Fish & Shellfish Immunology 31 (2011) 1218–1223

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

Molecular cloning and characterization of two isoforms of cyclophilin A gene from *Venerupis philippinarum*

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ARTICLE INFO

Article history:
Received 5 April 2011
Received in revised form
23 June 2011
Accepted 2 July 2011
Available online 18 July 2011

Keywords:
Venerupis philippinarum
Cyclophilin A
Gene expression

ABSTRACT

Cyclophilin A (CypA) is a ubiquitously distributed intracellular protein belonging to the immunophilin family, which is recognized as the cell receptor for the potent immunosuppressive drug cyclosporine A. In the present study, two isoforms of cyclophilin A gene (named as VpCypA1 and VpCypA2) were isolated and characterized from *Venerupis philippinarum* by RACE approaches. Both VpCypA1 and VpCypA2 possessed all conserved features critical for the fundamental structure and function of CypA, indicating that the two isoforms of cyclophilin A should be new members of CypA family. The expression of VpCypA2 mRNA in haemocytes was significantly up-regulated and the highest expression level was detected at 96 h post-infection with 7.7-fold increase compared with that in the blank group. On the contrary, the relative expression level of VpCypA1 mRNA was down-regulated rapidly at 6 h post-infection and reached 0.4-fold of the control group. They exhibited different expression profile and identical effect of immune modulation, which might suggest the two VpCypA isoforms exert their function in a manner of synergy. These results provide valuable information for further exploring the roles of cyclophilin A in the immune responses of *V. philippinarum*.

1. Introduction

Cyclophilins (Cyps), which contain a single conserved peptidyl-prolyl cis-trans isomerases (PPIase) domain [1,2], are widely distributed in organisms as constitutive proteins. Due to their PPIase activity, Cyps carry out a wide range of functions, such as protein folding [3,4], receptor complex stabilization [5], apoptosis participation [6] and receptor signaling [7]. Various Cyps have been found in diverse organisms ranging from bacteria to humans [8–12]. In mammals, five classic Cyp isoforms (cyclophilin A, B, C, D and cyclophilin 40) have been reported with size ranging from 18 to 40 kDa [13–15].

Cyclophilin A (CypA) is a ubiquitously distributed intracellular protein belonging to the immunophilin family [16] and recognized as the host cell receptor for the potent immunosuppressive drug cyclosporin A [17]. CypA–CsA complex can bind intracellular proteins and play important roles in the immune system of mammals [18]. For example, CypA–CsA complex can bind and inhibit calcineurin in T-lymphocytes, which blocks the T cell signal transduction to achieve immunosuppression [19]. Although CypA was originally believed to exist solely as an intracellular protein, later studies have revealed that it can be secreted by cells in response to inflammatory stimuli [20]. The clinical importance of Cyps has been implicated in diverse pathological conditions, such as HIV [21], hepatitis B and C viral infection [22], atherosclerosis [23], vascular diseases [24] and Rheumatoid Arthritis pathogenesis [25]. Recent studies also revealed that CypA could modulate HIV-1 capsid disassembly and that changes in capsid stability could influence HIV-1 sensitivity to the inhibition of CypA binding [26]. In addition, some results suggested that CypA played an important role in the innate immune system of some aquatic animals. For example, CypA was deduced to be involved in the early infection of *Edwardsiella ictaluri* in channel catfish *Ictalurus punctatus* [27]. In shrimp *Penaeus monodon*, the expression of CypA in the hepatopancreas was up regulated after stimulated with lipopolysaccharide, indicating that CypA was involved in the defense response against the bacterial infections [28].

The Manila clam, *Venerupis philippinarum*, is an important marine bivalve for commercial fisheries, accounting for about 80% of mudflat fishery production in China (China Bureau of Fisheries, 2004). However, clam culture in china has been severely plagued by pathogenic microorganisms, which has caused serious economic losses [29]. Therefore, understanding the immune responses of clams against pathogen challenge has become essential. Previous studies

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doi:10.1016/j.fsi.2011.07.001

on CypA mainly focused on its structure analysis [5–7,30–32]. Rare knowledge is available about the molecular characteristic and expression patterns, especially in invertebrates. In the present study, two full-length cDNAs of CypAs were cloned from *V. philippinarum*, and the expression profiles of VpCypA1 and VpCypA2 transcript characterization after bacterial challenge were also investigated, hopefully shedding light on the roles of CypAs in the immune responses of marine invertebrate.

2. Materials and methods

2.1. Clams and challenge

The clams *V. philippinarum* (shell length: 3.0–4.0 cm, Zebra pedigrees) were purchased from local culturing farm (Bohai Sea, Yantai, China) and acclimatized for 10 days before processing. The seawater was aerated continuously, and salinity and temperature maintained at 32 psu and 25 °C throughout the experiment. Clams were fed with *Chlorella vulgaris* Beij daily and the seawater was renewed daily. After the acclimatization, the clams were randomly divided into six flat-bottomed rectangular tanks and each tanks contained 50 individuals.

For *Listonella anguillarum* challenge experiment, one tank served as control. The other five tanks were challenged with high density of *L. anguillarum* with final concentration of 10^7 CFU mL^{-1}. Four individuals were randomly sampled at 6, 12, 24, 48 and 96 h respectively. The haemolymph from the control and the infected groups was collected using a syringe and centrifuged at 2000 × *g*, 4 °C for 10 min to harvest the haemocytes.

2.2. Cloning the full-length cDNA of VpCypA1 and VpCypA2

Two ESTs which shared homology to the previously identified CypA, were identified from the *V. philippinarum* haemocytes cDNA library. The 5′ and 3′ ends of VpCypA1 and VpCypA2 were obtained by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech, USA) according to manufacturer's recommendations. The PCR products were gel-purified and subcloned into pMD18-T simple vector (Takara, Japan). After transformed into the competent cells of *Escherichia coli* Top10F′, three positive clones were sequenced on an ABI3730 Automated Sequencer (Applied Biosystem).

2.3. Sequence analysis

The cDNA and amino acid sequences of VpCypA1 and VpCypA2 were analyzed by using the BLAST algorithm at NCBI website (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). The protein domain features were determined by using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). The percentage of similarity and identity of VpCypA1 and VpCypA2 with CypA proteins from other organisms was calculated by the Identity and Similarity Analysis program (http://www.biosoft.net/sms/index.html). Multiple alignments of VpCypA1 and VpCypA2 were performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) and Multiple Alignment Show program (http://www.bio-soft.net/sms/index.html).

2.4. Phylogenetic analysis

The deduced amino acid sequences of VpCypA1 and VpCypA2 were aligned with the corresponding cyclophilin sequences from various organisms using the Clustal X software. Based on this alignment, a phylogenetic tree was constructed with MEGA 4.0

Table 1
Primers used in the present study.

Primer Sequence	(5′–3′)	Sequence information
P1(forward)	CTCCCTTGAGAAGAGCTACGA	Real time β-actin primer
P2(reverse)	GATACCAGCAGATTCCATACCC	Real time β-actin primer
P3(forward)	AGGAGTCGGTTCCCAAAGTG	Real time VpCypA1 primer
P4(reverse)	TATTGCATCCGGGGGTAGGT	Real time VpCypA1 primer
P5(forward)	CTGGAGGCAAGAGCATTTATGG	Real time VpCypA2 primer
P6(reverse)	TCGTGACTACATCCATACCCTC	Real time VpCypA2 primer

A

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1                M  V  F  H  V  R  I  A  H  I  L  R  T  Q  G  V  R
1    AACGAAACCTCATGGTCTTCCACGTGCGAATCGCTCATATTTTGCGAACACAAGGAGTGC
18     Y  S  S  S  A  K  M  P  I  Q  T  F  F  D  I  T  I  G  G  K
61   GCTATTCATCATCTGCAAAAATGCCGATTCAGACATTCTTCGACATAACAATTGGTGGCA
38     P  A  G  R  I  V  M  E  L  N  N  E  V  V  P  K  T  A  E  N
121  AACCAGCTGGCAGAATTGTCATGGAGCTGAACAATGAAGTTGTTCCAAAAACTGCTGAAA
58     F  R  A  L  C  T  G  E  K  G  F  G  F  K  N  S  P  F  H  R
181  ATTTCCGTGCCTTGTGCACCGGAGAAAAAGGATTTGGATTTAAGAATTCCCCATTCCACC
78     V  I  P  G  F  M  C  Q  G  G  D  F  T  K  R  N  G  T  G  G
241  GTGTAATTCCAGGCTTCATGTGCCAAGGTGGAGATTTCACTAAACGAAATGGCACCGGAG
98     K  S  I  Y  G  N  K  F  A  D  E  N  F  I  L  K  H  D  G  E
301  GCAAAAGCATATATGGGAACAAATTTGCAGATGAAAACTTCATTCTCAAGCACGATGGTG
118    G  I  L  S  M  T  N  A  G  P  N  T  N  G  S  Q  F  F  L  C
361  AAGGAATTCTGTCCATGACAAATGCAGGTCCAAATACAAATGGATCACAGTTTTTCTTGT
138    T  A  K  T  P  W  L  D  G  K  H  V  V  F  G  R  V  T  S  G
421  GTACAGCAAAAACACCATGGTTGGATGGTAAACATGTTGTATTTGGTAGAGTTACAAGTG
158    M  D  V  V  K  K  I  E  G  V  G  S  Q  S  G  K  T  S  Q  A
481  GTATGGATGTTGTGAAGAAAATTGAAGGAGTCGGTTCCCAAAGTGGCAAAACTAGCCAGG
178    V  E  V  V  N  C  G  E  L  *
541  CTGTGGAGGTTGTTAACTGTGGAGAATTATAAATTTATTGTCTTCGAAGGATGTAGGACT
601  CACTAGGAGCAGTATTGTAAGCAAGAAAACTTTCATTGCCTACAATTTTTTTGCAATCAA
661  AATTTGCTATAAAATTGATCCAAATAATTGTTGGTATTTTATCTTTTAATTATATCTCAT
721  CTTTGCTAACAATACCTACCCCCGGATGCAATACCTTTTTTACTTACTTTTGTTTTTATA
781  AAAATTTATTATGGAATTCTATTCGTGTATCACGCGTTTTACAATAAATGACTCTTTACT
841  TGACCAAAAACAACTAATTAATCAAGTGAACGAAAAAAACATAAAATCAAGTTAAACGCT
901  TGTTGATTGATACTGTGAAAAATGAAATCCTGAAATATATATAATATAATTCTATTGATT
961  CATTTGGTACAATTCATTAAATTGCACCCTTCTTTTCTCTGTTTGTACTTTAATCCACCC
1021 AAAAAAGGAAATAAAAAAAAAGGAACAAAAAAAAAAAAAAAAAAAAAAAAAA
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B

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1    GCTTACATCNAAAAGAATTCGACAAAAAGTACAACCCAACATGGCATTGCATTGTGGGAA
61   GAAACTTTGGCAGCTACGTCACTCATGAAACCAAGCATTTTATTTACTTCTACCTGGGAC
121  AAGTAGCCATCCTCCTATTTAAATCGGGGTGAACAAAAAGTGACAAGGAAAAAAGGAAAA
1                M  A  N  P  K  C  Y  F  D  I  T  I  G  G  K  P
181  TAAGAAAAGCAATCATGGCAAACCCAAAATGCTACTTTGATATTACTATTGGTGGAAAGC
17     A  G  R  I  V  M  E  L  R  A  D  V  V  P  K  T  A  E  N  F
241  CAGCTGGTAGAATTGTCATGGAGTTGAGAGCTGATGTTGTACCTAAAACTGCAGAAAATT
37     R  A  L  C  T  G  D  K  G  F  G  F  K  G  S  K  F  H  R  V
301  TCCGTGCATTGTGCACTGGGGATAAGGGATTTGGTTTCAAGGGCAGCAAATTCCACCGTG
57     I  P  G  F  M  C  Q  G  G  D  F  T  R  G  N  G  T  G  G  K
361  TAATTCCTGGTTTCATGTGCCAAGGTGGTGATTTTACTCGTGGTAACGGTACTGGAGGCA
77     S  I  Y  G  E  K  F  A  D  E  N  F  N  L  K  H  T  G  S  G
421  AGAGCATTTATGGAGAGAAATTTGCTGATGAGAACTTCAATCTGAAGCATACTGGATCAG
97     I  L  S  M  A  N  A  G  P  N  T  N  G  S  Q  F  F  L  C  T
481  GTATTCTTCAATGGCAAATGCTGGTCCTAACACAAATGGGTCGCAGTTTTCTTGTGTA
117    A  K  T  T  W  L  D  G  K  H  V  V  F  G  S  V  V  E  G  M
541  CAGCAAAGACCACATGGCTTGATGGGAAACATGTAGTGTTTGGTTCAGTAGTTGAGGGTA
137    D  V  V  T  K  I  E  S  V  G  S  D  S  G  K  T  K  V  D  V
601  TGGATGTAGTCACGAAAATAGAAAGTGTAGGAAGTGATAGTGGAAAGACAAAAGTGGATG
157    G  I  A  D  C  G  Q  L  *
661  TTGGTATTGCTGATTGTGGACAGCTATAAAGCCTACAATGGTCATTGTGTGACTATCCAT
721  CATAAACAATTTATGTTCTTACATTTTCTGGATCAAAATATGACTTATTTAAATTACTAA
781  TAGCTTGTTAAACAGCTTTTTTGAATTTGATTTTGAAATCTTTTCAATATTTGAGATAAT
841  CGCGTCAACAAATTTATTATTCCTTACATCACACTATTTATAATTTTTTATAGATAATTT
901  CATGTCAGAGGTGATTTTCTTTTTTCTAGGAATTCATTTGGATTATGTATTCTTTTTCAA
961  GGAAAACTGTCTGCTTCTGAAATAAATGTATGATCTTAAAAAAAAAAAAAAAAAAAAAAA
1021 AAAAA
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Fig. 1. A. Nucleotide and deduced amino acid sequences of the CypA1 gene from *Venerupis philippinarum*. Nucleotide and deduced amino acid residues are numbered on the left. The signature of peptidyl-prolyl cis-trans isomerase is shadowed. The start and stop codons are marked in bold and the classical polyadenylation signal in the 3′-UTR is boxed. The conserved amino acids residues are underlined. B. Nucleotide and deduced amino acid sequences of the CypA2 gene from *V. philippinarum*. Nucleotide and deduced amino acid residues are numbered on the left. The signature of peptidyl-prolyl cis-trans isomerase is shadowed. The start and stop codons are marked in bold and the classical polyadenylation signal in the 3′-UTR is boxed. The conserved amino acids residues are underlined.

software by using the neighbor-joining (NJ) method [33]. Bootstrap analysis was used with 1000 replicates to test the relative support for the branches produced by the NJ analysis. All the analyzed sequences were retrieved from GenBank and SWISS-PROT database.

2.5. Temporal expression profile of VpCypA1 and VpCypA2 transcript post Vibrio challenge

The temporal expression of VpCypA1 and VpCypA2 transcript in haemocytes after *L. anguillarun* challenge was measured by quantitative real-time RT-PCR in an Applied Biosystem 7500 Real-time PCR System, and performed in a total volume of 20 μL, containing 10 μL of 2 × SYBR Green Master Mix (Applied Biosystems), 6 μL of the diluted cDNA mix, 0.25 μL of each primer (10 μmol L^{-1}), 3.5 μL of DEPC-treated water. The thermal profile for real-time PCR was 50 °C for 2 min and 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. To maintain consistency, the baseline was set automatically by the software. The expression level of VpCypA1 and VpCypA2 was analyzed using $2^{-\Delta\Delta C}{}_T$ method [34]. Two clam β-actin primers, P1 and P2 (Table 1) were used to amplify a 121 bp fragment as an internal control to verify the successful reverse transcription and to calibrate the cDNA template. Two sets of gene-specific primers, P3 and P4 (Table 1) for VpCypA1, P5 and P6 (Table 1) for VpCypA2, were designed to amplify products of 248 bp and 204 bp, respectively. In a 96-well plate, each sample was run in triplicate along with the internal control. The reaction component, thermal profile and the data analysis were conducted according to previously reported [35]. All data were given in terms of relative mRNA expression as means ± S.E. One-way analysis of variance (one-way ANOVA) was performed on all data and $P < 0.05$ was considered statistically significant.

3. Results

3.1. cDNA cloning and sequence analysis of the VpCypA1 and VpCypA2 gene

Two nucleotide sequences of 1070 bp and 1026 bp representing the complete cDNA sequence of VpCypA1 (Fig. 1A) and VpCypA2 (Fig. 1B) were obtained by overlapping EST and the amplified fragments. The sequences of VpCypA1 and VpCypA2 were deposited in GenBank under accession no. GQ384405 and GQ384406, respectively. The deduced amino acid sequences of VpCypA1 and VpCypA2 were shown in the corresponding nucleotide acid sequence in Fig. 1.

The complete sequence of VpCypA1 cDNA encoded a polypeptide of 186 amino acids with the predicted molecular weight of 20.15 kDa and the theoretical isoelectric point of 9.39. The full-length cDNA of VpCypA2 encoding a polypeptide of 164 amino acids with the predicted molecular weight of 17.34 kDa and the theoretical isoelectric point of 8.60. SMART program analysis revealed that both VpCypA1 and VpCypA2 contained the typical pro isomerase domain ranging from Gln^{27} to Leu^{186} and Lys^{5} to Leu^{164}, respectively. Multiple alignments revealed that the cyclophilin family signature sequences (YKGSTFHRIIPGFMCQGG) could be identified in both isoforms of CypA, and the residues involved in CsA binding and PPIase activities were well conserved (Fig. 2).

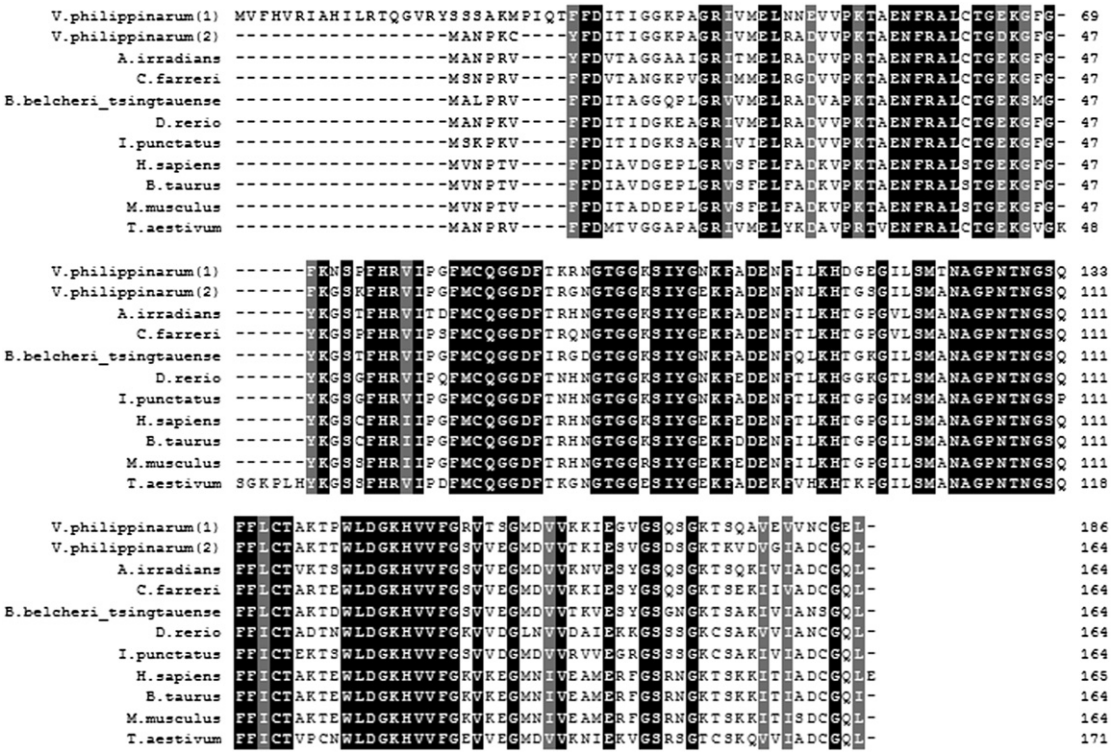

Fig. 2. Multiple sequence alignment of the VpCypA with other registered counterparts. The CypA sequences from other organisms were obtained from GenBank database: *V. philippinarum* (*V. philippinarum*, ACU83229, ACU83230), *A. irradians* (*Argopecten irradians*, ABM92916), *C. farreri* (*Chlamys farreri*, AAR11779), *B. belcheri tsingtauense* (*Branchiostoma belcheri tsingtauense*, AAQ24380), *D. rerio* (*Danio rerio*, AAQ91263), *I. punctatus* (*Ictalurus punctatus*, AAY86951), *H. sapien* (*Homo sapien*, AAH05982), *B. taurus* (*Bos taurus*, AAW82121), *M. musculus* (*Mus musculus*, NP_032933), *T. aestivum* (*Triticum aestivum*, AAS17067). The amino acid residues with black background are totally conserved, and the grey background are conserved more than eighty percentage.

3.2. Phylogenetic analysis

A phylogenetic tree was constructed based on the amino acid sequences of 29 Cyps by the NJ method (Fig. 3). According to the phylogenetic tree, the cyclophilin members were mainly clustered into three groups by their category. VpCypA1 was firstly clustered with VpCypA2 from *V. philippinarum*, and then formed a sister group with CypAs from invertebrates and further grouped with those from vertebrates. The relationships displayed in the phylogenic tree were in generally agreement with traditional taxonomy.

3.3. The expression level of VpCypA1 and VpCypA2 after Vibrio challenge

The temporal expression of VpCypA1 transcript post-*Vibrio* challenge was shown in Fig. 4. During the first 6 h after pathogen challenge, the expression level of VpCypA1 mRNA significantly decreased down to 0.4-fold of the blank group ($P < 0.05$). After that, the expression level was obviously up-regulated at 12 h and fluctuated from 24 h to 96 h. However, no significant difference was observed in these time points compared with the blank group ($P > 0.05$). As illustrated in Fig. 4, the expression level of VpCypA2 was obviously up-regulated throughout the experiment and increased significantly at 24 h (3.4-fold, $P < 0.05$) and 96 h (7.7-fold, $P < 0.01$).

4. Discussion

Cyclophilin A (CypA), a receptor for the immunosuppressive agent cyclosporin A (CsA), is a cis–trans peptidyl-prolyl isomerase (PPIase) which catalyzes the cis–trans isomerization of prolyl-peptide bonds, regulates activities of a variety of proteins by interacting with them [17–19]. However, knowledge on the function, expression and regulation of immune effectors in mollusk, especially in the innate immune system, are still insufficient for cyclophilin A. In the present study, two isoforms of cyclophilin A gene were identified from *V. philippinarum* (designated as VpCypA1 and

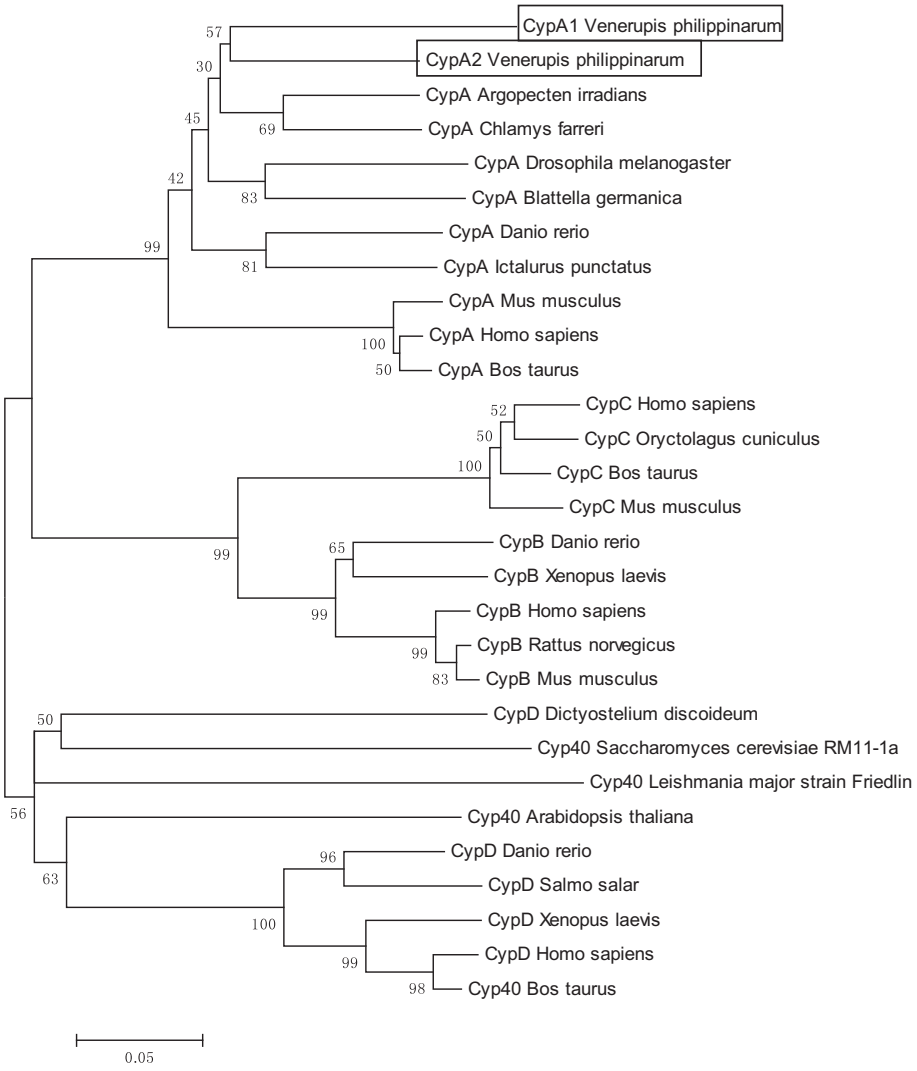

Fig. 3. A phylogenetic tree constructed by the neighbor-joining method. Numbers next to the branches indicated bootstrap value of each internal branch in the phylogenetic tree nodes from 1000 replicates. Cyclophilin sequences include *Venerupis philippinarum* (ACU83229 and ACU83230), *Argopecten irradians* (ABM92916), *Chlamys farreri* (AAR11779), *Drosophila melanogaster* (AAB03701), *Blattella germanica* (CAA60869), *Danio rerio* (AAQ91263), *Ictalurus punctatus* (AAY86951), *Homo sapien* (AAH05982), *Bos taurus* (AAW82121), *Mus musculus* (NP_032933). CypB sequences include *Homo sapiens* (CAG33110), *Mus musculus* (NP_035279), *Rattusnorvegicus* (AAH61971), *Xenopus laevis* (NP_001080505), *Danio rerio* (NP_998184). CypC sequences include *Homo sapiens* (AAB31350), *Mus musculus* (AAA37511), *Bos taurus* (NP_001070378), *Oryctolagus cuniculus* (ABB45383). CypD sequences include *Homo sapiens* (NP_005029), *Xenopus laevis* (NP_001087854), *Danio rerio* (AAH71388), *Salmo salar* (ACH70718), *Dictyostelium discoideum* (AAD50375). Cyp40 sequences include. *Leishmania major strain Friedlin* (CBZ13107), *Arabidopsisthaliana* (AAK02067), *Bos taurus* (AAA30484), *Saccharomyces cerevisiae* (EDV08549).

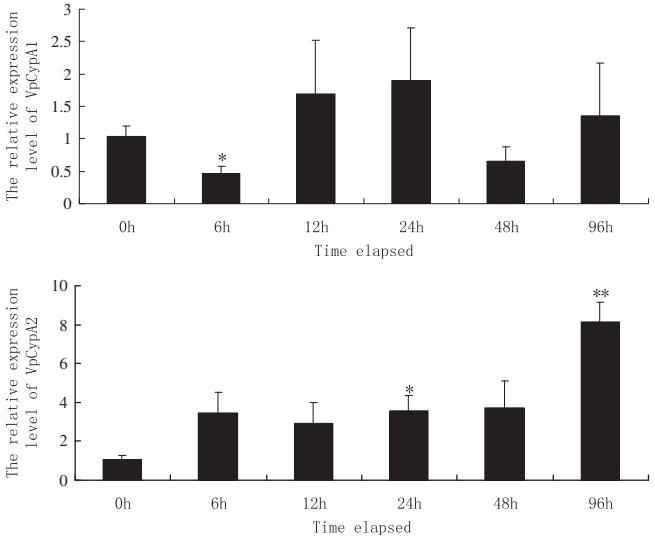

Fig. 4. Temporal expression of VpCypA1 and VpCypA2 transcript in haemocytes of clams after *Listonella anguillarum* infection measured by quantitative real-time PCR at 0, 6, 12, 24, 48 and 96 h. Each bar represented mean ± S.E. Significant difference was indicated with an asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.

VpCypA2), and they both possessed the conserved features critical for the fundamental structure and function of cyclophilin A. The Cyp-type PPIase signature and conserved amino acid residues were identical with previously reported CypA [36–38]. Phylogenetic analysis showed that VpCypA1 was first clustered with VpCypA2 and had a close relationship with *Argopecten irradians*, *Chlamys farreri* and *Branchiostoma belcheri tsingtauense* counterparts, which suggested that they might share similar biological functions.

In mammals, cyclophilin A was suggested to play a role in defending the viral infection [39]. In human, CypA had proinflammatory effects on endothelial cell (EC) and might play an important role in the pathogenesis of inflammatory diseases [40]. Studies also showed that CypAs from aquatic animals were not only a constitutive protein, but also an inducible protein, and perhaps involved in defending the pathogen infection [41]. In the present study, the temporal expression of VpCypA1 and VpCypA2 mRNA in haemocytes were examined at different time points after bacterial stimulation. At early stages of infection, the resulting decline in the transcript of VpCypA1 at 6 h might be closely related to the mRNA consuming. This phenomenon was similar to the discovery in *Bombyx mori*, where mRNA is consumed for the synthesis of more protein to defend the invasion of pathogens [42]. The mRNA expression level of VpCypA1 in haemocytes was almost stable during the process of challenge at other time points compared with blank group. On the contrary, the mRNA expression level of VpCypA2 was up-regulated in the mass and increased significantly at 24 h and 96 h, which indicated that VpCypA2 was perhaps involved in the clam immunity responses to pathogen infection. Although different expression profile were exhibited, both of the two isoforms played the role in immunization against the bacteria, which might suggest that the two VpCypA isoforms exert their function in a manner of synergy. However, the explicit role of two VpCypA isoforms in immunization is still confusing and requires great effort to acquire a comprehensive understanding.

In conclusion, the full-length cDNAs of two VpCypA isoforms were identified from *V. philippinarum*, and both possessed highly conserved amino acid residues to counterparts of other species. Especially, the VpCypA isoform 2 was inducibly expressed in haemocytes, suggesting its involvement in the defense response against the bacterial infections. These results provide valuable information for further exploring the roles of cyclophilins in aquatic animal immune responses to infection. To further broaden our understanding of the immune roles of VpCypA in *V. philippinarum*, future efforts will be focused on the correlation between the expression profile and corresponding mechanism.

Acknowledgement

The authors were grateful to all the laboratory members for continuous technical advice and helpful discussion. This research was supported by Innovation Research Team in University (IRT0734), Doctoral Fund of Ministry of Education of China (20103305110003, 20103305120002), Nature Fund of Ningbo (2010A610004) and K.C.Wong Magana Fund in Ningbo University.

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