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E-mail:xbywb@ouc.edu.cn

Construction of cDNA Library from Intestine, Mesentery and Coelomocyte of *Apostichopus japonicus* Selenka Infected with *Vibrio* sp. and a Preliminary Analysis of Immunity-Related Genes

LIU Hongzhan[1)], ZHENG Fengrong[2), *], SUN Xiuqin[2)], and CAI Yimei[3)]

1) *College of Marine Life Sciences*, *Ocean University of China*, *Qingdao* 266003, *P. R. China*
2) *The First Institute of Oceanography*, *SOA*, *Qingdao* 266061, *P. R. China*
3) *Beijing Institute of Genomics*, *Chinese Academy of Sciences*, *Beijing* 100029, *P. R. China*

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Abstract The aquaculture of sea cucumber *Apostichopus japonicus* (Echinodermata, *Holothuroidea*) has grown rapidly during recent years and has become an important sector of the marine industry in Northern China. However, with the rapid growth of the industry and the use of non-standard culture techniques, epidemic diseases of *A. japonicus* now pose increasing problems to the industry. To screen the genes with stress response to bacterial infection in sea cucumber at a genome wide level, we constructed a cDNA library from *A. japonicus* Selenka (Aspidochirotida: Stichopodidae) after infecting them with *Vibrio* sp. for 48 h. Total RNA was extracted from the intestine, mesentery and coelomocyte of infected sea cucumber using Trizol and mRNA was isolated by Oligotex mRNA Kits. The ligated cDNAs were transformed into DH5α, and a library of 3.24×10^5 clones (3.24×10^5 cfu mL^{-1}) was obtained with the sizes of inserted fragments ranging from 0.8 to 2.5 kb. Sequencing the cDNA clones resulted in a total of 1106 ESTs that passed the quality control. BlastX and BlastN searches have identified 168 (31.5%) ESTs sharing significant homology with known sequences in NCBI protein or nucleotide databases. Among a panel of 25 putative immunity-related genes, serum lectin isoform, complement component 3, complement component 3-like genes were further studied by real-time PCR and they all increased more than 5 fold in response to *Vibrio* sp. challenge. Our library provides a valuable molecular tool for future study of invertebrate immunity against bacterial infection and our gene expression data indicates the importance of the immune system in the evolution and development of sea cucumber.

Key words *Apostichopus japonicus*; cDNA library; expressed sequence tags; immunity-related genes; real-time PCR

1 Introduction

Sea cucumber *A. japonicus* belongs to *Holothuroidea* of *Echinodermata*. It has been consumed by Chinese and Japanese since ancient times. Its aphrodisiac and curative properties serve as a traditional agent healing various internal and external wounds (Stephen *et al.*, 1999). With our lifted living standard and health care awareness, sea cucumber has attracted more and more attention in the food industry and science for its health benefits and its cultivation has become a big industry (Zhang *et al*., 2006).

However, in recent years, because of rapid expansion and intensification of cultivation, sea cucumber in China has suffered from frequent disease outbreaks. Bacteria, especially vibrios, are often the cause of major diseases such as skin ulcer and bacterial ulceration syndrome in *A. japonicus* at breeding, aestivation and outdoor cultivation stages (Wang *et al*., 2004), and black mouth and red spotting diseases in *S. intermedius* and other species under aquaculture conditions (Li *et al*., 2000; Wang *et al*., 2005). The plague disease, a bacteria-caused disease, can be fatal to sea urchin. It poses a threat to the aquaculture industry and can lead to heavy economic losses (Wang *et al*., 2006).

The intestine's eviscerateing from coelome of *A. japonicus* is the most important reaction to stimulus and the intestine organization consists of protease, amylase, lipase, lysozyme and superoxide dismutase. During viral particles infection, histological examination of diseased sea cucumbers indicates that the intestine tissue is the primary target tissue (Wang *et al*., 2007). But most of the research on *A. japonicus* Selenka has been focused on traditional breeding and cultivation, few on function genes. To defend against bacterial infection, the immune system plays the most critical role. The genes encoding the proteins mediating the immunity have been an active research subject. Many of the function genes have been identified by traditional strategies, in which the genes of

* Corresponding author. E-mail: zhengfr@fio.org.cn

interest are individually identified and their functions determined by characterizing proteins. Since the number of genes participating in the stress response is very large, novel high-throughput screening technologies, such as cDNA library, expressed sequence tag (EST) sequencing, microarrays, and proteomics, have been developed to facilitate gene profiling.

To facilitate the study on immune response of sea cucumbers at the genome wide level, in this study, we constructed a cDNA library using the intestinal samples from *A. japonicus* infected with *Vibrio* sp. for 48 h. Totally 1106 ESTs were identified and 15 sequences were the same with genes related to the immune response in other organisms. We further analyzed five genes for their expression patterns in coelomocytes of the sea cucumber before and after bacterial infection.

2 Materials and Methods

2.1 Animals and Bacterial Infection

The sea cucumber specimens (10−12 cm long) were purchased from a local market in Qingdao, and kept in a tank with a flowing filtered water system. The water temperature was approximately 18−20℃ and the water salinity was 31.0. The water quality was monitored daily. The sea cucumbers were acclimated for one week in the laboratory before experiment.

The bacteria *Vibrio* sp. were isolated from sea cucumbers with visible skin ulceration. They were resuspended in seawater sterilized by filtration, and were injected into the wall muscle of sea cucumber at different sites with a syringe and a 29 G needle. The concentration was 1×10^8 cells mL^{-1} and the skin ulceration and peristome edema could be observed after 4 d.

2.2 RNA Preparation, cDNA Library Construction and Sequencing

The intestine, mesentery and coelomocyte were collected from eight sea cucumber individuals 48 h after injection with 1 mL *Vibrio* sp. (1×10^8 cells mL^{-1}) and then rinsed in seawater and immediately frozen in liquid nitrogen. They were then transferred in a liquid nitrogen container to the laboratory and stored at –170℃ until RNA preparation.

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) from infected intestine, mesentery and coelomocyte samples of sea cucumber specimens. The mRNAs were isolated using the PolyATtract™ mRNA isolation system (Promega, Madison, WI) according to the manufacturer's instructions. The RNA concentration was determined by spectrophotometry, and its integrity was assessed *via* electrophoresis in 1% formaldehydeagarose gel (Sambrook *et al*., 1989).

cDNA library was constructed by using the Superscript II system and the directional pBluescript II XR vector (Stratagene, La Jolla, CA) exploiting the *Eco*RI and *Xho*I restriction sites. The quality of the cDNA library was assessed by colony polymerase chain reaction (PCR) and sequencing for insert size and *E. coli* contamination. Sequencing templates were prepared from plasmids in *E. coli* DH10B (Invitrogen) and sequenced on MegaBase 1000 sequencers with a universal primer from a single end.

2.3 Clustering, Annotation, and Bioinformatic Analysis of ESTs

A Phred-Phrap-Consed package with default parameters was used to determine sequence qualities (Q20 or 99% accuracy) and to assemble the sequences. A cross-match program (University of Washington, Seattle, WA) was used to trim off vector sequences, and any sequences longer than 100 bp were saved in FASTA format for sequence comparisons (Ewing and Green, 1998; Gordon *et al*., 1998). The assembled contigs and singlets were manually examined. Assembled ESTs or unigenes were annotated by comparison with NCBI nonredundant (nr) or dbESTs (NEIBank) databases with BLASTn (e-value cutoff $1e^{-10}$) or BLASTx (e-value cut off $1e^{-5}$). Functional classes were assigned according to GO (Gene Ontology) mapping provided by the Uniprot database. KEGG analysis was based on the comparative results between our unigenes and the updated KEGG database. The network address is http://www.genome.jp/kaas-bin/kaas_main?mode=est_b (May 1st, 2009).

2.4 Assessing the Expression of EST Representing Immunity-Related Genes in the Infected Sea Cucumber by Real-Time PCR

The mRNA expression patterns of five selected EST sequences were determined in the control and infected coelomocytes of *A. japonicus* using real-time PCR. Twenty sea cucumber individuals (10−13 cm long) were randomly selected and assigned into two groups ($n=10$) to receive the injection of 100 μL sterile seawater (control) or bacteria suspension (10^8 cells mL^{-1}, *Vibrio* sp. group). Then the animals in each group were kept in different tanks under the same condition. The coelomic fluids of sea cucumbers were sampled from ten individuals in each group at two time points: on day 3 when no visible skin lesions were seen and on day 5 when skin ulceration, peristome edema and evisceration occurred in the infected individuals. The fluid samples were centrifuged at 500 *g* for 10 min (Beckman, SW41) and coelomocytes were collected and immediately frozen in liquid nitrogen for RNA extraction.

Total RNA was extracted from coelomocytes using TRIzol reagent (Invitrogen) and DNA contamination was removed by RNase-free DNase I. Total RNA was dissolved in 60 μL sterilized water containing 1U RNAase inhibitor. The first strand cDNA was synthesized from 2 μL total RNA using a cDNA synthesis kit according to the manufacturer's instruction (Promega, USA). The cDNA was then diluted to 100 μL and stored at –20℃.

The expression of immunity-related genes were quantified with real-time PCR while β-actin was employed as the reference gene. The reaction mixture contained 2 μL

of diluted cDNA, 0.8 μL, 20 pmol μL^{-1} primers (Table 1), 21.4 μL H_2O and 25 μL SYBR Green PCR core reagent (TaKaRa, Dalian, China). Amplification was carried out in triplicate as follows: pre-denaturing at 95℃ for 1 min, followed by 42 cycles of 10 s at 95℃ and 20 s at 60℃, 80 cycles of 15 s at 60℃. During the PCR amplification, the fluorescence signals were scanned and registered at every cycle, among which those higher than baseline10 times were used as positive signals and those of total cDNA from the coelomic fluids of sterile seawater group sea cucumbers were used as standard controls. All samples were run in triplicate. The threshold cycle (CT) represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. All data were analyzed by the method of $2^{-\Delta\Delta CT}$ and variance analysis (one-way ANOVA) and F test (P<0.05) were performed using SPSS 13.0. The relevant relations are as follows:

$$\triangle C_t(\text{sterile seawater injection group}) = C_t(\text{target gene}) - C_t(\beta\text{-actin}); \quad (1)$$

$$\triangle C_t(100\,\mu\text{L bacteria injection group}) = C_t(\text{target gene}) - C_t(\beta\text{-actin}); \quad (2)$$

$$\triangle\triangle C_t = \triangle C_t(100\,\mu\text{L bacteria injection group}) - \triangle C_t(\text{sterile seawater injection group}). \quad (3)$$

Table 1 PCR primer pairs used in the real-time PCR experiments

Target gene	Primer sequence (5'-3')	Product length (bp)
Actin	F5'-CAACTGGGATGACATGGAGAAG-3' R5'-TTGGCTTTGGGGTTCAGG -3'	114
Serum lectin isoform	F5'- GAACCTTTGTTTGGACCGATG -3' R5'- TCGGTGCCTTGATTTTGTTTTC -3'	83
Ubuiquitin/ribosomal L40 fusion protein	F5'- TCCTCCCGACCAACAGAGA -3' R5'- CACGGAGACGAAGAACCAGA -3'	116
Complement component C3	F5'- AGAACCAGAGGTCTTACTTGGTG -3' R5'- AAACTGCTCCCTGACTCTCCTTT -3	150
Complement component 3-like gene	F5'-CCATCAAGGAAGATTTAGAACAGGT -3' R5'-GTTCTTGGCTTTGTGTAGGGATG -3'	117
Apostichopus japonicus ferritin	F5'- CGTGGAACGGTCGGACTACTT -3' R5'- TCGGTGCCTTGATTTTGTTTTC -3'	83

3 Results

3.1 cDNA Library

For the library of infected intestines of sea cucumber, a total of 3.24×10^5 clones were obtained. They were each assigned an identifier indicating their number and position in a 96-well plate. All selected clones were sequenced from 5'-end in a single-pass manner. The generated ESTs were evaluated using Phred program, and vector and low quality sequences were trimmed. A total of 1106 high-quality ESTs were obtained in the sea cucumber intestinal cDNA library and 75.9% of them were longer than 500 bp (Table 1). The EST sequences have been deposited in GenBank at NCBI under the accession numbers from GO269754 to GO270859.

3.2 Clustering of ESTs

These ESTs were assembled into 533 unigenes, including 165 contigs and 368 singlets (Table 2). To explore the biochemical functions of the genes represented by the ESTs sequences, we searched their homologous proteins in the NCBI non-redundant protein databank (NR) using Blastx (Altschul *et al*., 1997). All the 168 annotation unigene sequences (100%) matched at least one known protein when the e-value threshold was set at 1×10^{-3}. Among these sequences, 44% had scores $<1\times10^{-50}$, while 93% had scores $<1\times10^{-10}$.

The percentage of these unigenes that matched sequences from the nonredundant library in GenBank was 31.5% in sea cucumber. The remaining 68.5% unigenes were not found in ESTs of NEI Bank. The unannotated unigenes were also analyzed according to the interproscan database and this combined procedure increased the annotation rate for the unigenes. Fig.1 shows the distribution of ESTs in contigs. Approximately 15.1% of the annotated contigs have more than ten clones, and 31.4% have more than fives clones, indicating that the sampling size is adequate for a qualitative test to discover new

Table 2 Expressed sequence tags and annotations were summarized from libarary of *Apostichopus japonicus*

Description		Number
Number of high-quality ESTs		1106
Number of unigenes		533
Number of contigs		165
Number of singlets		368
Number of ESTs in Contigs		759
Number of annotation (nr+nt)	contigs	85(51.5%)
	singlets	83(22.6%)
Number of unannotation (nr+nt)	contigs	80
	singlets	285

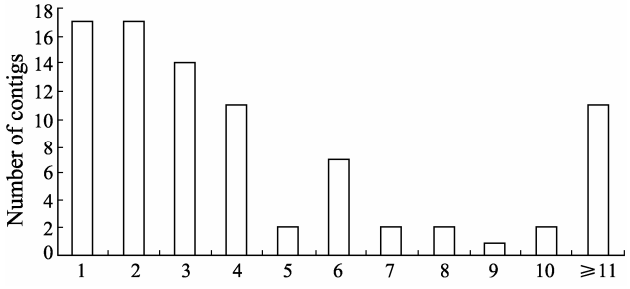

Fig.1 Number of ESTs in each contigs.

genes. According to GO analysis (Conesa *et al*., 2005) the major annotated groups were further classified into three categories: cellular component (39.53%), molecular function (22.92%), and biological process (37.54%) (Fig.2). The annotated unigenes with known functions and the immunity-related genes are listed in Table 3.

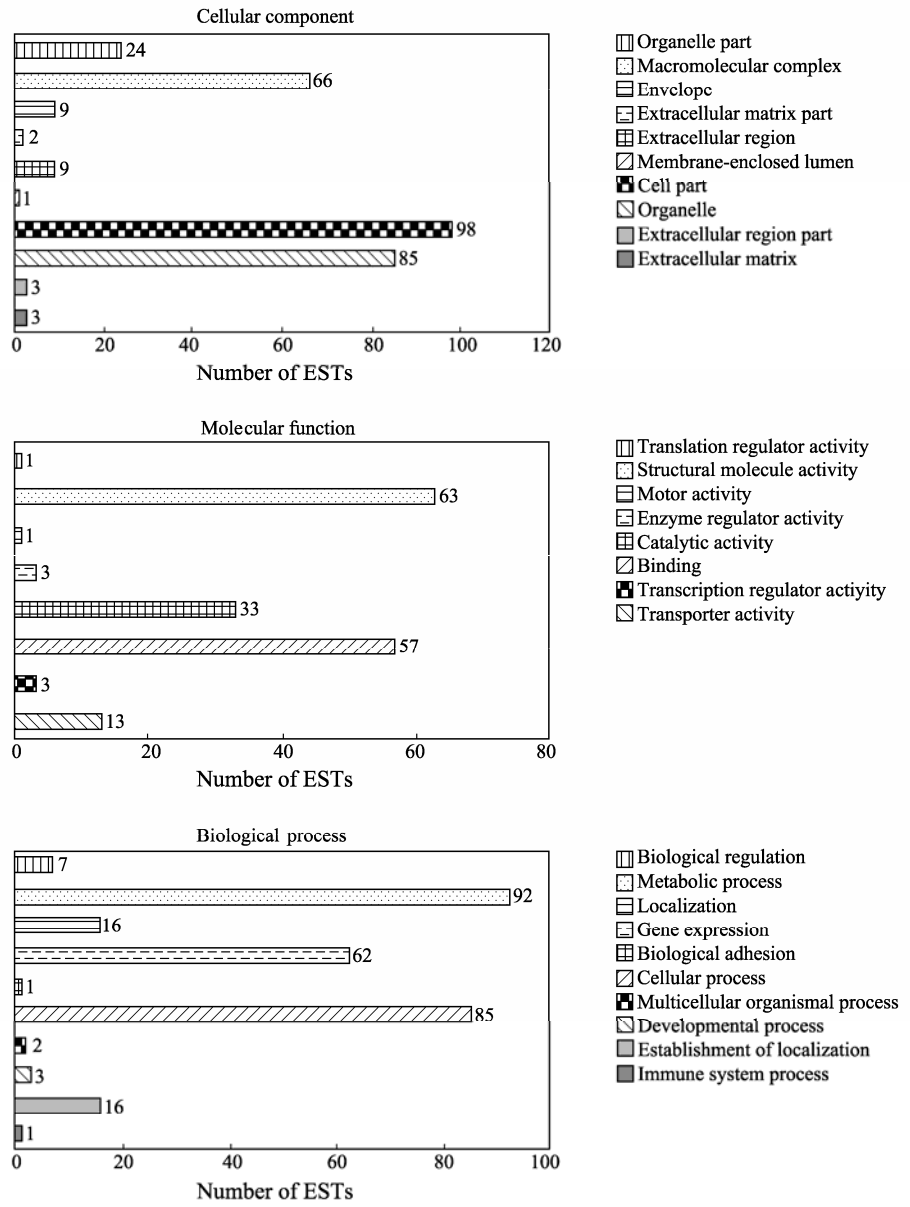

Fig.2 Abundance of transcripts represented by the ESTs in each functional category.

Table 3 One hundred and sixty-eight genes with known functions

Contig or single	Length (bp)	E-value distribution	Identity (%)	Gene names
∗Contig106	477	3.00E-33	94	*Apostichopus japonicus* ferritin mRNA, complete cds
Contig119	711	1.00E-50	58	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide, isoform CRA_b (*Homo sapiens*)
Contig148	516	5.00E-55	71	similar to 60S ribosomal protein L27A-related (*Strongylocentrotus purpuratus*)
Contig15	555	1.00E-53	96	Actin, non-muscle
Contig156	760	6.00E-70	72	hypothetical protein LOC549444 (*Xenopus tropicalis*)
Contig162	547	7.00E-30	49	predicted protein (*Nematostella vectensis*)
Contig163	531	3.00E-42	66	ribosomal protein S15 (*Solea senegalensis*)
∗Contig168	576	2.00E-27	71	similar to cytochrome c oxidase subunit VIa (*Strongylocentrotus purpuratus*)
Contig171	605	2.00E-75	97	B-actin (*Pagrus major*)

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Contig or single	Length (bp)	E-value distribution	Identity (%)	Gene names
Contig173	587	2.00E-96	89	similar to QM-like protein (*Strongylocentrotus purpuratus*)
Contig177	508	7.00E-11	30	tropomyosin 1 (*Saccoglossus kowalevskii*)
Contig18	632	2.00E-65	91	*Apostichopus japonicus* clone AH210 microsatellite sequence
Contig182	508	3.00E-28	56	similar to ribosomal protein L28 (*Strongylocentrotus purpuratus*)
Contig187	494	2.00E-49	75	similar to ribosomal protein L31 (*Strongylocentrotus purpuratus*)
Contig189	669	2.00E-31	59	60S ribosomal protein L22 (Development-specific protein 217)
*Contig190	1052	2.00E-19	31	similar to DD104 protein, upregulated upon bacterial challenge and trauma (*Strongylocentrotus purpuratus*)
Contig192	608	1.00E-65	62	ribosomal protein rpl15 (*Lineus viridis*)
Contig193	602	2.00E-69	81	similar to putative ribosomal protein L19e (*Strongylocentrotus purpuratus*)
Contig194	697	2.00E-61	70	cyclophilin A; rotamase (*Branchiostoma belcheri tsingtaunese*)
Contig207	638	6.00E-72	68	ribosomal protein LP0 (*Solea senegalensis*)
Contig211	508	7.00E-52	92	similar to 40S ribosomal protein S2 (*Gallus gallus*)
Contig22	687	4.00E-30	97	*Stichopus japonicus* mRNA for arginine kinase, complete cds
Contig221	562	1.00E-42	65	similar to Ribosomal protein L14, partial (*Strongylocentrotus purpuratus*)
Contig225	762	3.00E-63	63	ribosomal protein L13 (*Danio rerio*)
*Contig231	1514	4.00E-56	78	cytochrome c oxidase subunit I (*Cucumaria miniata*)
Contig242	1932	7.00E-30	84	TPA: putative 40S ribosomal protein S25 isoform 1 (*Spadella cephaloptera*)
Contig249	398	7.00E-10	37	40S ribosomal protein S13 (*Lumbricus rubellus*)
Contig250	502	2.00E-30	69	similar to 40S ribosomal protein S24 (*Strongylocentrotus purpuratus*)
Contig251	273	1.00E-21	62	putative ribosomal protein S24 (*Sipunculus nudus*)
Contig255	736	1.00E-25	82	hypothetical protein Bm1_07595 (*Brugia malayi*)
Contig260	608	6.00E-21	77	hypothetical protein LOC734491 (*Xenopus laevis*)
Contig262	668	2.00E-35	43	NADH dehydrogenase subunit 4 (*Cucumaria miniata*)
Contig263	566	2.00E-71	93	ribosomal protein S13 (*Danio rerio*)
*Contig267	708	1.00E-86	75	cytochrome oxidase 1 (*Parastichopus californicus*)
Contig272	1052	1.00E-119	70	cytochrome b (*Cucumaria miniata*)
Contig275	549	5.00E-52	89	similar to 40S ribosomal protein S20 (S22) (*Strongylocentrotus purpuratus*)
Contig280	573	1.00E-60	73	ribosomal protein S10 (*Branchiostoma belcheri tsingtaunese*)
Contig282	583	2.00E-16	70	putative ribosomal protein P2 (*Barentsia elongata*)
Contig284	449	2.00E-24	83	40S ribosomal protein S29 (*Scarabaeus laticollis*)
Contig285	762	1.00E-102	77	hypothetical protein LOC779385 (*Xenopus laevis*)
Contig286	605	2.00E-78	70	similar to ribosomal protein S8 (*Gallus gallus*)
Contig289	527	7.00E-52	90	similar to ribosomal protein L30 (*Strongylocentrotus purpuratus*)
Contig290	514	5.00E-28	65	similar to ribosomal protein L36 (*Strongylocentrotus purpuratus*)
Contig291	668	4.00E-86	88	hypothetical protein (Strongylocentrotus purpuratus)
*Contig296	707	1.00E-95	74	ferritin (*Apostichopus japonicus*)
Contig297	671	2.00E-69	64	similar to ribosomal protein L5 (*Strongylocentrotus purpuratus*)
*Contig300	604	5.00E-64	95	ubiquitin/ribosomal L40 fusion protein (*Scleronephthya gracillimum*)
Contig302	566	5.00E-45	92	similar to ribosomal protein L44 (*Strongylocentrotus purpuratus*)
*Contig306	825	1.00E-73	60	ATP synthase F0 subunit 6 (*Cucumaria miniata*)
Contig308	472	4.00E-26	68	putative ribosomal protein S21 (*Barentsia elongata*)
Contig309	599	7.00E-64	83	Unknown (protein for IMAGE:8375510) (*Rattus norvegicus*)
Contig31	641	1.00E-32	86	similar to Ribosomal protein S16 isoform 1 (*Strongylocentrotus purpuratus*)
Contig310	600	2.00E-69	89	similar to H3.3 histone (*Strongylocentrotus purpuratus*)
*Contig312	664	1.00E-93	78	cytochrome c oxidase subunit II (*Paracentrotus lividus*)
Contig314	538	1.00E-43	83	similar to ribosomal protein S26e (*Strongylocentrotus purpuratus*)

(to be continued)

(continued)

Contig or single	Length (bp)	E-value distribution	Identity (%)	Gene names
Contig316	515	6.00E-43	90	similar to 40S ribosomal protein S27-like protein (*Macaca mulatta*)
Contig317	419	2.00E-40	83	60S ribosomal protein L37A (*Branchiostoma belcheri*)
Contig320	739	2.00E-66	57	NADH dehydrogenase subunit 1 (*Cucumaria miniata*)
Contig321	617	2.00E-54	75	similar to Ribosomal protein L27 isoform 1 (*Strongylocentrotus purpuratus*)
Contig322	595	5.00E-59	73	ubiquitin/40S ribosomal protein S27a (*Ornithodoros parkeri*)
Contig323	565	8.00E-26	66	predicted protein (*Nematostella vectensis*)
Contig324	577	1.00E-71	72	similar to 60S ribosomal protein L12 (*Monodelphis domestica*)
Contig325	619	2.00E-27	40	similar to Myosin light chain 1, skeletal muscle isoform (MLC1F) (A1 catalytic)
Contig326	615	4.00E-16	35	similar to fibropellin Ia (*Strongylocentrotus purpuratus*)
Contig327	909	5.00E-60	44	NADH dehydrogenase subunit 2 (*Cucumaria miniata*)
*Contig328	632	2.00E-90	76	cytochrome c oxidase subunit II (*Cucumaria miniata*)
*Contig332	881	2.00E-99	72	cytochrome c oxidase subunit III (*Cucumaria miniata*)
Contig333	652	3.00E-53	58	similar to calponin (*Strongylocentrotus purpuratus*)
Contig334	531	8.00E-17	69	60S ribosomal protein L29 (*Brugia malayi*)
Contig335	510	7.00E-57	87	similar to S17 (*Strongylocentrotus purpuratus*)
*Contig336	1142	7.00E-83	90	translationally-controlled tumor protein (*Apostichopus japonicus*)
Contig338	576	2.00E-13	88	*Argopecten irradians* clone ScaE_6721 microsatellite sequence
Contig339	722	4.00E-27	83	Pearsonothuria graeffei isolate 28 12S ribosomal RNA gene, partial sequence; mitochondrial
Contig34	543	1.00E-72	72	similar to Rpl13a-prov protein (*Strongylocentrotus purpuratus*)
Contig343	615	8.00E-27	99	*Apostichopus japonicus* clone 1 16S ribosomal RNA gene, partial sequence; mitochondrial
Contig344	1206	6.00E-30	99	*Apostichopus japonicus* clone 2 16S ribosomal RNA gene, partial sequence; mitochondrial
Contig61	658	1.00E-72	81	40S ribosomal protein S2 (*Ornithodoros parkeri*)
Contig64	613	6.00E-12	63	similar to myosin regulatory light chain (*Strongylocentrotus purpuratus*)
Contig76	705	3.00E-06	42	hypothetical protein PC103780.00.0 (*Plasmodium chabaudi chabaudi*)
Contig77	688	3.00E-62	90	*Stichopus japonicus* DNA, microsatellite, clone:Psj2409
Contig78	537	2.00E-36	83	putative 60S ribosomal protein RPL12 (*Flustra foliacea*)
Contig93	674	3.00E-46	87	*Apostichopus japonicus* clone AH210 microsatellite sequence
Contig95	595	3.00E-44	90	similar to 40S ribosomal protein S27-like protein (*Canis familiaris*)
Contig97	537	3.00E-58	89	*Apostichopus japonicus* clone AH210 microsatellite sequence
hsaa_0001_A04.ab1	612	1.00E-06	48	CG30281 CG30281-PA (*Drosophila melanogaster*)
hsaa_0001_A05.ab1	565	3.00E-59	67	aldolase A, isoform CRA_d (*Rattus norvegicus*)
hsaa_0001_A09.ab1	393	6.00E-27	81	similar to mCG19129 (*Homo sapiens*)
hsaa_0001_B09.ab1	329	7.00E-24	86	Lvariegatus 26S rRNA 3' region
*hsaa_0001_B11.ab1	542	1.00E-29	49	similar to KH domain containing, RNA binding, signal transduction associated 2 (E*quus caballus*)
hsaa_0001_C09.ab1	543	1.00E-64	65	similar to MGC89105 protein (*Strongylocentrotus purpuratus*)
hsaa_0001_C11.ab1	471	3.00E-29	56	hypothetical protein LOC752411 (*Strongylocentrotus purpuratus*)
hsaa_0001_E02.ab1	265	3.00E-07	50	similar to apolipoprotein B (*Strongylocentrotus purpuratus*)
hsaa_0001_F02.ab1	183	5.00E-26	95	hypothetical protein TVAG_199440 (*Trichomonas vaginalis* G3)
hsaa_0001_F10.ab1	534	8.00E-51	62	similar to ribosomal protein L23a (S*trongylocentrotus purpuratus*)
hsaa_0001_G12.ab1	447	4.00E-68	84	similar to sea urchin Arp3 (SUArp3) isoform 1 (*Strongylocentrotus purpuratus*)
hsaa_0001_H04.ab1	547	1.00E-19	55	predicted protein (*Nematostella vectensis*)
hsaa_0002_B06.ab1	196	6.00E-20	93	*Apostichopus japonicus* clone AH181 microsatellite sequence
hsaa_0002_B11.ab1	185	2.00E-72	96	*Stichopus japonicus* gene for 18S rRNA, partial sequence
hsaa_0002_D02.ab1	498	3.00E-56	82	ribosomal protein S12 (*Branchiostoma belcheri*)
hsaa_0002_F03.ab1	561	5.00E-41	55	similar to testis enhanced gene transcript (BAX inhibitor 1) (*Equus caballus*)

(to be continued)

(continued)

Contig or single	Length (bp)	E-value distribution	Identity (%)	Gene names
hsaa_0003_A01.ab1	661	5.00E-08	27	similar to GAC-1 (*Strongylocentrotus purpuratus*)
hsaa_0003_C08.ab1	630	1.00E-179	96	12S rRNA (*Parastichopus californicus* = *holothuroids*, Mitochondrial, 1366 nt)
hsaa_0003_D01.ab1	633	2.00E-55	87	similar to Ribosomal protein S2 (*Strongylocentrotus purpuratus*)
hsaa_0003_D09.ab1	665	2.00E-10	27	similar to fibrosurfin, partial (*Strongylocentrotus purpuratus*)
*hsaa_0003_E010.ab1	644	3.00E-56	73	similar to Chain A, Crystal Structure Of Recombinant Chicken Sulfite Oxidase With Arg At Residue 161 (*Strongylocentrotus purpuratus*)
*hsaa_0003_E08.ab1	682	6.00E-09	49	collagen type I alpha 1 (*Oryzias latipes*)
*hsaa_0003_F01.ab1	639	1.00E-34	72	mitochondrial ATP synthase alpha subunit precursor (*Strongylocentrotus purpuratus*)
hsaa_0004_B02.ab1	621	1.00E-56	62	ribosomal protein L6 (*Gallus gallus*)
hsaa_0004_C07.ab1	666	3.00E-36	40	Actin-53| actin (*Nicotiana tabacum*)
hsaa_0004_C11.ab1	597	1.00E-66	74	predicted protein (*Nematostella vectensis*)
hsaa_0004_G02.ab1	654	2.00E-69	81	similar to ribosomal protein L17 isoform 2 (*Strongylocentrotus purpuratus*)
hsaa_0004_H010.ab1	561	2.00E-30	42	similar to connectin/titin (*Strongylocentrotus purpuratus*)
hsaa_0004_H11.ab1	604	9.00E-59	75	CG9836 CG9836-PA (*Drosophila melanogaster*)
hsaa_0005_D010.ab1	610	3.00E-12	51	similar to ORF2-encoded protein (S*trongylocentrotus purpuratus*)
hsaa_0005_D09.ab1	498	1.00E-77	99	Family T1, proteasome beta subunit, threonine peptidase (*Trichomonas vaginalis* G3)
hsaa_0005_E03.ab1	554	2.00E-17	51	Hypothetical protein CBG19793 (*Caenorhabditis* briggsae AF16)
*hsaa_0005_E04.ab1	536	1.00E-27	43	complement component 3-like protein (*Carcinoscorpius rotundicauda*)
hsaa_0005_E11.ab1	704	2.00E-67	67	hypothetical protein (*Ornithorhynchus anatinus*)
hsaa_0005_G05.ab1	349	1.00E-10	46	putative HMG-like protein (*Dermacentor variabilis*)
*hsaa_0005_G07.ab1	633	1.00E-28	38	similar to complement component C3 (*Strongylocentrotus purpuratus*)
hsaa_0005_H01.ab1	582	6.00E-67	64	similar to filamin, partial (*Strongylocentrotus purpuratus*)
hsaa_0005_H010.ab1	534	2.00E-57	59	hypothetical protein, partial (*Strongylocentrotus purpuratus*)
hsaa_0006_C05.ab1	566	1.00E-20	42	similar to fibropellin Ia (*Strongylocentrotus purpuratus*)
hsaa_0006_E01.ab1	671	5.00E-11	94	*Apostichopus japonicus* clone AH165 microsatellite sequence
hsaa_0006_E02.ab1	626	2.00E-23	35	similar to transmembrane protein UO-44D (*Strongylocentrotus purpuratus*)
hsaa_0006_E09.ab1	588	6.00E-36	51	hypothetical protein, partial (*Strongylocentrotus purpuratus*)
hsaa_0006_F11.ab1	546	6.00E-12	47	similar to CG14483-PA (*Tribolium castaneum*)
hsaa_0007_B03.ab1	613	2.00E-18	39	similar to alpha macroglobulin (*Strongylocentrotus purpuratus*)
hsaa_0007_C01.ab1	561	1.00E-67	95	Similar to ribosomal protein S23 (*Oncorhynchus mykiss*)
hsaa_0007_C06.ab1	680	6.00E-63	63	similar to Chain A, The Structure Of Alpha-N- Acetylgalactosaminidase (*Strongylocentrotus purpuratus*)
*hsaa_0007_C08.ab1	486	7.00E-21	55	similar to forkhead transcription factor C (*Strongylocentrotus purpuratus*)
hsaa_0007_C11.ab1	583	7.00E-44	87	*Apostichopus japonicus* clone AH210 microsatellite sequence
hsaa_0007_G06.ab1	676	1.00E-35	77	glutaredoxin 5 (*Xenopus tropicalis*)
hsaa_0007_G07.ab1	528	5.00E-08	35	similar to TPA-induced protein 11B-mouse isoform 2 (*Strongylocentrotus purpuratus*)
hsaa_0007_H01.ab1	532	3.00E-75	73	arginine kinase (*Stichopus japonicus*)
hsaa_0007_H03.ab1	614	4.00E-86	94	elongation factor 1 alpha (*Mytilus galloprovincialis*)
hsaa_0008_A01.ab1	485	8.00E-12	34	cellular nucleic acid binding protein mutant delta-RGG (*Synthetic construct*)
hsaa_0008_A10.ab1	592	2.00E-11	75	hypothetical protein LOC541328 (*Danio rerio*)
hsaa_0008_D01.ab1	597	7.00E-59	83	similar to putative ribosomal protein S14e (*Strongylocentrotus purpuratus*)
*hsaa_0008_D10.ab1	605	6.00E-17	39	serum lectin isoform 4 (*Verasper variegatus*)
hsaa_0008_E03.ab1	615	1.00E-22	46	predicted protein (*Nematostella vectensis*)
hsaa_0008_E11.ab1	620	4.00E-34	59	basic transcription factor 3 (*Ciona intestinalis*)
hsaa_0009_A04.ab1	583	1.00E-43	60	similar to signal sequence receptor, beta (*Equus caballus*)
hsaa_0009_B07.ab1	699	1.00E-85	83	Hypothetical protein CBG16945 (*Caenorhabditis briggsae*)
hsaa_0009_C03.ab1	631	3.00E-13	53	similar to HrTT-1 (*Strongylocentrotus purpuratus*)
hsaa_0009_F08.ab1	642	4.00E-41	65	unnamed protein product (*Tetraodon nigroviridis*)

(to be continued)

(continued)

Contig or single	Length (bp)	E-value distribution	Identity (%)	Gene names
*hsaa_0009_G11.ab1	585	1.00E-08	28	similar to complement component C3 (*Strongylocentrotus purpuratus*)
*hsaa_0009_H04.ab1	616	3.00E-19	52	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9) (*Danio rerio*)
hsaa_0009_H07.ab1	535	1.00E-55	62	ribosomal protein L7 (*Spodoptera frugiperda*)
hsaa_0010_B09.ab1	642	1.00E-24	53	hypothetical protein LOC574826 (*Strongylocentrotus purpuratus*)
hsaa_0010_C07.ab1	555	6.00E-11	26	predicted protein (*Nematostella vectensis*)
hsaa_0010_D11.ab1	532	5.00E-66	80	ribosomal protein S11 (synthetic construct)
hsaa_0010_G09.ab1	428	2.00E-42	75	similar to putative ribosomal protein L34 (*Strongylocentrotus purpuratus*)
hsaa_0010_H01.ab1	695	3.00E-22	49	coiled-coil-helix-coiled-coil-helix domain containing 2 (*Danio rerio*)
hsaa_0011_B02.ab1	572	6.00E-09	74	putative ribosomal protein L35a (*Sipunculus nudus*)
*hsaa_0011_B03.ab1	613	9.00E-35	38	similar to cAMP response element modulator tau alpha gamma (*Strongylocentrotus purpuratus*)
hsaa_0011_B07.ab1	582	4.00E-13	57	hypothetical protein LOC569582 (*Danio rerio*)
*hsaa_0011_D06.ab1	515	3.00E-48	71	similar to ATP synthase G chain (*Strongylocentrotus purpuratus*)
*hsaa_0011_E03.ab1	598	6.00E-21	45	similar to matrix metalloproteinase 14 (*Strongylocentrotus purpuratus*)
hsaa_0012_C07.ab1	592	8.00E-81	84	similar to vacuolar proton pump delta polypeptide (*Strongylocentrotus purpuratus*)
hsaa_0012_D08.ab1	602	1.00E-73	80	similar to spermatogenesis associated 4 (*Strongylocentrotus purpuratus*)
hsaa_0012_D11.ab1	651	5.00E-49	84	FKBP12 (*Bombyx mori*)
hsaa_0012_E06.ab1	597	1.00E-77	68	hypothetical protein (*Monodelphis domestica*)
hsaa_0012_E12.ab1	620	1.00E-36	44	similar to Legumain precursor (Asparaginyl endopeptidase) (Protease, cysteine 1) isoform 1 (*Canis familiaris*)
hsaa_0012_F10.ab1	550	6.00E-16	63	similar to MGC85582 protein (*Strongylocentrotus purpuratus*)
hsaa_0012_H04.ab1	538	8.00E-46	68	similar to ribosomal protein L32 (*Strongylocentrotus purpuratus*)
hsaa_0012_H07.ab1	516	1.00E-41	55	predicted protein (*Nematostella vectensis*)

Note: * immune-related genes.

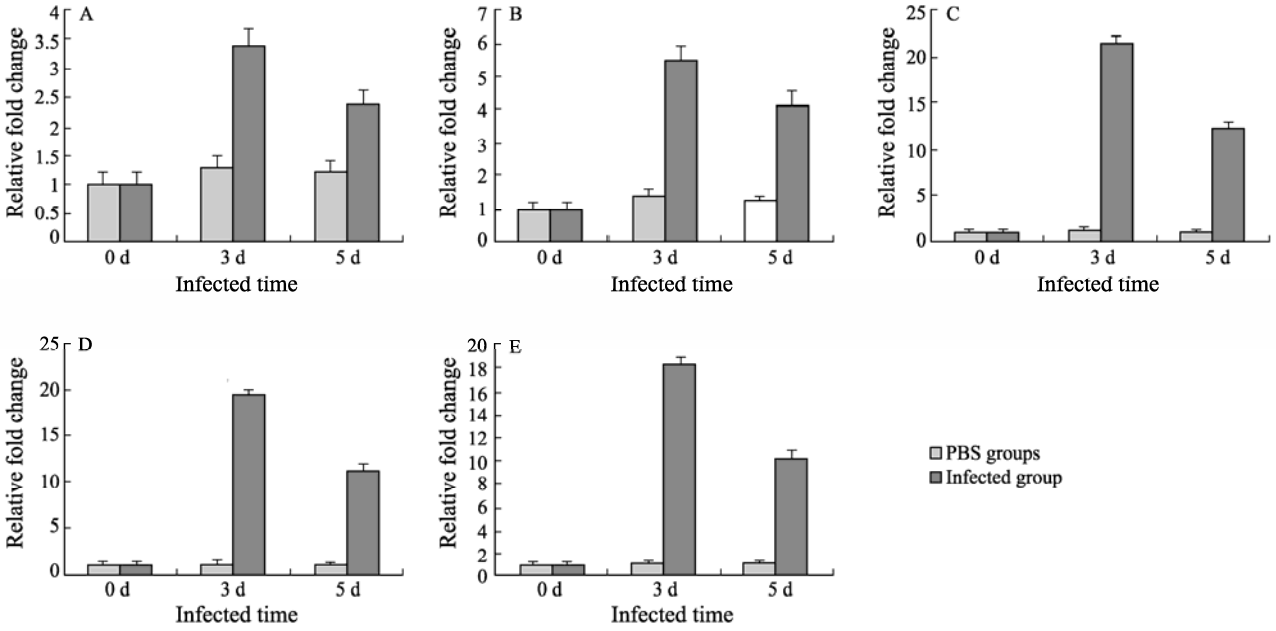

Fig.3 Relative fold changes of transcripts of five immune function-related genes in sea cucumber after *Vibrio* sp. infection. A. ferritin; B. ubuiquitin/ribosomal L40 fusion protein; C. serum lectin isoform; D. complement component C3; E. complement component 3-like protein.

3.3 Highly-Expressed Genes in the cDNA Library

The abundant genes were categorized into 10 major functional groups based on the GO categories: organele part, structural molecule activity, macromolecular com-

plex, binding, gene expression, cellular process, catalytic activity, metabolic process, cellular process, and cell component. The top five functional categories include organele part, metabolic process, cellular process, cell component, and macromolecular complex. The third level GO categories provided more information on cellular functions and subcellular locations. Genes encoding the proteins involved in protein synthesis, native immunity and cell division were abundant in the cDNA library. For example, the genes encoding complement component 3, ferritin, fibropellin and serum lectin isoform were among the highly expressed genes, suggesting their up-regulation during infection. The genes encoding cytochrome oxidase, ribosomal protein and NADH dehydrogenase were also highly enriched in the cDNA library after infection.

3.4 The Expression of Immunity-Related Genes

To investigate the immune gene responses to bacterial infection, the expression patterns of five immunity-related genes were assayed by real-time PCR before and 3 d, 5 d after sea cucumber was infected with *Vibrio* sp. As shown in Fig.3, the expressions of serum lectin isoform, complement component C3 gene and complement component 3-like gene were elevated more than 5 folds 3 d after *Vibrio* sp. infection. The changes in the expression of ferritin and ubuiquitin/ribosomal L40 fusion protein genes were not significant (Fig.3).

4 Discussion

A. japonicus is an economically important sea cucumber species in the marine culture industry. However, with the overdevelopment and nonstandard operation, diseases caused by bacterial and viral infection have broken out frequently in the past several years and resulted in serious economic losses. Therefore, studying the immune defense mechanism of *A. japonicus* is essential for finding effective cures.

With the advances in molecular biology, sequencing expressed sequence tag (EST) has greatly facilitated gene profiling of the immune responses in invertebrates. Ramirez-Gómez *et al.* established three cDNA libraries of normal and regenerating intestinal tissues of the sea cucumber *Holothuria glaberrima*. They analyzed 5173 expressed sequence tags (ESTs) and found 22 putative immunity-related genes (Ramírez-Gómez *et al.*, 2008). The cDNA libraries of normal and regenerating intestines at two different regeneration stages were also established (Zheng *et al.*, 2006; Ortiz-Pineda *et al.*, 2009). In addition, the completion of S. purpuratus genome project has provided valuable sources of information for several research fields including comparative immunology (Hibino *et al.*, 2006; Rast *et al.*, 2006).

In the current study, we obtained 1106 high-quality ESTs that were clustered into 533 unigenes from the cDNA library made from *Vibrio* sp.-infected intestine, mesentery and coelomocyte samples of *A. japonicus*. BlastX and BlastN searching identified 168 (31.5%) ESTs that share significant homologies with known sequences in the NCBI protein or nucleotide databases, and many of them, such as serum lectin, complement component 3, ferritin, and complement component 3-like gene, are related to immunology. These EST sequences have been deposited in GenBank of NCBI under the accession numbers from GO269754 to GO270859. They not only contribute novel sequences to the public databases but also provide information on the different gene expression patterns of sea cucumber in response to *Vibrio* infection.

We have classified most of the unigenes into three functional categories or distinct pathways and identified a panel of 25 immunity-related genes. Among the five genes selected for further assayed by real-time PCR, genes encoding serum lectin isoform, complement component C3 and complement component 3-like protein showed most remarkable increase in *A. japonicus* after they were infected by *Vibrio* sp. for 3 d. Since they are the most abundantly expressed immune functional genes in our experiment, they may play important roles in the immune defense mechanism of sea cucumber against bacterial infection. The expression of ferritin and ubuiquitin/ribosomal L40 fusion protein were also induced by the bacterial infection but to a lesser extent. These results indicate that the echinoderm immunity system is not only an archaic set of genes. The response to bacterial infection may be more complex than previously hypothesized.

The library we constructed in this study is a valuable molecular tool for future study of host defense of sea cucumber against bacterial infection. The gene expression data may offer new evidence to support the importance of the immune system in the evolution and development of sea cucumber.

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