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KM Beale

DW Towle

N Jayasundara

CM Smith

JD Shields

Virginia Institute of Marine Science

See next page for additional authors

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Authors

KM Beale, DW Towle, N Jayasundara, CM Smith, JD Shields, HJ Small, and SJ Greenwood



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Anti-lipoplysaccharide factors in the American lobster *Homarus americanus*: Molecular characterization and transcriptional response to *Vibrio fluvialis* challenge

K.M. Beale¹, D.W. Towle^{1,*}, N. Jayasundara¹, C.M. Smith¹, J.D. Shields², H.J. Small², and S.J. Greenwood³

¹Center for Marine Functional Genomics, Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672 USA

²Virginia Institute of Marine Science, Gloucester Point, VA 23062 USA

³AVC Lobster Science Centre, Atlantic Veterinary College, Charlottetown, PEI C1A 4P3 Canada

Abstract

Two partial mRNA sequences predicted to encode anti-lipoplysaccharide factors (ALFs) were identified among expressed sequence tags generated from the American lobster *Homarus americanus* and complete cDNA sequences were obtained from library clones. Comparison of the translated amino acid sequences to those publicly available confirmed similarity to arthropod anti-lipoplysaccharide factors. Both protein sequences, designated ALFH_a-1 and ALFH_a-2, contained an N-terminal signal peptide and two half-cysteines participating in a disulfide bridge, features conserved in other ALFs. Predicted secondary structures were similar to that described for the ALF from the horseshoe crab *Limulus polyphemus*. As part of an exploratory study of immunity in *H. americanus*, lobsters were injected with the bacterium *Vibrio fluvialis* and gill, hematopoietic, and hepatopancreas tissues were sampled for analysis of gene expression of ALFH_a-1 and ALFH_a-2 by quantitative PCR. The relative abundance of ALFH_a-2 mRNA was not significantly affected by *Vibrio* injection in any of the three tissues tested. In contrast, ALFH_a-1 mRNA levels in gills were increased by the treatment some 17-fold. Our results support a molecularly specific regulation of antimicrobial proteins in response to bacterial infection in *H. americanus*.

Keywords

crustacean; antimicrobial peptides; real-time quantitative PCR; DNA sequencing; gill tissue; hepatopancreas tissue; hematopoietic tissue

1. Introduction

Species and strains of *Vibrio* bacteria have become an increasing problem in the crustacean mariculture industry, affecting lobster, crab, and shrimp. In the American lobster *Homarus americanus*, the subgroup *Vibrio fluvialis* has been implicated in limp lobster disease (Tall et

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*Author for correspondence, dtowle@mdibl.org, phone (207-288-9880), fax (207-288-2130).

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al. 2003) and as a member of the bacterial community associated with epizootic shell disease (Smolowitz et al. 2005). This gram-negative bacterium has caused significant economic detriment to the lobster industries of the eastern coasts of the US and Canada, with infections resulting in losses exceeding \$2.5 million annually (Tall et al. 2003). Although antibiotics could be a possible means of treatment, they are only feasible in holding facilities where antibiotic resistance could become an issue. The industry prides itself on being sustainable and environmentally friendly, making the use of broad spectrum antibiotics a particularly unattractive option.

The immune system of arthropods does not exhibit adaptive immunity in response to potential pathogens, but uses instead a system of innate immunity that includes pathogen recognition, phagocytosis, encapsulation, cytotoxicity, and melanization (Söderhäll et al. 1992; Muta et al. 1996). Much of this activity takes place in hemocytes, where antimicrobial peptides and proteins are synthesized, stored, and released into the hemolymph upon exposure to infective agents (Iwanaga et al. 1998; Johansson et al. 2000).

Although a number of antimicrobial proteins have been identified in crustaceans, primarily in shrimp species, little research has been conducted to characterize these factors in the American lobster *Homarus americanus*. One antimicrobial protein from lobster, crustin, has been characterized in our laboratories (Christie et al. 2007). The present study identified two additional antimicrobial proteins related to known crustacean anti-lipopolysaccharide factors and examined their expression in lobster tissues in response to challenge by *Vibrio fluvialis*.

2. Materials and Methods

2.1 Identification and sequencing of cDNA clones encoding anti-lipopolysaccharide factors

Expressed sequence tags predicted to encode two different anti-lipopolysaccharide factors were identified in a normalized multiple tissue cDNA library prepared from *H. americanus* (Towle et al. 2006). The library clones selected for complete sequencing were HA_MX0_13f11 (Accession No. **CN853488**), designated as encoding anti-lipopolysaccharide factor Ha-1 (ALFHa-1), and HA_MX2_15b07 (**FC556430**), designated as encoding anti-lipopolysaccharide factor Ha-2 (ALFHa-2). Plasmids were isolated from overnight cultures of each clone and the inserts were sequenced using universal primers SP6 and M13F with a capillary-based sequencer (ABI 3100, Applied Biosystems, Foster City, CA, USA). Contiguous sequences were produced with the SeqMan component of Lasergene software (DNASTAR, Madison, WI, USA) and open reading frames were predicted and translated with DNASIS (Hitachi, S. San Francisco, CA, USA). Hydrophobicity indices (Kyte et al. 1982) of the resulting amino acid sequences were analyzed through the ExPaSy ProtScale server (Swiss Institute of Bioinformatics) and secondary structure predictions were made via PSIPRED (McGuffin et al. 2000). Alignment with arthropod anti-lipopolysaccharide protein sequences and a neighbor-joining tree of evolutionary relationships were generated via MEGA4 (Tamura et al. 2007).

2.2 Animals and tissue sampling

At the Virginia Institute of Marine Science, American lobsters (*Homarus americanus*) were obtained from a local supplier and acclimated overnight in individual aerated 9.5-liter tanks containing 35 psu seawater at 10–11°C. Three lobsters were injected at the base of a walking leg with 1 mL *Vibrio fluvialis* (10^8 cfu/mL) in tryptone soya broth (TSB) medium and 3 control lobsters were injected with 1:10 dilution of TSB. After 24 hours, all lobsters were euthanized. Gill, hematopoietic, and hepatopancreas tissues were aseptically removed and stored in RNAlater (Sigma-Aldrich, St. Louis, MO, USA) at –20°C for subsequent transport to the Mount Desert Island Biological Laboratory.

2.3 Total RNA isolation and cDNA synthesis

Total RNA was extracted from hepatopancreas, hematopoietic tissue, and gill of 3 control and 3 experimental lobsters under RNase-free conditions (Chomczynski et al. 1987) using RNeasy Total RNA Isolation System (Promega, Madison, WI, USA). To determine quantity and quality of the RNA, samples were analyzed on a nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and by microfluidic electrophoresis (Agilent 2100 Bioanalyzer, Santa Clara, CA, USA), showing complete absence of degradation products or genomic DNA. Aliquots of 2 µg of each sample of total RNA were reverse transcribed to single-stranded cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT)₂₀ as a primer according to the manufacturer's protocol. cDNA was stored at -20°C between uses.

2.4 PCR amplification of cDNA templates, electrophoresis, and DNA sequencing

Two sets of non-degenerate primers were designed to target specifically ALFH_a-1 or ALFH_a-2 using Primer Premier software (Premier Biosoft, Palo Alto, CA, USA) and were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Polymerase chain reactions were carried out using RedTaq Ready Mix PCR Reaction Mix (Sigma-Aldrich) under the following conditions: 30 cycles of 92°C for 1 min, 55°C for 1min, and 72°C for 2 min, then 72°C for 5 min and 4°C for storage. Following amplification, PCR products were analyzed by agarose gel electrophoresis (1% agarose in TBE buffer, 100 V, 30 min), stained with SYBR Green, and viewed over a UV light source. Amplification products of the correct size were excised from the agarose gel using a sterile scalpel blade and purified using a QiaQuick gel extraction kit (Qiagen, Valencia, CA, USA). Resulting nucleotide sequences were analyzed and edited using ChromasPro 1.3 (Technelysium, Tewantin, QLD, Australia). Forward and reverse sequences for each target were aligned using SeqMan (Lasergene, DNASTAR, Madison, WI, USA). BLASTN and BLASTX (Altschul et al. 1997) comparisons with non-redundant sequence databases (National Center for Biotechnology Information, Bethesda, MD, USA) were performed to confirm the presence of the intended target cDNAs.

2.5 Quantification of gene expression

Analysis of ALFH_a-1 and ALFH_a-2 mRNA expression was carried out via real-time quantitative PCR (QPCR) using Brilliant SYBR Green QPCR mix and MX4000 instrumentation (Stratagene, LaJolla, CA, USA). The primer combinations selected for ALFH_a-1 were Ha-1-3F (5'-TAAAGTGGTTGGGTTATGGC-3') and Ha-1-4R (5'-TGAGTAGAGGTTAAAGGGTGA-3'), producing an amplicon of 289 bp. For ALFH_a-2, the primers were Ha-2-7F (5'-AGCGAACACTACAAGATGCG-3') and Ha-2-6R (5'-CACCTTCTTATCTTGGGTTTCA-3'), producing an amplicon of 215 bp. The thermal profile for QPCR included an activation step at 95°C for 15 min and then 40 cycles of 94°C for 40 sec, 55°C for 40 sec, and 72°C for 60 sec. Following amplification, a thermal denaturation profile was generated to assess product homogeneity. For both targets, a single inflection point of the denaturation curve at 82.5° C for ALFH_a-1 and 85.0° C for ALFH_a-2 indicated a homogenous product. To enable calculation of relative expression values, a dilution series of cDNA prepared from control hematopoietic tissue was analyzed in parallel with the other samples, all of which were run in triplicate. Amplification efficiency was 89.8% for ALFH_a-1 and 87.9% for ALFH_a-2. Expression values were normalized to total RNA content (2.0 µg) in each sample employed in the reverse transcription step, a method well accepted in systems lacking a validated housekeeping gene {Bustin et al. 2005}. Means and SD were calculated for each triplicate and then pooled to obtain relative expression data for control and experimental gill, hepatopancreas and hematopoietic tissues (n=3). Statistical analysis using two-way ANOVA and Bonferroni posttests was accomplished with Prism GraphPad (LaJolla, CA, USA).

3. Results

Two partial nucleotide sequences encoding putative anti-lipoplysaccharide factors were identified in a normalized multiple-tissue cDNA library prepared from the American lobster *Homarus americanus* (Towle et al. 2006). Sequencing the inserts contained in the respective library clones yielded complete cDNA sequences with open reading frames encoding a 123-amino-acid protein for ALFH_a-1 (Accession No. [EU625516](#), Figure 1) and a 124-amino-acid protein for ALFH_a-2 ([EU625517](#), Figure 2). Calculated molecular weights for the two proteins are 13,683 for ALFH_a-1 and 13,909 for ALFH_a-2. The predicted ALFH_a-1 protein is substantially more cationic than ALFH_a-2, its calculated isoelectric point being 10.65 compared to 8.85 for ALFH_a-2. Contributing to this difference, the ALFH_a-1 protein contains 10 arginine residues and 6 lysine residues, compared with 7 arginine residues and 4 lysine residues for ALFH_a-2.

Both protein sequences contain a pair of conserved cysteine residues, at positions 55 and 76 in ALFH_a-1 and positions 56 and 77 in ALFH_a-2 (Figure 1 and Figure 2). Between these positions lies a cationic region containing a conserved KPXXRR motif, comprising the looped portion of the sequence that is thought to interact with target lipopolysaccharides in bacterial cell walls (Hoess et al. 1993). Hydrophobic signal peptides were predicted by SignalP 3.0 (Bendtsen et al. 2004) for both protein sequences, encompassing the N-terminal 25 amino acid residues in ALFH_a-1 and 26 amino acid residues in ALFH_a-2 (Figure 3). A second hydrophobic region lies adjacent to the signal peptide, but the remainder of the sequence is generally hydrophilic in both proteins.

Secondary structure predictions using PSIPRED (McGuffin et al. 2000) identified α -helical regions in the signal peptide and the adjacent hydrophobic region, as well as two segments at the C-terminus (Figure 4). Between these regions are segments of β -strand and random coil that include the amphipathic loop defined by the conserved cysteine residues at positions 55 and 76 (ALFH_a-1) or 56 and 77 (ALFH_a-2). These predictions are surprisingly consistent with the known crystal structure of the mature anti-lipoplysaccharide factor of *Limulus polyphemus* in which the corresponding amphipathic loop includes amino acids 32–50, the position coinciding precisely with the cleavage of a 25-amino-acid signal peptide (Hoess et al. 1993).

Alignment via MEGA4 (Tamura et al. 2007) of ALFH_a-1 and ALFH_a-2 amino acid sequences with representative arthropod anti-lipoplysaccharide factor amino acid sequences revealed several regions of sequence conservation, particularly around the conserved cysteine residues (Figure 5). Although the signal peptides of each sequence are consistently hydrophobic, amino acids within these regions are not highly conserved. A neighbor-joining tree based on this alignment sorted the chelicerates *Limulus polyphemus* and *Tachypleus tridentatus* into a group separate from the crustaceans, as expected (Figure 6). ALFH_a-1 of *Homarus americanus* appears to be most similar to the ALFs of shrimps *Litopenaeus vannamei* and *Farfantepenaeus paulensis* and ALFPm3 of giant tiger prawn *Penaeus monodon*, while ALFH_a-2 is grouped with an ALF from the kuruma prawn *Marsupenaeus japonicus*.

To investigate the transcriptional response of ALFH_a-1 and ALFH_a-2 to a pathogen, we injected adult lobsters with *Vibrio fluvialis* and sampled tissues 24 hours later for analysis of the respective RNAs by quantitative PCR. Employing sequence-specific primers that amplified the respective cDNAs, as affirmed by direct sequencing of PCR products, we found very low levels of the two mRNAs in hepatopancreas (Figure 7). However, hematopoietic tissue and gill showed substantial levels of both ALFH_a-1 and ALFH_a-2 mRNA. Injection of *V. fluvialis* elicited no response in the abundance of ALFH_a-2, but induced a statistically significant 17-fold increase in ALFH_a-1 mRNA in gill tissue (Figure 7). A high degree of

specificity in the immune response to *V. fluvialis* was indicated since one of the genes encoding an anti-lipopolysaccharide factor (ALFH_a-1) appeared to be transcriptionally upregulated, while the other (ALFH_a-2) was not.

4. Discussion

We report the finding of two putative anti-lipopolysaccharide factors in the tissues of the American lobster, *Homarus americanus*. Anti-lipopolysaccharide factors (ALFs) are small basic proteins of approximately 100 amino acids that were initially characterized from the hemolymph of distantly related arthropods, the horseshoe crabs *T. tridentatus* (Aketagawa et al. 1986) and *L. polyphemus* (Muta et al. 1987). ALFs bind to cell walls of gram-negative bacteria and inhibit the lipopolysaccharide-mediated coagulation cascade (Chaby 2004). The cDNA nucleotide sequence encoding ALFs includes a 5' section encoding a hydrophobic signal peptide of approximately 25 amino acid residues, producing a nascent protein prior to signal cleavage of approximately 125 amino acids (Supungul et al. 2002). The mature protein consists of a single domain with three α -helices crowded against a four-strand β -sheet (Hoess et al. 1993). Two of the β -strands are linked by a disulfide bond to form an amphipathic loop rich in cationic amino acid side chains. Synthetic cyclic versions of this loop are functionally active against gram-negative bacteria including *Vibrio harveyi* (Imjongjirak et al. 2007) and *Pseudomonas aeruginosa* (Pan et al. 2007) and against gram-positive bacteria such as *Micrococcus luteus* (Imjongjirak et al. 2007). A recombinant ALF from the giant tiger prawn *Penaeus monodon* is highly effective against *Vibrio* species, including several that are pathogenic to shrimp (Somboonwiwat et al. 2005). Injection of double-stranded RNA corresponding to the mRNA encoding an anti-lipopolysaccharide factor from the Pacific white shrimp *Litopenaeus vannamei* into shrimp challenged with low doses of *Vibrio penaeicida* lead to knock-down of the ALF message and increased mortality (de la Vega et al. 2008). It is clear from this evidence that anti-lipopolysaccharide factors are potent antimicrobial agents in marine arthropods, with important implications in prevention of disease in aquacultured and farmed species.

A variety of ALF isoforms have been described in shrimp, including five (ALFPm1-5) in the giant tiger prawn *P. monodon* discovered during the course of expressed sequence tag analysis (Supungul et al. 2004; Tassanakajon et al. 2006). Two of these (ALFPm1 and 2) appear to be transcribed from a single gene locus and arise as a result of differential exon splicing (Tharntada et al. 2008). The remaining three ALF mRNAs are transcribed from a second gene locus, with ALFPm3 and 5 being essentially identical and ALFPm4 derived via differential exon splicing. In the present study, it is apparent that the two ALFs described in *H. americanus* are transcribed from separate gene loci and are not the result of differential splicing, since the nucleotide sequences, though closely related, show differences throughout their lengths.

Studies of the transcriptional response to pathogen exposure have shown increased abundance of anti-lipopolysaccharide factor mRNA in several crustacean species. In the Chinese shrimp *Fenneropenaeus chinensis*, infection with *Vibrio anquillarum* resulted in enhanced transcription of ALF mRNA in hemocytes within the first 24 hours (Liu et al. 2005). Anti-lipopolysaccharide factor mRNA was also abundant in gill tissue of *F. chinensis*, but not in hepatopancreas, in agreement with the present study in *H. americanus*. Injection of *Vibrio harveyi* into the giant tiger prawn *P. monodon* led to increased abundance of ALF mRNA in hemocytes, while mRNAs for other antimicrobial proteins including penaeidin and crustin decreased (Supungul et al. 2004; Somboonwiwat et al. 2006). Interestingly, treatment of Pacific white shrimp *Litopenaeus vannamei* with white spot syndrome virus resulted in upregulation of an ALF gene in hepatopancreas, suggesting that there may be some overlap between the antiviral response and the antibacterial response (Robalino et al. 2007).

We have shown in this study that one of the two anti-lipopolysaccharide factors identified in the American lobster, ALFH_a-1, is transcriptionally upregulated in response to bacterial challenge with *Vibrio fluvialis*, but the other, ALFH_a-2, is not (Figure 7). Messenger RNAs for both ALFH_a-1 and ALFH_a-2 are present in gill and hematopoietic tissue, but only ALFH_a-1 responds to infection. Gills of bacteria-infected crustaceans are known to accumulate hemocytes (Burnett et al. 2006), and thus it is not surprising that gills would exhibit high levels of mRNAs that may be derived from hemocytes. However, the observation that ALFH_a-2 transcription is not induced by *Vibrio* challenge, while ALFH_a-1 is upregulated, indicates some degree of ALF specificity in the immune response to *V. fluvialis*.

Structural differences between the ALFH_a-1 and ALFH_a-2 proteins may suggest a mechanism by which they are capable of selecting a specific target. The amphipathic loop that is thought to interact with lipopolysaccharides in bacterial cell walls (Kloczewiak et al. 1994) contains one additional cationic residue in ALFH_a-2 compared with ALFH_a-1, possibly supporting interaction with more highly acidic regions of its target. On the other hand, ALFH_a-1 contains a single pair of half-cysteines, compared to two pairs in ALFH_a-2, likely producing a different 3-dimensional conformation of the protein that may further dictate specificity of binding.

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1	CC CGT CAA GGC TCA CTC ACA TCT CCC TAC TAC GAC GAC ACC AGC GAG CAC TAC AGG ATG	59
1		<u>M</u> 1
60	CGT CAG TCT GTG TTA GTG AGT GTG TTG GTG GTG TCG CTG TTA GTG ACA ACC ATC ACG CCA	119
2	<u>R Q S V L V S V L V V S L L V T T I T P</u>	21
120	CAA TGC AAC GCC CAG GGA TGG GCG GCA GTA GCA GCA GCT GTC GCC TCT AAA GTG GTT GGG	179
22	<u>Q C N A</u> Q G W A A V A A A V A S K V V G	41
180	TTA TGG CAA AAC GGA CAT GTT GAC CTG CTT GAC CAC CCC TGC CGG TTC AGT GTC AAG CCC	239
42	L W Q N G H V D L L D H P C R F S V K P	61
240	ACC GTC AGA AGG TTT CAG CTG TAC TTC AAG GGG AGG ATG TGG TGC CCA GGC TGG ACA AGT	299
62	<u>T V R R</u> F Q L Y F K G R M W C	81
300	ATT AGG GGA GAA GCT AAG ACC CGC AGT CGT TCT GGT GTT GTT GGG AAA ACT ACC AGG GAT	359
82	I R G E A K T R S R S G V V G K T T R D	101
360	TTT GTC AAC AAG GCT TTC CAA GCT GGT CTT ATA ACC GAG AGG GAT GCC CAA CAA TGG CTG	419
102	F V N K A F Q A G L I T E R D A Q Q W L	121
420	AGT CAT TGA AGG CTT TAG TCA CCC TTT ACC TCT ACT ACT CAT CAG GAA TAT AAA CAA AAT	479
122	S H	123
480	ATA TCT GGA AAT TTT GTC GTT GTG TTT AAT ATA GCG GCC ATC TTT TTT GTG TTT GAA TGT	539
540	GGC GGC CAT CTT TGT GTG TTT GAA TGT GGC GGC CAT CTT GTT ATA TTG AAT GTG GTG GCC	599
600	ATC TTA AAT TTT TCC GAC TTT TGT TAT TGT GTA ATT TAA TGT TTG TCT TGG TAG AGA ATG	659
660	AAG TTT TCA ACA AAA GTA TAA AAA AAA AAA AAA AAA AAA AAA AAA A	708

Figure 1.

Nucleotide and amino acid sequence of anti-lipopolysaccharide factor cDNA ALFH α -1 from the lobster *Homarus americanus*, derived from the library clone identified by expressed sequence tag **CN853488**. The predicted amino acid sequence encoded by the open reading frame is indicated by single letter abbreviations, and the 25-amino-acid signal peptide predicted by SignalP 3.0 (Bendtsen et al. 2004) is underlined. Conserved cysteine residues are indicated by arrows, defining a predicted loop region containing the highly cationic KPXXRR motif (~~~~). (Accession No. **EU625516**).

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1   GA ATT CCC GGA AAA TGG TTC CCG CAG TTT ACA CTC TAC TGC TAA CAA GAA ACC ATC AGC   59
60  GAA CAC TAC AAG ATG CGG CAG TGT GTG TTG TTG GTG AGC GTG TTG GTG GTG GGA GTG TTG   119
1   M   R   Q   C   V   L   L   V   S   V   L   V   V   G   V   L
120 TTG GCA CCC TTC GCC CCC CAG TGC CAC GCC CAG GGC TGG GAA ACC TTA GTA GCG GGC GTC   179
17  L   A   P   F   A   P   Q   C   H   A   Q   G   W   E   T   L   V   A   G   V
180 TCT TCG CAA CTT GTC AGT CTG TGG CAT CAG GGA GAG CTG GAG CTG ATG GGT CAC TAC TGT   239
37  S   S   Q   L   V   S   L   W   H   Q   G   E   L   E   L   M   G   H   Y   C
240 AAC TTC CAG GTG AAA CCC AAG ATA AGA AGG TGG CAG CTG TAC TTC GTG GGG TCC ATG TGG   299
57  N   F   Q   V   K   P   K   I   R   R   W   Q   L   Y   F   V   G   S   M   W
300 TGC CCA GGC TGG ACC AAC ATC AGG GGG TCA GCC CAG ACC AGC AGC CGG TCT GGC GTG GTC   359
77  C   P   G   W   T   N   I   R   G   S   A   Q   T   S   S   R   S   G   V   V
360 GGG AAG ACT ACC ACT GAC TTC GTG AGG AAG GCC TTC AGA GCA GGT CTC ATC ACC CAG CAG   419
97  G   K   T   T   T   D   F   V   R   K   A   F   R   A   G   L   I   T   Q   Q
420 GAC GCC CAG GAG TGG CTG GAC AAC TGA GTG TCG TCC ACC ACA GTG GCT CAG TGT GGA GTC   479
117 D   A   Q   E   W   L   D   N
480 CTA AAG GTG TTA ACG GAT CAT CCC GAG AAG ATT AAA CCA TCA TCG CCA AAA TGT YTT CAT   539
540 GAC CAT TAT ACG TAT ATC TCT GTA CTG TAT CCG TAC TTG TGT ATA CAA CGA GTA ATA AAA   599
600 CAA CTT GTA TAT TTT YGA AAA AAA AAA AAA AAA AAA AAA AAA A                               642

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Figure 2.

Nucleotide and amino acid sequence of anti-lipopolysaccharide factor cDNA ALFH α -2 from the lobster *Homarus americanus*, derived from the library clone identified by expressed sequence tag **FC556430**. The predicted amino acid sequence encoded by the open reading frame is indicated by single letter abbreviations, and the 26-amino-acid signal peptide predicted by SignalP 3.0 is underlined. Conserved cysteine residues are indicated by arrows, defining a predicted loop region containing the highly cationic KPXXRR motif (~~~~) (Accession No. **EU625517**).

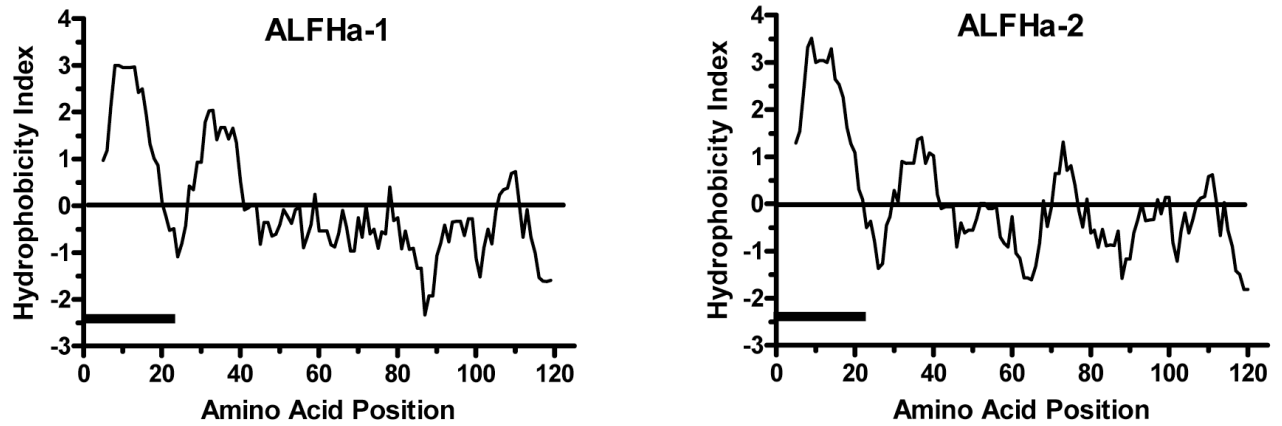


Figure 3. Hydrophobicity plots (Kyte et al. 1982) of amino acid sequences of anti-lipopolysaccharide factors ALFHα-1 and ALFHα-2 translated from cDNA sequences from *Homarus americanus*. The hydrophobicity index was calculated over a window of 9 residues by the ExPaSy ProtScale server (Swiss Institute of Bioinformatics). The signal peptide predicted by SignalP 3.0 (Bendtsen et al. 2004) is indicated by a solid bar.

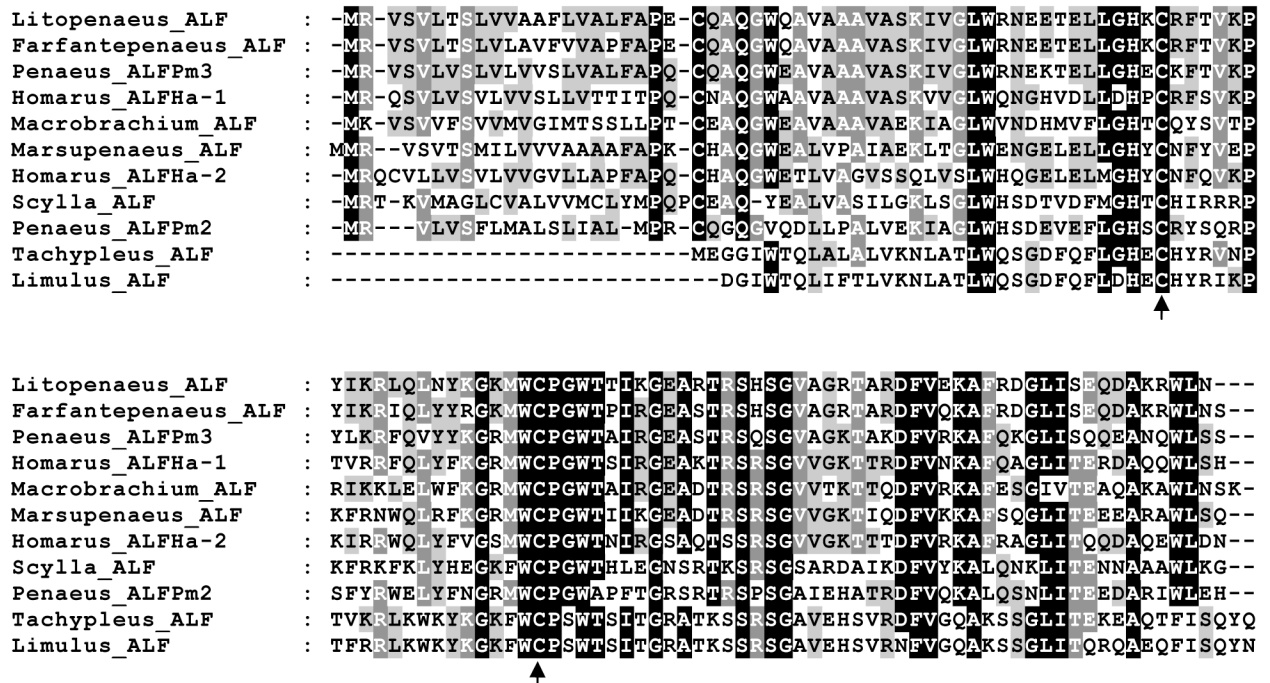


Figure 5.

Alignment of representative arthropod anti-lipopolysaccharide protein sequences, selected from chelicerate and decapod crustacean examples. Intensity of color reflects percentage identity calculated via MEGA4 (Tamura et al. 2007) and displayed via GeneDoc (Nicholas et al. 1997). ALFs presented include those from Pacific white shrimp *Litopenaeus vannamei* (DQ208703), pink shrimp *Farfantepenaeus paulsenis* (EF601051), giant tiger prawn *Penaeus monodon* (ALFPm2 EF523561, ALFPm3 EF523559), American lobster *Homarus americanus* (ALFH1 EU625516, ALFH2 EU625517, this study), freshwater shrimp *Macrobrachium olfersii* (EU289220), kuruma prawn *Marsupenaeus japonicus* (AB210110), mud crab *Scylla paramamosain* (EF207786), and mature ALF peptides in the chelicerates Japanese horseshoe crab *Tachypleus tridentatus* (AF227150) and Atlantic horseshoe crab *Limulus polyphemus* (P07086). Arrows indicate conserved cysteine residues participating in a disulfide bridge that forms a cationic loop in the protein.

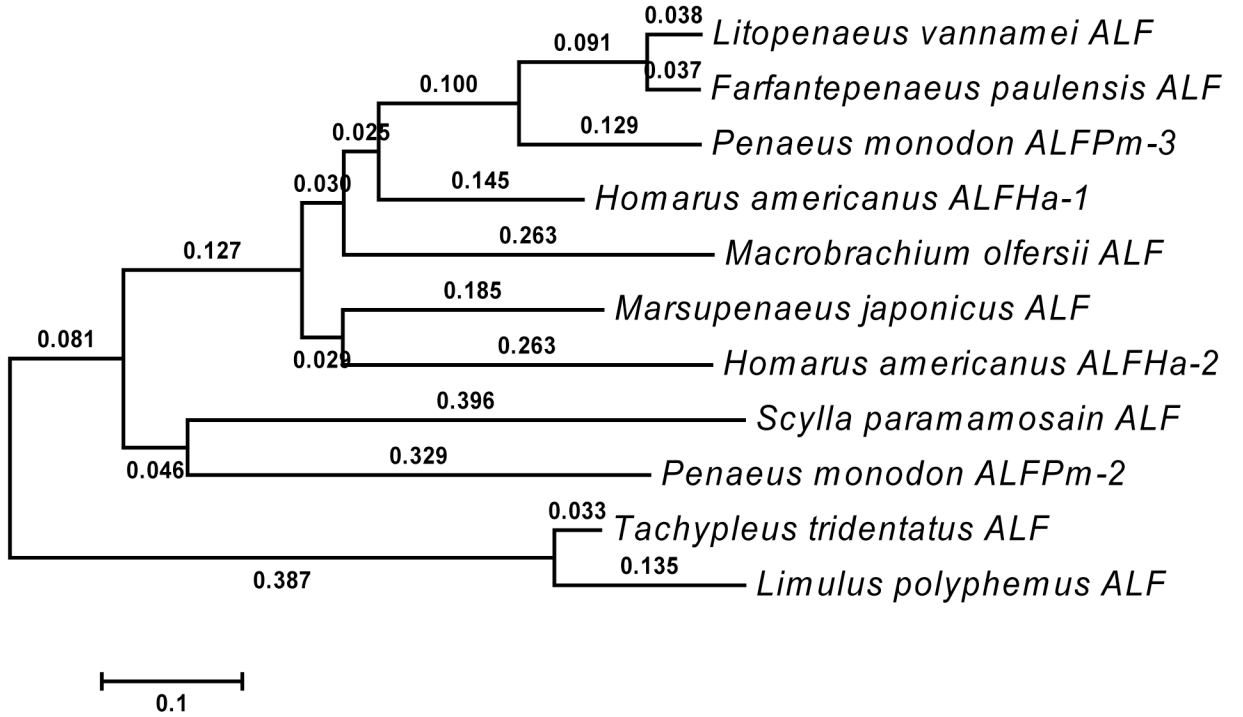


Figure 6.

Neighbor-joining tree of representative arthropod anti-lipopolysaccharide protein sequences based on the alignment in Figure 5, as calculated via MEGA4 (Tamura et al. 2007). Branch lengths were computed using the Poisson correction method and are expressed as number of amino acid substitutions per site. Accession numbers associated with the indicated species of origin are listed in the legend of Figure 5.

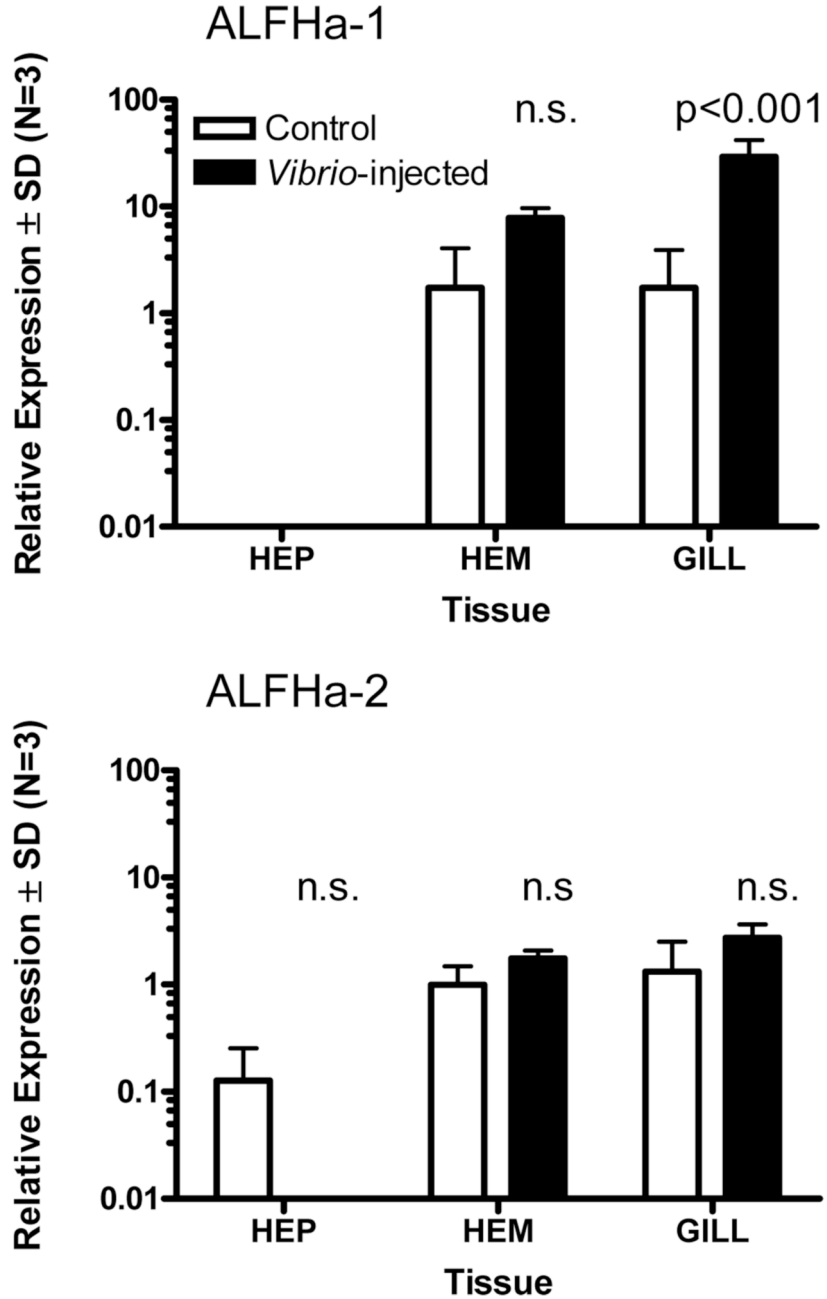


Figure 7. Relative expression of two anti-lipopolysaccharide factor mRNAs, ALFHα-1 and ALFHα-2, in hepatopancreas (HEP), hematopoietic tissue (HEM), and gill of *Homarus americanus* 24 hours following injection with tryptone soya broth medium (TSB) (light bars) or *Vibrio fluvialis* in TSB (filled bars), determined by quantitative PCR. A statistically significant effect of *Vibrio* injection was observed only for ALFHα-1 in gill tissue (ANOVA with Bonferroni posttests, N=3).