Molecular cloning of partial 14-3-3 genes in the marine sponge *Hymeniacidon perleve* and its role in differentiating infectious and non-infectious bacteria

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Sponges (phylum Porifera) are the phylogenetically oldest metazoa and highly efficient filter feeders. In the marine ecosystem, they are unconditionally exposed to environmental stresses. Understanding the sponge-bacteria interaction is hence of both ecological and biological significance. This study investigated the specific interaction between the sponge *Hymeniacidon perleve* and the non-infectious bacteria, *Escherichia coli* and infectious bacteria, *Vibrio* spp. by measuring the 14-3-3 mRNA expression of *H. perleve*. Three partial cDNAs of 14-3-3 proteins and partial 18S RNA in *H. perleve* were cloned and sequenced. Using Reverse-transcription real-time PCR, the 14-3-3 mRNA expression of *H. perleve* was examined when exposed to three common bacteria in aquatic water—*E. coli* and two *Vibrio* spp. for different time and dosages. *H. perleve* could efficiently remove *E. coli* from the water column without self-infection; however *Vibrio* at higher dosages infected *H. perleve*. When *H. perleve* was exposed to *E. coli* (1.1×10^7 CFU mL⁻¹), *V. anguillarum* II (1.2×10^6 CFU mL⁻¹) and *V. alginolyticus* (3.6×10^5 CFU mL⁻¹) for 6 h, the 14-3-3 mRNA expression in the *V. anguillarum* II and *V. alginolyticus* groups was down-regulated by 2.67- and 2.36-fold, respectively. The 14-3-3 mRNA expression in the *E. coli* group was not significantly different. However, no clear trend was observed on the 14-3-3 transcript levels of *H. perleve* in response to different doses of *V. anguillarum* II for different time. The results demonstrated that infectious bacteria can be discriminated by 14-3-3 mRNA expression of sponge *H. perleve*.

marine sponge *Hymeniacidon perleve*, 14-3-3 mRNA, RT-PCR, infectious bacteria

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Sponges (Porifera) are the oldest metazoan phylum and still exist today [1,2]. As sessile filter feeders, sponges have the ability to filter large volumes of water ($0.002\text{--}0.84$ mL s⁻¹ cm⁻³ of sponge tissue) through their aquiferous canal system to extract edible material [3–5], including bacteria, with about

96% retention efficiency [6]. Therefore, these animals are constantly exposed to many different microorganisms in seawater, such as bacteria [7] and viruses [8]. To face threats from different pathogenic bacteria in seawater, sponges have evolved with immune/defense mechanisms against these invaders over about 580 million years of their evolution [9]. Meanwhile sponges synthesize a huge number of bioactive

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secondary metabolites that show anti-bacterial activity [10–13]. Sponges are also reported to possess efficient humoral and cellular defense/immune mechanisms similar to those found in humans [14].

However, the precise relationship/interactions between sponge and microorganisms remain to be clarified for different types of microorganisms. Metchnikoff [15] firstly demonstrated that sponges eliminated bacteria by means of phagocytosis through their archeocytes. Wilkinson et al. [16] illustrated that marine sponges could discriminate between food bacteria and their bacterial symbionts. In the past 10 years, Perović-Ottstadt and colleagues [17] have done excellent work to document molecular responses to bacteria and fungi infection using the demosponges *Suberites domuncula* as the model system. For example, *S. domuncula* recognizes fungi through its receptor, the (1-3)- β -D-glucan-binding protein, on the cell surface. With respect to bacteria, the lectin of *S. domuncula* displays antibacterial activity against both Gram-negative and Gram-positive bacteria [18]. Thakur et al. [19] further reported that *S. domuncula* responded to Gram-positive bacteria with a rapid activation of endocytosis and increased synthesis of lysozyme. For Gram-negative bacteria, Bohm et al. [20] reported molecular biomarkers of sponge upon bacterial infection. p38 protein kinase and JNK protein kinase of the mitogen-activated protein kinase (MAPK) pathway were activated (phosphorylated) after the binding of lipopolysaccharide (LPS) to *S. domuncula* cells [20]. Wiens et al. [21] demonstrated that the receptor (LPS-interacting protein) on the surface of *S. domuncula* recognized Gram-negative bacteria and interacted with downstream molecules (MyD88 and macrophage-expressed protein). To the best of our knowledge, existing studies on sponges responding to bacterial infection used model inducer compounds, such as (1-3)- β -D-glucan, peptidoglycan (PPG) and LPS, rather than directly using live bacteria. Furthermore, whether sponges have the ability to differentiate between infectious and non-infectious bacteria and how this occurs, remain unknown. It would be fundamentally important because sponges do not have filtering selectivity of bacteria types.

The 14-3-3 protein is a family of ~30 kD highly conserved proteins within all eukaryotic cells [22]. This family of proteins, which includes 7 isotypes in human cells and up to 15 in plants [23], plays a critical role in many cellular processes such as signal transduction, cell-cycle control, apoptosis, viral and bacterial pathogenesis [24–26]. Through the specific phosphorylation interaction with its binding proteins, 14-3-3 proteins may respond to environment stresses at multiple levels [26,27], in particularly the defense response to viral and bacterial pathogen attack in plants [28–30]. In mammalian systems, 14-3-3 proteins block JNK and p38 activation (phosphorylation) [31,32]. Bohm et al. [20] found that the phosphorylation of the MAP kinase p38 and JNK in sponge *S. domuncula* was strongly increased after bacterial infection. In addition, Wiens et al. [33]

reported that PCB 118 (2,3,4,4,5-pentachlorobiphenyl) induced the expression of 14-3-3 proteins in the marine sponge *Geodia cydonium*. Therefore, it may be logical to investigate if 14-3-3 proteins in sponges can be used as a biomarker to study the sponge-bacteria interaction.

The marine sponge, *Hymeniacidon perleve*, is a common intertidal demosponge species living on the rocky shores of Chinese Yellow Sea around Dalian, which was heavily polluted by microbial pathogen (Reports on Marine Pollution in China, 2006–2009). *E. coli* from human and animal waste pollute the coastal environment off the growing city of Dalian. In China, the concentrations of *E. coli* in seawater and some commercial bivalve products are more than that of allowable standard [34]. Fu et al. [34] has demonstrated that *E. coli* did not infect sponge and were retained efficiently by *H. perleve*. Chinese aquaculture products are approximately 70% of the world's products [35]. *Vibrio* spp. are common and major pathogens in Chinese marine aquaculture waters [34]. Fu et al. [34] also has demonstrated that *H. perleve* only can keep *V. anguillarum* II with lower initial concentration growth. To test the hypothesis that sponges may have the ability to differentiate between infectious and non-infectious bacteria by its molecular responses, we used *H. perleve* as a model sponge. In this study, we isolated and cloned three partial cDNAs of 14-3-3 proteins from *H. perleve*; used the 14-3-3 mRNA expression as the specific molecular biomarker to study the interactions between *H. perleve* and the two types of bacteria, *E. coli* as non-infectious bacteria, *V. anguillarum* II and *V. alginolyticus* as infectious bacteria.

1 Materials and methods

1.1 Chemicals, bacterial strains and growth conditions

All chemicals used were of analytical grade. LB medium, 2216E medium, bacteriological agar and TCBS agar were purchased and prepared as previously described [34]. *Escherichia coli* strain AS 1.1017 was obtained from Dalian Polytechnic University and was maintained in LB agar medium at 4°C. *Vibrio anguillarum* II and *Vibrio alginolyticus* which were respectively obtained from Liaoning Institute of Aquaculture Science and Environment and Dalian Ocean University, were maintained in 2216E medium at 4°C. Before tests, a 250-mL flask containing 100 mL LB medium was inoculated with a single colony of *E. coli* and placed on a rotary shaker at 200 r min⁻¹ and 37°C for 17 h. For *V. anguillarum* II and *V. alginolyticus*, all the culture conditions were the same as *E. coli*, except that 2216E medium and 28°C were used for the enrichment cultures.

1.2 Sponge and seawater

The marine sponge, *Hymeniacidon perleve*, was collected from the rocky shores of Chinese Yellow Sea around Dalian.

The sampling, maintenance and acclimation of the sponge specimens before experiments were described previously [34]. The sterilized natural seawater (SNSW) was used and prepared as per [34].

1.3 Interaction between sponge *H. perleve* and bacteria (*E. coli*, *V. anguillarum* II and *V. alginolyticus*)

In the test of removal of *E. coli* and *V. anguillarum* II by *H. perleve* respectively, we adopted the experimental procedure of the previous study [34]. In the test of removal of *V. alginolyticus* by *H. perleve*, we only substituted *V. anguillarum* II with *V. alginolyticus*, otherwise the procedure was the same as previously [34].

In tests to validate if *E. coli* infects *H. perleve*, 500 μL *E. coli* culture grown in LB medium for 17 h was added to 6 flasks containing 200 mL SNSW. Sponge clones of approximately 1.7 g were put into 5 flasks; one flask without sponge clones was used as the control. The 6 flasks were together placed on a rotary shaker at 100 r min^{-1} to keep water in constant movement. In 1.5 h, one flask of treatment group and the control flask were taken away. Aliquots of seawater (100 μL) were taken to dilute by 10-fold and plated on the LB agar media in triplicate. The control group flask was placed back on the rotary shaker. Plates were incubated for 24 h at 37°C for CFU counting. Meanwhile the sponges in the flask which was taken away were taken to briefly wash twice by SNSW and homogenized with SNSW to obtain a final volume of 15 mL. The number of *E. coli* in sponge clones was detected according to GB 17378.4-1998. In the same procedures, samples were taken and analyzed at 3, 6, 9 and 24 h.

In the test of *V. anguillarum* II and *V. alginolyticus* infecting respectively *H. perleve*, 200 μL *V. anguillarum* II cultures and 200 μL *V. alginolyticus* cultures grown in 2216E medium for 17 h were respectively added to 6 flasks containing 200 mL SNSW. The other test conditions were the same as the tests on *E. coli*. To count *V. anguillarum* II and *V. alginolyticus* in SNSW, the seawater samples were 10-fold diluted and plated on 2216E agar media in 3 replicates for 2 d at 28°C . To count *V. anguillarum* II and *V. alginolyticus* in sponge clones, the sponge tissue was homogenized with SNSW to obtain a final volume of 15 mL. The resultant solution was plated on TCBS media [34] in 3 replicates for 2 d at 28°C for counting colonies.

A sponge clone of 1.59 g was taken to be washed and homogenized with SNSW to also obtain a final volume of 15 mL to detect *E. coli* and *Vibrio* spp. concentration, which was regarded as the concentrations of *E. coli* and *Vibrio* spp. in sponge tissue at 0 h. The analysis methods are the same as that described above.

1.4 Extraction of Total RNA from *H. perleve* tissues and cells

Total RNA which was used for cloning 14-3-3 mRNA of *H.*

perleve was extracted from the dissociated mixed-cell population prepared with the protocol using TRIzol Reagent (Invitrogen) [36], according to [37]. Total RNA for all other studies was extracted from liquid nitrogen-pulverized sponge tissue samples with TRIzol Reagent (Invitrogen), also according to [37]. Total RNA was treated with RNase-free DNase I (TaKaRa) to remove DNA contamination according to the manufacturer's protocol. The concentration of RNA samples was determined on GeneQuant pro UV/Vis Spectrophotometer (Amersham Pharmacia Biotech). The quality and integrity of RNA samples was checked by agarose gel electrophoresis. The RNA was stored at -80°C .

1.5 Cloning and sequencing three partial 14-3-3 mRNA from *H. perleve*

The purified Total RNA derived from the dissociated mixed-cell population was reverse transcribed into cDNA on a Rotor-Gene 2000 Real Time Cycler (Corbett Research, Australia) at 30°C for 5 min, then 42°C for 40 min, finally RNase inactivation at 99°C for 5 min. The reactor mixture included 1 μL 10 $\times$ RNA PCR buffer, 2 μL 25 mmol L^{-1} MgCl_2 , 1 μL 10 mmol L^{-1} dNTP mixture, 0.5 μL Oligo dT-Adaptor Primer (TaKaRa), 0.25 μL 40 U μL^{-1} RNase inhibitor, 2 μL Total RNA as template and 0.5 μL 100–200 U μL^{-1} AMV Reverse Transcriptase (TaKaRa), and then adjusted to 10 μL with RNase Free H_2O . The cDNA was stored at -20°C .

The procedures for amplification of partial 14-3-3 cDNA of *H. perleve* were carried out using a Rotor-Gene 2000 Real Time Cycler (Corbett Research) at an initial denaturation at 94°C for 3 min, followed by 35 amplification cycles at 94°C for 50 s, 54°C for 40 s and 72°C for 50 s. The reaction mixture included 4 μL 10 $\times$ PCR buffer, 3 μL 25 mmol L^{-1} MgCl_2 , 0.5 μL 20 $\mu\text{mol L}^{-1}$ Forward Primer and Reverse Primer respectively, 0.25 μL LA *Taq* (5 U μL^{-1}) (TaKaRa) in 10 μL reverse transcript reaction, then adjusted to 50 μL with RNase free H_2O . Primers (F_1/R_1) were designed against the highly conserved region (forward 5'-GTGTGG-CCTACAAGAACGTG-3' against aa 50–56: SVAYKNV; reverse 5'-TGCATGATGAGAGTGGAGTC-3' against aa 218–224: DSTLIMQ) within the Demospongiae *Geodia cydonium* 14-3-3 (GenBank accession No. Y15900). The PCR samples were sent to TaKaRa Company to sequence.

For 3'-RACE, the poly(A)⁺ mRNA was reverse transcribed as described above, Oligo dT-Adaptor Primer in conjunction with the 3'-end vector-specific primer M13M14. According to sequence results in this study, the forward primer (F_2) (5'-TCAGTAGCTTACAAGAATG-3') was designed against the highly conserved aa region (SVAYKNV), the reverse primer was M13M14 (5'-GTT-TTCCAGTCACGAC-3'). The PCR conditions were as described above, except annealing temperature at 42°C for 40 s. PCR samples were also sent to TaKaRa Company to sequence.

1.6 Cloning and sequencing of partial 18S RNA from *H. perleve*

The purified Total RNA was reverse transcribed on the GeneAmp PCR systems 2700 (Applied Biosystems) first at 30°C for 10 min, then 42°C for 15 min, finally RNase inactivation at 99°C for 5 min. The reactor mixture, except 1 μL Total RNA as template and 0.5 μL Random 9mers (50 $\mu\text{mol L}^{-1}$) representing Oligo dT-Adaptor Primer, was described above.

H. perleve partial 18S RNA was obtained by PCR. Primers (F_3/R_3) were designed on the consensus conserved nucleotides region (forward primer 5'-TTGACGGAAGGG-CACCA-3' and reverse primer 5'-CAAAGGGCAGGGAC-GTAATC-3') of 18S RNA in several sponge species (GenBank accession Nos. AJ620112, AJ627184, AY348885, AY737639). The PCR was performed on the GeneAmp PCR systems 2700 (Applied Biosystems) at an initial denaturation at 94°C for 2 min, then 35 amplification cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 60 s. The reaction mixture included 10 μL 5 $\times$ PCR Buffer, 0.5 μL 20 $\mu\text{mol L}^{-1}$ Forward Primer and Reverse Primer respectively, 0.25 μL *Ex Taq* HS (5 U μL^{-1}) (TaKaRa) in 10 μL reverse transcript reaction, then adjusted to 50 μL with RNase free H_2O . PCR sample were sent to TaKaRa Company to sequence.

1.7 Sequence analysis

The sequences were analyzed using BLAST (<http://www.ncbi.nih.gov/blast>). Multiple alignments were performed using Clustal X (1.8). Phylogenetic analysis was constructed on the basis of aa sequence alignments with Clustal X (1.8) and the TreeView software. A motif/pattern search was performed using PROSITE (<http://www.Expasy.org/prosite/>).

1.8 Exposure of *H. perleve* to bacterial pathogens (*E. coli*, *V. anguillarum* II and *V. alginolyticus*)

Vibrio spp. are common and major pathogens in Chinese marine aquaculture waters. In four 500 mL flasks, 500 μL *E. coli* culture, 200 μL cultures of *V. anguillarum* II and *V. alginolyticus* each, were respectively added to 3 flasks containing 200 mL SNSW, one remaining flask without bacteria as control. The final concentration of *E. coli*, *V. anguillarum* II, and *V. alginolyticus* were approximately 1.1×10^7 , 1.2×10^6 and 3.6×10^5 CFU mL^{-1} , respectively. Sponge clones of approximate 1.5 g were separately put into four flasks. The 4 flasks were together placed on a rotary shaker at 100 r min^{-1} to keep water in constant movement. After 6 h, aliquots of seawater (100 μL) were taken from each flask for counting CFU [34]. Meanwhile the sponges were taken and placed respectively on 4 sterilized plates. Three small clones approximately 0.3 g were respectively cut from the 4 sponges for extracting Total RNA to detect 14-3-3 mRNA expression.

1.9 Exposure of *H. perleve* to *V. anguillarum* II at different doses and times

In the tests of exposure of *H. perleve* to *V. anguillarum* II with varying inoculum density (dose response) and varying time period (time course), the 14-3-3 mRNA expression of *H. perleve* was detected. In twelve 500-mL flasks containing 200 mL SNSW each, the flasks in each groups, three flasks as one group, were respectively added 0, 5, 20 and 200 μL *V. anguillarum* II cultures to obtain a density of 0, 1.1×10^4 , 3.6×10^4 and 5.6×10^5 CFU mL^{-1} . The group without bacteria addition was the control. The 12 flasks were respectively put into sponge clones about 1.5 g and then were together placed on a rotary shaker at 100 r min^{-1} to keep water in constant movement. In 1.5, 3 and 6 h, one flask of each group was taken away. Aliquots of seawater (100 μL) for the CFU counting and the sponge clones for total RNA extraction were sampled as above.

1.10 Reverse transcript real-time PCR

Partial 14-3-3 cDNA of *H. perleve*, containing 443 bp (GenBank accession No. DQ218450), was amplified by PCR on the GeneAmp PCR systems 2700 (Applied Biosystems) at an initial denaturation at 94°C for 3 min, then 35 amplification cycles at 94°C for 50 s, 56°C for 35 s and 72°C for 40 s, using reversed transcript mRNA as template (the condition was the same as above). The new primers (F_4/R_4) were designed against the un-conserved region (forward, 5'-ATGCCAAGAGGTCATTGATC-3' against aa 64–70: ICQEVID; and reverse, 5'-CTTGCTCAGCGTC-AACGTC-3' against aa 206–212: DVDAEQE) within *H. perleve* 14-3-3 (GenBank accession No. DQ218450). *H. perleve* partial 18S ribosomal RNA of 451 bp (GenBank accession No. DQ206445) was amplified by PCR as describe above. The amplified partial 18S RNA and 14-3-3 cDNA were respectively T-A cloned into the expression plasmid pT7Blue (Novagen), and transfected to JM109 *E. coli* strain, selected on TB/Amp agar plates and verified by sequencing. The plasmids were used for *in vitro* transcription of the corresponding RNA by TaKaRa Company. After isolation and purification from the DNase RNase-free treated transcription solutions, the sense RNA transcripts were *in vitro*-transcribed. The RNA was quantitated accurately on GeneQuant Pro (Amersham Pharmacia Biotech) to construct standard curve for 14-3-3 mRNA as target gene and for 18S RNA as housekeeping gene.

Reverse transcription was then performed on the GeneAmp PCR systems 2700 (Applied Biosystems) as described above, 2 μL total RNA as template and specific primer (5'-GTCAGCAGTCCACAAAGTCAGG-3') for 14-3-3 mRNA evaluation, 2 μL of 10^{-3} -diluted total RNA and specific primer (5'-CAATCCCCAGCAGCAGACAA-3') for 18S RNA. For Real time PCR, SYBR[®] RT-PCR Kit (Code: DRR041A) (TaKaRa) was used. Real time PCR was carried

out using a Rotor-Gene 2000 Real Time Cycler (Corbett Research) at an initial denaturation at 94°C for 20 s, then 35 amplification cycles at 94°C for 10 s and 60°C for 25 s. The specific primers (F₅/R₅) for 14-3-3 were designed (forward 5'-TGCATATACAGATGCCACTAG-3' against aa 123–129: SAYTDAT; reverse 5'-GTCAGCAGTCCACAAAGTCA-GG-3' against aa 199–206: NLTLWTAD) within *H. perleve* 14-3-3 (GenBank accession No. DQ218450). The specific primers (F₆/R₆) for 18S RNA were designed (forward: 5'-AATTCCGATAACGAACGAGACC-3'; reverse: 5'-CA-ATCCCCAGCACGACAA-3') within *H. perleve* 18S RNA (GenBank accession No. DQ206445). The reaction mixture contained 12.5 µL 2× Concentration SYBR Premix *Ex Taq* (including SYBR Green PCR Buffer, TaKaRa *Ex Taq* HS, dNTP Mixture, Mg²⁺, SYBR Green I) (TaKaRa), 0.5 µL 10 µmol L⁻¹ each primer for 14-3-3 or for 18S RNA, 2 µL of reverse transcribed product as template, then adjusted to 25 µL with RNase free H₂O. The PCR reactions were carried out in duplicates.

A standard curve was respectively generated for each RT-PCR reaction (18S RNA and 14-3-3 mRNA) by known copy numbers (diluted to 10³–10⁹ for 18S RNA and 10⁴–10⁹ copies for 14-3-3 mRNA) of reverse transcribed product *in vitro*. Samples of unknown concentration were plotted onto the standard curve to calculate their concentration according to Operator's Manual on Rotor-Gene 2000 Real Time Cycler (Corbett Research). Reaction specificity for 18S RNA and 14-3-3 mRNA was respectively determined from the melt curve of the PCR product. All runs included a no template control, a no AMV reverse transcriptase control and a no primer control, and no PCR product was detected there.

1.11 Nucleotide and amino acid sequence accession numbers

The nucleotide sequence for *H. perleve* 18S ribosomal RNA was deposited in GenBank (DQ206445). The amino acid sequences for *H. perleve* were designated as HP 14-3-3 A, HP 14-3-3 B and HP 14-3-3 H (GenBank accession Nos. DQ218448, DQ218449, DQ218450).

1.12 Statistical analysis

Triplicates were used in each treatment and the values throughout this article were presented as mean±standard deviation (SD). An independent Student's *t*-test was used to determine the statistical significance of differences.

2 Results

2.1 Interaction between bacteria (*E. coli*, *V. anguillarum* II and *V. alginolyticus*) and sponge *H. perleve*

For the tests of removing *E. coli*, *V. anguillarum* II and *V.*

alginolyticus in SNSW by *H. perleve*, the time courses of bacteria concentration are shown in Figure 1. The initial concentration of *E. coli*, *V. anguillarum* II and *V. alginolyticus* was 8.3×10⁶, 6.3×10⁵ [34] and 3.9×10⁵ CFU mL⁻¹ (Figure 1), respectively.

During the tests, *E. coli* concentrations in the treatments declined markedly with time (Figure 1). At 9 h, the average *E. coli* concentration was about 8.6% of the initial concentration. Accordingly, an average 9.13×10⁷ bacterial cell h⁻¹ g⁻¹-fresh sponge was retained by the sponge [34]. The *E. coli* concentrations in the control flask have no significant difference with time (*P*<0.02). Therefore *H. perleve* has an extremely high ability of removing *E. coli* in SNSW.

In the course of the tests, typical bacterial growth curves were observed in both the control and treatment cultures of *V. anguillarum* II and *V. alginolyticus* (Figure 1). At 10.5 h, *Vibrio* spp. in the control was still in exponential phase, with bacteria concentration of 1.3×10⁷ CFU mL⁻¹ for *V. anguillarum* II and 1.1×10⁷ CFU mL⁻¹ for *V. alginolyticus*; however, *Vibrio* spp. in the treatment entered stationary phase at 9 h, reaching 4.5×10⁶ CFU mL⁻¹ for *V. anguillarum* II and 4.6×10⁶ CFU mL⁻¹ for *V. alginolyticus*. It was clear that *H. perleve*, to some extent, has ability of controlling *Vibrio* spp. growth [34]. However, it is unknown how *H. perleve* interacts with bacteria (*E. coli* and *Vibrio* spp.) in seawater.

To validate whether bacteria have the ability of infecting *H. perleve*, sponge clones were respectively exposed to *E. coli*, *V. anguillarum* II and *V. alginolyticus* cultures for 1.5, 3, 6, 9 and 24 h. The time courses of *E. coli*, *V. anguillarum* II and *V. alginolyticus* concentration in SNSW and in sponge clones are shown in Table 1. The weight of fresh sponge clones exposed to *E. coli* for 1.5, 3, 6, 9 and 24 h was 1.38, 1.02, 1.38, 1.17 and 1.32 g, respectively. The weight of fresh sponge clones exposed to *V. anguillarum* II and *V. alginolyticus* was 1.85, 1.91, 2.37, 2.26, 1.93 and 1.83, 1.57, 1.84, 1.56, 1.73 g, respectively.

Figure 1 Removal kinetics of *E. coli* AS 1.1017 strain, *V. anguillarum* II and *V. alginolyticus* in sterile natural seawater (SNSW) supplemented with cultured *E. coli*, *V. anguillarum* II and *V. alginolyticus* over 10.5 h. The error bars represent the standard deviation of three counting replicates.

For the tests of *E. coli* infecting *H. perleve*, *E. coli* concentration in the control fluctuated among samples, but no significant change was observed ($P < 0.05$) (Table 1). *E. coli* concentrations in both SNSW and sponge clones in the treatments decreased markedly with time. These results demonstrated that *H. perleve* did not get infected by *E. coli*, but digested efficiently the *E. coli* retained from SNSW [34]. For the tests of *V. anguillarum* II and *V. alginolyticus* infecting *H. perleve*, the *Vibrio* spp. in the treatments quickly grew with time. This fitted in with the characteristics of *Vibrio* spp. In the treatment flasks, the concentration of *Vibrio* spp. in SNSW at 1.5 and 3 h was lower than initial concentration, respectively. The reason lies in the filtration of *H. perleve*. At 6 h, *Vibrio* spp. in SNSW in the treatments were respectively 3.1-fold for *V. anguillarum* II and 4.7-fold for *V. alginolyticus* higher than initial concentration, and increased over time. The concentrations of *Vibrio* spp. in sponge only at 1.5 h were slightly lower than initial concentration. From 3 h onward, *Vibrio* spp. in sponge increased markedly with time.

2.2 Clone and sequence analysis of 18S RNA and 3 partial 14-3-3 cDNAs of *H. perleve*

To obtain nucleotide sequences of partial 18S RNA and 14-3-3 mRNA from *H. perleve*, the total RNA purified was reverse transcribed and was amplified through PCR to sequence. A nucleotide sequence of 451 bp was obtained for 18S RNA from *H. perleve* tissue using PCR amplification with specific primers (F_3/R_3). The results from BLAST nucleotides sequence analysis show high similarity to other sponges: *Halichondria melanodocia* 18S RNA (AY 737639) (99% identities), *Suberites ficus* (AJ 627184) (99% identities), *Suberites domuncula* (AJ 620112) (99% identities). The fragment was termed *Hymeniacidon perleve* 18S ribosomal RNA (GenBank accession No. DQ206445).

Three partial sequences of 14-3-3 cDNA from the dissociated mixed-cell population of *H. perleve* were obtained

using PCR amplification with primers (F_1/R_1). Three fragments with 506, 518 and 583 bp, encoded partial sequences of three new 14-3-3 proteins from sponge *H. perleve*. According to three sequences obtained, the primer (F_2) was designed to amplify by PCR for 3'-RACE with the 3'-end vector-specific primer (M13M14). The three cDNA fragments was respectively comprised of 711 nucleotides, 749 nucleotides and 786 nucleotides, including the poly(A) tail. The deduced amino acids sequences (Figure 2), termed respectively HP 14-3-3 A, HP 4-3-3 B and HP 14-3-3 H (GenBank accession Nos. DQ218448, DQ218449, DQ218450 respectively), comprise 198 amino acids residues with a stop codon (TAA) at nucleotide position 592; 204 amino acids residues with a stop codon (TAA) at nucleotide position 610; and 219 amino acids residues with a stop codon (TAG) at nucleotide position 657. Through BLAST analysis, the three polypeptides HP 14-3-3 A, HP 14-3-3 B and HP 14-3-3 H, share respectively significant identity to *G. cydonium* 14-3-3 protein (CAA75860) of 56%, 62% and 61%.

Further analysis of motif search reveals the presence of several motifs, patterns, and signatures in the three 14-3-3 polypeptides: Signature 1 in HP14-3-3 H, [RA]-N-L-[LIV]-S-[VG]-[GA]-Y-[KN]-N-[IVA] (Prosite database PS000796) at the N-terminus; Signature 2 in all three 14-3-3 polypeptides, Y-K-[DE]-[SG]-T-L-I-[IML]-Q-L-[LF]-[RHC]-D-N-[LF]-T-[LS]-W-[TANS]-[SAD](PS000797) at the C-terminus. The two regions, particular signature 2 as nuclear export signal (NES) motif, are highly conserved in all 14-3-3 protein family [28,38].

A motif/pattern search (PROSITE) revealed protein kinase C phosphorylation sites ([ST]-x-[RK]; PS00005), Casein kinase II phosphorylation sites ([ST]-x(2)-[DE]; PS00006), a Tyrosine kinase phosphorylation site ([RK]-x(2)-[DE]-x(3)-Y or [RK]-x(3)-[DE]-x(2)-Y; PS00007) in all three proteins; a cAMP- and cGMP-dependent protein kinase phosphorylation site ([RK]-(2)-X-[ST]; PS00004) in *H. perleve*-A and *H. perleve*-H; a cell attachment sequence (RGD; PS00016).

Table 1 Growth kinetics of bacteria (*E. coli*, *V. anguillarum* II and *V. alginolyticus*) in the seawater and the sponges when infecting sponge *H. perleve*

Concentration of bacteria	Exp group	Sample time (h)					
		0	1.5	3	6	9	24
<i>E. coli</i> in water (10^4 CFU mL ⁻¹)	Cont	827	947	750	1060	677	757
	Treat	827	756	455	132	71	24
<i>E. coli</i> in sponge (cells g ⁻¹)		31.1	35.9	33.8	25.0	29.5	7.2
<i>V. anguillarum</i> II in water (10^4 CFU mL ⁻¹)	Cont	63	55	96	166	370	1069
	Treat	63	39	36	196	448	1170
<i>V. anguillarum</i> II in sponge (10^4 cells g ⁻¹)		12.3	8.1	39.3	82.3	159.3	352.3
<i>V. alginolyticus</i> in water (10^4 CFU mL ⁻¹)	Cont	39	43	66	157	396	1106
	Treat	39	28	33	185	459	1249
<i>V. alginolyticus</i> in sponge (10^4 cells g ⁻¹)		12.3	12.2	33.4	138.6	288.5	346.8

Figure 2 The deduced amino acid sequence from the *Hymeniacidon perleve* cDNA, termed respectively HP 14-3-3 A, HP 14-3-3 B and HP 14-3-3 H (GenBank accession Nos. DQ218448, DQ218449, DQ218450 respectively).

The three polypeptides were aligned with 6 isotypes of human cells and 2 isotypes of yeast (Figure 2). A rooted phylogenetic tree was constructed to show sequence relations of 14-3-3 polypeptides (Figure 3). The three polypeptides and the Demosponge *G. cydonium* 14-3-3 protein form the basis of the metazoan 14-3-3 proteins group, which share the highest sequence similarity with mammals. Analysis of other polypeptides showed that sponge genes displayed predominantly the closest similarity/homology to corresponding human genes [39].

2.3 Discrimination of infectious bacteria (*Vibrio* spp.) and non-infectious bacteria (*E. coli*) by 14-3-3 mRNA differential expression in *H. perleve*

The 14-3-3 mRNA expression was investigated when sponge *H. perleve* was exposed to infectious bacteria (*Vibrio* spp.) and non-infectious bacteria (*E. coli*). 14-3-3 mRNA expression levels were normalized to the house-keeping gene 18S RNA determined by RT-PCR. When *H. perleve*

was exposed to *E. coli* (1.0×10^7 CFU mL⁻¹), *V. anguillarum* II (1.2×10^6 CFU mL⁻¹) and *V. alginolyticus* (3.6×10^5 CFU mL⁻¹) for 6 h, the 14-3-3 mRNA expression is shown in Figure 4. The 14-3-3 mRNA expression of *H. perleve* in the *E. coli* group was almost the same as that of the control group. The 14-3-3 mRNA expression in the *V. anguillarum* II and in the *V. alginolyticus* groups were down-regulated by 2.67- and 2.36-fold lower, respectively, when compared with the control group. However, the 14-3-3 mRNA expression level was not statistically different between the *V. anguillarum* II group and *V. alginolyticus* group ($P < 0.05$). The results show for the first time that differential expression in 14-3-3 mRNA was induced by infectious (*Vibrio* spp.) and non-infectious bacteria (*E. coli*).

2.4 Dose and time-dependent responses of 14-3-3 mRNA expression of *H. perleve* to *V. anguillarum* II

When *H. perleve* was exposed to *V. anguillarum* II at different concentrations and time periods, the 14-3-3 mRNA

Figure 3 The rooted phylogenetic tree was constructed from the three sequences together with 14-3-3 protein from other organisms. (1) Sponge: *Hymeniacidon.p-H* (DQ218450), *Hymeniacidon.p-A* (DQ218448), *Hymeniacidon.p-B* (DQ218449), *Geodia.c* (CAA75860); (2) Human: *Homo-Beta* (2BQ0A), *Homo-Eta* (Q04917), *Homo-Gamma* (P61981), *Homo-Sigma* (AAC52030), *Homo-Theta* (P27348), *Homo-Zeta* (P63104); (3) Yeast: *Yeast-BMH1* (P29311), *Yeast-BMH2* (P34730); (4) Evolution: *Caenorhabditis elegans* (P41932), *Candida albicans* (O42766), *Chlamydomonas reinhardtii* (P52908), *Dictyostelium discoideum* (P54632), *Drosophila melanogaster* (P92177), *Echinococcus multilocularis* (AAC48315), *Eimeria tenella* (AAD02687), *Entamoeba histolytica* (P42649), *Fritillaria* (AAC04811), *Fucus vesiculosus* (Q39757), *Hordeum vulgare* (CAA74592), *Hypocrea lixii* (Q99002), *Oryza sativa* (AAB07456), *Picea glauca* (AAD27827), *Plasmodium knowlesi* (AAC17515), *Schistosoma japonicum* (AAD56715), *Schizosaccharomyces* (P42656), *Solanum tuberosum* (CAA72384), *Tetrahymena pyriformis* (BAA83080), *Xenopus laevis* (AAC41252), *Zea mays* (P49106).

Figure 4 The 14-3-3 gene expression (mean±SD) in *H. perleve* exposed to *E. coli* (1.1×10^7 CFU mL⁻¹), *V. anguillarum* II (1.2×10^6 CFU mL⁻¹) and *V. alginolyticus* (3.6×10^5 CFU mL⁻¹) for 6 h, respectively.

Figure 5 The 14-3-3 gene expression (mean±SD) in *H. perleve* exposed to *V. anguillarum* II at 1.1×10^4 CFU mL⁻¹, 3.6×10^4 CFU mL⁻¹ and 5.6×10^5 CFU mL⁻¹ for 1.5, 3 and 6 h, respectively.

expression level is shown in Figure 5. For the control group, the 14-3-3 mRNA expression at 1.5, 3 and 6 h was not significantly different ($P < 0.05$). In the test with *V. anguillarum* II at 1.1×10^4 CFU mL⁻¹, the 14-3-3 mRNA expression

at 1.5, 3 and 6 h were not significantly different ($P < 0.05$), but slightly declined when compared with that of the control. In the test with *V. anguillarum* II at 3.6×10^4 CFU mL⁻¹, significant decrease in 14-3-3 mRNA expression level did

not occur until *H. perleve* was exposed for 3 h. The 14-3-3 mRNA expression level at 3 and 6 h was 2.71-fold and 2.65-fold lower than that of the control. In the test with *V. anguillarum* II at 5.6×10^5 CFU mL⁻¹, the 14-3-3 gene expression level at 1.5, 3 and 6 h was respectively 2.16-, 2.82- and 2.66-fold lower than that of the control.

3 Discussion

In the recent two decades, outbreaks of diseases in wild and cultured marine organisms, due to microbial pathogens, have been frequent [40,41], particularly in China (Reports on Marine Pollution in China, 2001–2005). This has resulted in economic losses for the aquaculture industry [42]. As sessile filter feeders, sponges (phylum Porifera) are exposed to a large amount of microorganisms, including pathogenic bacteria. Besides their ability to remove microorganisms through phagocytosis [34] and characteristics to produce chemicals with anti-bacterial activity [13], sponges possess an efficient system of immune/defense mechanism [43]. In nearly a decade, the demosponge *S. domuncula* was used as the model system to study the molecular responses to bacteria [18–21] and fungi [17]. From 2002 to now, the marine sponge *H. perleve* maintains a healthy state in the field around Dalian City, but aquacultured sea cucumber and some bivalve molluscs, which are also filter feeders cultured in open seawater column in the same sea areas have suffered from infectious bacterial diseases. Through microbiology and molecular biology approaches, we have demonstrated for the first time that *H. perleve* could differentiate between infectious bacteria (*Vibrio* spp.) and non-infectious bacteria (*E. coli*) through molecular response (14-3-3 mRNA) to bacteria.

In the infection tests, *E. coli* as non-indigenous bacteria had been alive, but did not grow in seawater [34]. Hence, *E. coli* was used as the most common traced bacteria in the sponge filtering experiments [44,45]. Whereas the indigenous bacteria, *Vibrio* spp. reproduce rapidly in seawater [46] and are extremely infectious to aquacultured and wild animals, and humans [47]. Our results show that *H. perleve* has the ability to effectively retain *E. coli* in seawater, and the ability to control the growth of *V. anguillarum* II, to some degree. Here *H. perleve* exhibit a similar ability of controlling the growth of *V. alginolyticus*. Furthermore, the tests demonstrated that *E. coli* does not infect *H. perleve*. However, it was demonstrated that both *V. anguillarum* II and *V. alginolyticus* have extremely high ability of infecting *H. perleve* (Table 1).

14-3-3 proteins are evolutionarily well conserved from yeast to human [38] and play critical roles in regulation of many cellular processes, such as signal transduction, cell cycle, and stress response [24,26,27,48]. Here we isolated three partial cDNA (including poly(A) tail) from the marine sponge *H. perleve*. The two signature regions of 14-3-3

proteins are highly conserved: signature 1 is a peptide of 11 residues located in the N-terminal section; signature 2, a 20-amino acid region located in the C-terminal section. The polypeptides of *H. perleve*-A and *H. perleve*-B do not include signature 1, because of shorter amino acid sequences than that of *H. perleve*-H. In addition, most of the motifs found in the three 14-3-3 polypeptides are located at highly conserved residues (Figure 2). These findings support previously published results, that some highly conserved residues specifically bind to target proteins [49,50]. Nevertheless, there are different motifs among 14-3-3 isoforms of the same organism, such as a cell attachment sequence (RGD; PS00016) is only located at *HP*-H. This confirms that isoforms found in multicellular organisms have isoform-specific functions [38].

14-3-3 proteins have been examined as a biomarker for abiotic stress such as PCBs [33], however their expression patterns in response to biotic stress, such as bacterial infection, have not been studied. Using RT-PCR, the expression of 14-3-3 mRNA in *H. perleve* in response to live Gram-negative bacteria (*E. coli* and *Vibrio* spp.) was examined, rather than lipopolysaccharide (LPS) as a model inducing compound. The challenge of sponges with *E. coli* did not affect the 14-3-3 mRNA expression. When *H. perleve* was exposed to *V. anguillarum* II and *V. alginolyticus*, the 14-3-3 mRNA expression was down-regulated by 2.67- and 2.36-fold, respectively (Figures 4 and 5). Both *E. coli* and *Vibrio* spp. are Gram-negative bacteria with LPS, a major component of the outer membrane of Gram-negative bacteria [18], but 14-3-3 mRNA expression of *H. perleve* was different between the *E. coli* and the *Vibrio* spp. treatment. *E. coli* as non-indigenous bacteria did not grow in seawater and were rapidly digested by *H. perleve* [34]. Previous research has shown that *E. coli* is as a food source for sponge [6,44,51]. *E. coli* may only be phagocytosed by sponges and digested as food. In contrast, *Vibrio* spp. as indigenous marine bacteria, possess the ability to infect sponge (Figure 1 and Table 1). Our result for the first time demonstrated that infectious bacteria could be discriminated from non-infectious bacteria by the differential expression of 14-3-3 mRNA of the sponge *H. perleve* in response to bacteria.

However, these results may explain why the MAP kinases p38 and JNK in *S. domuncula* when exposed to LPS were strongly activated (phosphorylated) [20]. It was reported that 14-3-3 blocked p38 and JNK MAP activation (phosphorylation) in human [31] and in mice [32]. The 14-3-3 proteins [38] and Mitogen-activated protein kinases p38 and JNK [52] are evolutionarily well conserved from yeast to human. These 14-3-3 residues involved in binding p38 and JNK are 100% conserved in all eukaryotic organisms (Figure 2, [38]). The MAP kinases p38 and JNK are activated by phosphorylation at the dual phosphorylation TXY motif, which is highly conserved from yeast to human [53,54]. Hence the 14-3-3 proteins in sponges may have the ability

of inhibiting phosphorylation of MAP kinases p38 and JNK. The present study showed that the expression of 14-3-3 mRNA of *H. perleve* was down-regulated during the exposure to the infectious bacteria *Vibrio* spp. As a result, the down-regulation of 14-3-3 mRNA expression led to the decrease in the ability of 14-3-3 blocking p38 and JNK activation (phosphorylation). In the future, the expression of each isoform of 14-3-3 proteins from *H. perleve* should be detected when exposed to pathogenic bacteria. As multi-gene analysis is available, the underlying mechanism of interaction of 14-3-3 proteins and their binding proteins which are also involved in pathogen-resistance response, such as p38 protein kinase and JNK protein kinase, should be investigated after the sponge is exposed to infectious bacteria.

4 Conclusion

Here we understand that the marine sponge *H. perleve* has the ability to discriminate between infectious bacteria from non-infectious bacteria through molecular response to different-type bacteria, and that both *V. anguillarum* II and *V. alginolyticus* have high ability of infecting sponge *H. perleve*. In the process of infecting the sponge *H. perleve*, *Vibrio* spp. also induce down-regulation of 14-3-3 mRNA of sponge *H. perleve*. *E. coli* is retained and then is fed on by *H. perleve*. The results from this study demonstrated that infectious bacteria can be discriminated by 14-3-3 gene expression of sponge *H. perleve*.

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