



A new group of anti-lipopolysaccharide factors from *Marsupenaeus japonicus* functions in antibacterial response



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ABSTRACT

Anti-lipopolysaccharide factors (ALFs) are a group of critical effector molecules with a broad spectrum of antimicrobial activities in crustaceans. Four groups of ALFs (A, B, C, and D) have been identified in penaeid shrimp. In the study, we identified a new group of ALFs (designated as *Mj*ALF-E) from *Marsupenaeus japonicus*. This new group (group E) included *Mj*ALF-E1 and E2. *Mj*ALF-E1 was highly expressed in hemocytes, heart, and intestine, whereas E2 was highly expressed in gills, stomach, and intestine. Expressions of both *Mj*ALF-E1 and E2 were upregulated by bacterial challenge. Synthesized LPS-binding domain peptides of *Mj*ALF-E1 and E2 strongly bind to bacterial cell wall components lipopolysaccharide (LPS) and peptidoglycan (PGN). The recombinant r*Mj*ALF-E2 showed relatively weak binding activity to LPS and PGN. Both synthesized peptides and r*Mj*ALF-E2 exhibited antimicrobial activity against Gram-negative bacteria, whereas r*Mj*ALF-E2 could promote the clearance of bacteria in vivo. After knockdown of *Mj*ALF-E2 and infection with *Vibrio anguillarum*, shrimp showed high and rapid mortality compared with *GFPi* shrimp. These results suggest that *Mj*ALF-Es serves a protective function against bacterial infection in shrimp.

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1. Introduction

Antimicrobial peptides (AMPs) are integral components of the immune system. They are effector molecules induced by invading pathogens in both vertebrates and invertebrates (Hoffmann and Reichhart, 2002). Most AMPs are usually cationic, small peptides that exhibit a broad spectrum of activities against Gram-positive and Gram-negative bacteria, fungi, and viruses (Guani-Guerra et al., 2010). AMPs are widely identified in vertebrates, invertebrates, plants, and even microbes. Several families of AMPs, such as penaeidins, lysozymes, crustins, anti-lipopolysaccharide factors (ALFs), and styllicins have been identified and characterized in shrimp (Tassanakajon et al., 2013; Zhao and Wang, 2008). ALFs are broad-spectrum AMPs that show activity against Gram-positive and Gram-negative bacteria, fungi, and viruses. ALFs serve an important function in the innate immune system of crustaceans.

The first ALF was obtained from hemocytes of the horseshoe crab *Limulus polyphemus* and was named according to its ability to inhibit the lipopolysaccharide (LPS)-mediated activation of the *Limulus* coagulation system (Muta et al., 1987; Tanaka et al., 1982). This ALF has strong antibacterial activity against bacteria (Morita et al., 1985).

In recent years, many ALFs have been isolated and characterized in a variety of crustaceans, including shrimp, crab, lobster, and crayfish (Beale et al., 2008; Imjongjirak et al., 2011; Li et al., 2008; Liu et al., 2005; Sun et al., 2011; Supungul et al., 2008; Zhang et al., 2010). All known ALFs usually have a signal peptide and a conserved LPS-binding domain between two conserved cysteine residues. Usually, the conserved LPS-binding domain contains positively charged amino acids within the disulfide loop (Hoess et al., 1993; Muta et al., 1987; Somboonwiwat et al., 2008; Yang et al., 2009). These special characteristics are related to biological functions (Aketagawa et al., 1986; Hoess et al., 1993; Pristovsek et al., 2005). The structures of LALF and ALFPm3 have been resolved (Hoess et al., 1993; Yang et al., 2009). ALFs have a conserved three-dimensional structure, consisting of three α -helices packed against a four-stranded β -sheet. The LPS-binding domain (LPS-BD) is a β -hairpin structure linked by a conserved disulfide bridge.

Different ALFs serve varied functions in shrimp immune responses. In the black tiger shrimp, five different ALF isoforms (ALFPm1 to ALFPm5) have been found in hemocytes (Supungul et al., 2004; Tassanakajon et al., 2006). ALFPm3 was the major isoform found in both normal and *Vibrio harveyi*-infected shrimp hemocytes. Purified recombinant ALFPm3 displays a broad antimicrobial activity against filamentous fungi, and Gram-positive and Gram-negative bacteria, including *Vibrio*, a natural shrimp pathogen (Somboonwiwat et al., 2005). In the crayfish *Pacifastacus leniusculus*, it was first reported that ALF interferes with white spot syndrome

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virus (WSSV) propagation in vivo and in vitro (Liu et al., 2006). In another study, ALFPm3 protects against WSSV infection both in freshwater crayfish hematopoietic cell culture and shrimp, *Penaeus monodon* (Tharntada et al., 2009). Recently, a new ALFPm named ALFPm6 was identified in *P. monodon* hemocytes; the silencing of ALFPm6 revealed that ALFPm6 was essential for shrimp survival and protection from *Vibrio harveyi* and WSSV infections (Ponprateep et al., 2012). In *Litopenaeus vannamei*, LvALF1 was essential to shrimp survival after infection with *V. penaeicida* and *Fusarium oxysporum* (de la Vega et al., 2008).

ALFs have been identified from diverse crustaceans, such as penaeid shrimp and swimming crabs, and have been classified by different authors. The ALFs from penaeid shrimp were divided into three groups (groups A, B, and C), based on sequence similarities and phylogenetic analysis (Tassanakajon et al., 2011). All three ALF groups were found in one species of shrimp. Six different ALFs from *P. monodon* were classified into groups A, B, and C, based on their LPS-binding sites (Ponprateep et al., 2012). ALFPm1 and ALFPm2 belong to group A, ALFPm3 to ALFPm5 belong to group B, and ALFPm6 belongs to group C (Ponprateep et al., 2012; Supungul et al., 2004; Tharntada et al., 2008). A new group of shrimp ALFs, designated as group D, was identified recently by sequence and phylogenetic analyses (Rosa et al., 2013). Group D ALFs have a negative net charge ($pI \leq 6.2$), display impaired LPS-binding activity, and have weak antimicrobial activity.

Therefore, four groups of ALFs were identified in terms of the sequences and range of theoretical isoelectric points (pI) in penaeid shrimp. Group B is highly cationic peptides, group C is cationic peptides, group A is anionic and cationic and group D is very anionic peptides (Rosa et al., 2013) (Tassanakajon et al., 2014). We obtained more than 30 different unigene sequences of ALFs by the hemocyte and hepatopancreas transcriptome analyses of kuruma shrimp, *Marsupenaeus japonicus* (unpublished data). These sequences belong to nine different ALFs according to sequence alignment results. By phylogenetic analysis, we identified a new group of ALFs, and designated as ALF-E. In this study, we focused on the expression and function of this new group of ALFs. The tissue distribution, expression patterns, binding activity, and antibacterial activities of group E in vitro and in vivo were analyzed. Characterization and function analysis of the new group of ALFs will be helpful for understanding antimicrobial immune responses in shrimp.

2. Material and methods

2.1. cDNA sequence and phylogenetic analysis

Full-length cDNA of nine putative anti-lipopolysaccharide factors (ALFs) were obtained from 30 different unigene sequences in the hemocyte and hepatopancreas transcriptome sequencing of kuruma shrimp *M. japonicus*. Penaeid shrimp homologous ALF sequences were collected from the NCBI database for multiple alignment and phylogenetic analyses. Domain architectures were predicted with the SMART program (<http://smart.embl-heidelberg.de/>). A phylogenetic tree was constructed using MEGA 4 software based on peptide residues from 21 to 58, which are involved in LPS-binding activity (Tamura et al., 2007). Another tree was constructed using the same software based on the full mature peptides of ALFs.

2.2. Immune challenge of shrimp and collection of tissues

Kuruma shrimp, *M. japonicus* was purchased from a market in Jinan, Shandong province, China. *V. anguillarum* and *Staphylococcus aureus* were cultured overnight and collected by centrifugation at $4000 \times g$ for 5 min. Subsequently, bacterial cultures were washed with phosphate-buffered saline (PBS; containing 140 mM NaCl,

2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 ; pH 7.4). The shrimp (3–5 g/shrimp) were divided into three groups, with 12 shrimp for each group. The 50 μl of resuspended *V. anguillarum* and *S. aureus* (2×10^7 CFU/shrimp) solutions or PBS solutions was injected into the third abdominal segment of each shrimp. Untreated shrimp were used as control (normal shrimp). Hemocytes and other tissues (heart, hepatopancreas, gills, stomach, and intestine) of challenged or unchallenged shrimp were collected at 0, 6, 12, 24, and 48 h after injection by a method described previously (Jiang et al., 2013). For hemocyte collection, hemolymph was drawn out from ventral blood sinus of shrimp with 1 ml syringe preloading anti-coagulant buffer (0.45 M NaCl, 10 mM KCl, 10 mM EDTA and 10 mM HEPES, pH 7.45) and then centrifuged at $800 \times g$, 4°C for 10 min to isolate hemocytes.

2.3. Total RNA extraction and cDNA synthesis

Total RNAs were extracted from hemocytes and other tissues of shrimp, including heart, hepatopancreas, gills, stomach, and intestine, using Unizol reagent (Biostar, Shanghai, China) according to the manufacturer's protocol. The first strand cDNA was reverse-transcribed via a method described by Du et al. (2007), and used as templates for cDNA cloning and expression pattern analysis.

2.4. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qRT-PCR)

Tissue distributions of MjALFs were analyzed by RT-PCR with nine pairs of primers MjALF-RT-F and R (Table 1). β -actin was used as the internal control with a pair of primers (β -actinF and β -actinR). The PCR reaction protocol was performed as follows: 94°C for 3 min; 30 cycles of 94°C for 30 s, 56°C for 40 s, 72°C for 40 s, and 72°C for 10 min.

Table 1
Sequences of the primers used in this study.

Primers	Sequences (5'–3')	Direction
SMART F	TACGGCTGCAGAGACGACAGAA	Forward
Oligo-anchor R	GACCACGCGTATCGATGTGCAC	Reverse
MjALF-E1RT-F	TCCTAACCCAGCAGTCTTGCTAATG	Forward
MjALF-E1RT-R	GCTTTTCGGATTTCCTTCGATGTTG	Reverse
MjALF-E2RT-F	TGCCGTGTTCTCTGCTTAT	Forward
MjALF-E2RT-R	TTGGTGGGATTCGTGTGGT	Reverse
MjALF-A1RT-F	CTGGTCGGTTCTCTGTGGC	Forward
MjALF-A1RT-R	CCAACCTGGGCACCACATACTG	Reverse
MjALF-A2RT-F	TGACAGGATGACAGGCCAGAG	Forward
MjALF-A2RT-R	CATAGGAACAGTAGTCCCAAGA	Reverse
MjALF-B1RT-F	CGGTGGTGGCCCTGGTGGCACTCTTCG	Forward
MjALF-B1RT-R	GACTGGCTGCGTGTGCTGGCTTCCCTC	Reverse
MjALF-C1RT-F	CGCTTCAAGGTCGGATGTG	Forward
MjALF-C1RT-R	CGAGCCTCTTCTCCGTGATG	Reverse
MjALF-C2RT-F	TCCTGGTGGTGGCAGTGGCT	Forward
MjALF-C2RT-R	TGCGGGTCTCGGCTTCTCT	Reverse
MjALF-D1RT-F	CTTGGCGTGAACAAGGTAGAGGAT	Forward
MjALF-D1RT-R	GCTTTTATTTTGGGGTCACTGCTGT	Reverse
MjALF-D2RT-F	CGAGGCTATGAGGAC	Forward
MjALF-D2RT-R	AGGTGACAGTCCCGAGGA	Reverse
MjALF-E2EXF	TACTCAGAATTCATGATACGTCACCCAATCC	Forward
MjALF-E2EXR	TACTCACTCGAGTTACAGCACTCTGCCGCTT	Reverse
MjALF-E2RNAiF	CGCTAATACGACTACTATAGGTCAGTCAAC	Forward
	ATCGGCGGC	
MjALF-E2RNAiR	GCGTAATACGACTACTATAGGTCAGTGGC	Reverse
	TTAGCGGAGGC	
β -actinF	AGTAGCCGCCCTGGTTGTAGAC	Forward
β -actinR	TTCTCATGTCGTCCAGT	Reverse
GFP RNAi-F	TAATACGACTCACTATAGGGGGTGGTCCCA	Forward
	ATTCTCGTGAAC	
GFP RNAi-R	TAATACGACTCACTATAGGCTTGTACAGC	Reverse
	TCGTCCATGC	

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