, high expression was detected in hepatopancreas. After challenge, the shrimps showed higher expression level in all tested tissues except in hepatopancreas, which indicates that ALF*Pm*11 might play a very important role in the anti-*Vibrio* immune response of *P. monodon*. The relative expression level of *Litvan* ALF-G was found to vary in five different shrimps [19]. We also found that ALF*Pm*11 showed very distant expression levels in different individuals, *e.g.* some shrimps did not show any expression in the hepatopancreas (data not shown). This might because that the RNA of ALF*Pm*11 was quite unstable or the Group G ALF had large individual variation.

In this research, we found that ALF*Pm*11-LBD showed high inhibitory activity against several types of aquatic bacteria, which suggested that ALF*Pm*11 might play an important role in the innate immunity of shrimp to defend invading bacteria in the water environment. The antimicrobial activity of ALF*Pm*3 had been described in literatures. Recombinant ALF*Pm*3 showed strong inhibitory effects on varieties of bacteria and fungi. It inhibited the growth of several types of *Vibrios* (*V. alginolyticus*, *V. anguillarum* and *V. harveyi*) at 0.39–1.56 μM but displayed low activity against a highly pathogenic strain *V. penaeicida* (MIC = 25–50 μM) [21]. P. Supungul et al. reported that rALF*Pm*3 could efficiently kill virulent and non-virulent *V. parahaemolyticus*, with MIC values at 1.25–5.0 μM and 0.65–2.5 μM [37]. However, antimicrobial activity of the LBD region was not shown, except the region 35–51 [21]. In this research, we found that ALF*Pm*3-LBD could inhibit the growth of *Vibrio* sp. W28 at 64 μM, and could not kill *V. parahaemolyticus* OS4 at 128 μM, which indicated that full-length peptide was required to kill *Vibrios* effectively. Interestingly, the antimicrobial activity of ALF*Pm*3-LBD and ALF*Pm*11-LBD showed different strain specificity, *e.g.* ALF*Pm*3-LBD could kill *Exiguobacterium* sp. L33 more efficiently while ALF*Pm*11-LBD showed higher activity against *Acinetobacter* sp. L32, which indicated that sequence diversity of ALF could affect their activity and this provides more information for the bioengineering of ALFs to increase specific activity.

It has been shown previously with ELASA that ALF*Pm*3 could bind to *Vibrio harveyi* 639 cells [30]. However, agglutination function of ALF*Pm*3 or ALF*Pm*3-LBD has not been shown. A modified synthetic LBD peptide (LBDv) of FcALF2 was found to agglutinate both Gram-negative and Gram-positive bacteria quickly. The mechanism of agglutination was generally considered that the positively charged peptides neutralized the surface charge of bacteria and reduced the electrostatic repulsion between bacteria, resulting in agglutination [31]. In this research, ALF*Pm*11-LBD and ALF*Pm*3-LBD induced significant agglutination of both Gram-positive and Gram-negative bacteria, which indicated that both peptides could bind to some surface components of bacteria (LPS, LTA or perhaps peptidoglycan (PGN)), make them uncharged and then gather together.

AMPs normally interact with bacterial membrane and kill the bacteria in different modes [38]. Some directly disrupt the membrane causing leakage of cytoplasm by barrel stave/toroidal pore formation or carpet-like mechanism [39]. Some AMPs can translocate into the membrane and bind to an intracellular target to inhibit biological activity of microorganism [40,41]. Nucleotide leakage assay of *Bacillus* sp. T2 demonstrated that ALF*Pm*11-LBD could break the membrane of *Bacillus* sp. efficiently. Membrane permeabilization activity of ALF*Pm*3 was described previously by Phattarunda Jaree et al., and apparent increase of A260 was observed within 5 min [30]. This indicated that damaging the bacterial membrane and making the cellular contents overflow might be the bactericidal mechanism of ALF*Pm*11.

In this research, we characterized a new ALF in *P. monodon* and found that it was highly active against various bacteria and showed apparent *Vibrio* responses, which suggested that ALF*Pm*11 might play an important role in the innate immune responses of *P. monodon*. As antibiotics resistance is still a global problem, searching for alternatives remains necessary [42]. AMPs may become promising antimicrobial agents and be used in food industry and agriculture [43–45]. This study suggested that ALF*Pm*11 was an effective AMP of shrimp that could be developed as a safe antimicrobial agent and applied in aquaculture or as food preservative.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.066.

Conflicts of interest

The authors declare that there is no conflict of financial interest.

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