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Full Length Research Paper

Generation and analysis of cDNA library from lipopolysaccharide-stimulated gastropod abalone (*Haliotis diversicolor supertexta)* hemocytes

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A cDNA Library of lipopolysaccharide-stimulated gastropod abalone hemocytes was constructed using switching mechanism at 5' end of RNA transcript (SMART) approach. 300 random clones were selected and sequenced. 180 ESTs was gained. After assembling, we obtained 24 contigs and 119 singlets finally, some of which were immune- related genes. These immune-related genes include cytidine deaminase, ferritin, nonmuscle myosin essential light chain, cytochrome c oxidase subunit I, CD63 antigen-like protein and lysosomal-associated transmembrane protein. This study may contribute to the understanding of the immune mechanism of gastropod abalone *Haliotis diversicolor supertexta*.

Key words: *Haliotis diversicolor supertexta,* cDNA Library, gene expression, immune mechanism.

INTRODUCTION

Abalone is an important mollusc species for commercial production in the world including Australia, China, Japan, Korea, Mexico, South Africa, and the United States (Gordon and Cook, 2001). Since the late 1990s, the global industry of abalone has been gradually decreased due to diseases and environmental pollution (Gardner et al., 1995; Moore et al., 2000). Therefore, more investigation is required to aid in understanding the innate immune system of abalone for aquaculture development.

A critical step in any immune response is the recognition of invading organisms. It is mediated by many proteins, including pattern recognition proteins (PRPs), which recognize and bind to molecules present on the surface of microorganisms (Janeway, 1989). The binding of PRPs to lipopolysaccharide (LPS) triggers a series of responses which lead to the activation of the host defence system (Lee and Soderhall, 2002). LPS is an essential cell wall component of Gram-negative bacteria.

LPS stimulated polyclonal proliferation of salmonid lymphocytes (Warr and Simon, 1983), respiratory burst and phagocytic activity of macrophages (Solem et al., 1995; Dalmo and Seljelid, 1995), and elicited secretion of IL-1 like compounds in channel catfish (Clem et al., 1985). LPS injected into red sea bream, *Pagrus major*, enhanced macrophage phagocytic activity (Salati et al., 1987). Similarly, LPS stimulated the production of macrophage activating factor in goldfish lymphocytes (Neumann et al., 1995).

Some reports showed that the effect of LPS has been studied in some aquatic animals, such as: Red sea bream ((Salati et al., 1987), trout (MacKenzie et al., 2003) and yellow grouper (Wang and Wu, 2007). However, there is still little information about the molecular response of abalone to LPS. In this study, a cDNA library was constructed to identify expressed genes in LPS-stimulated gastropod abalone hemocytes in an attempt to understand the molecular processes involved in bacteria

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Abbreviations: **PRPs**, Pattern recognition proteins; **LPS**, lipopolysaccharide.

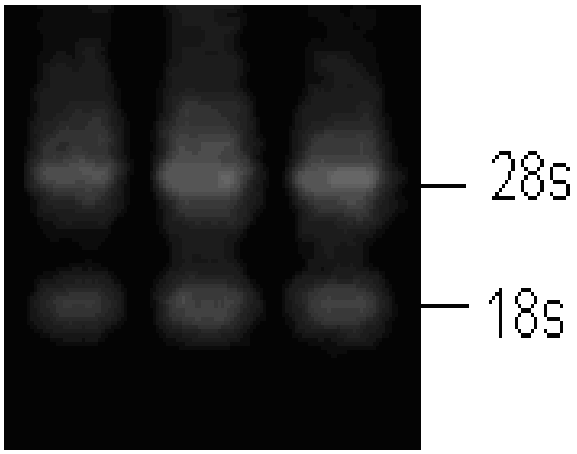

Figure 1. 1.2% agarose gel electrophoresis of the total RNA extracted from LPS-stimulated *H. diversicolor supertexta* hemocytes.

disease primary prevention.

MATERIALS AND METHODS

Animals, immune challenge and tissue collection

Healthy abalones (*Haliotis diversicolor supertexta*), 3 years of age, were collected from an abalone farm in Xiamen (Fujian, China) and kept in artificial seawater with a cycling system at 23°C (Jiang and Wu, 2007). Six abalones were challenged by injecting 50 uL *Escherichia coli* lipopolysaccharide (LPS) (1 mg/mL diluted in sterile 0.9% sodiumchloride). Hemocytes were collected at 12 h post injection by centrifugation (700 g, 10 min, 4°C).

Total RNA isolation

Total RNA was isolated from LPS -stimulated abalone hemocytes using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA integrity was checked using 1% formaldehyde agarose gel. The quantity and quality of RNA were determined by absorbance at A260 and at A260/280 ratios, respectively, using a Perkin-Elmer Lambda 3B spectrophotometer.

Construction of cDNA library

Abalone cDNA library was constructed by a SMART^{TM} cDNA library construction kit (Clontech, USA) according to the manufacture's instruction with a few modifications. Briefly, the isolated RNA was reverse transcribed to synthesize first strand cDNA at 42°C for 1 h. Then first strand cDNA was mixed with PCR primer and other PCR reagents at 95°C for 30 s followed by 20 cycles of 95°C for 10 s and 68°C for 6 min to synthesize double-strand DNA. After digestion and purification, double-strand DNA was ligated to TriplEx2 vectors. Then the resulting ligation reaction was packed using the Lambda DNA Packaging System (Promega, USA) following the manufacturer's specifications. Screening of the abalone cDNA library was based on polymerase chain reaction (PCR) with the pTriplEx2 sequencing primers. The forward primer is 5′-CTCCGAGATCTGGACGAGC-3′, and the reverse primer is 5′-TAATACGACTCACTATAGGGC-3′. Positive plasmid clones were grown in liquid cultures and induced to a high copy number for direct sequencing using an ABI PRISM 3730 automated sequencer (Applied Biosystems).

Sequence analysis

DNA traces were assembled with PHRED/PHRAP/CONSED package (www.phrap.org). Vector contamination sequences were removed with the program Cross_match. Low-quality bases (quality score <20) were trimmed from both ends of sequences using a PHRED. EST sequences corresponding to the same transcript were then assembled with the program PHRAP. Sequence similarity analysis was performed by using the BLASTX program (Altschul et al., 1997) with threshold E-value ≤1e-5, against the non-redundant database provided by National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Classification of annotated sequence was searched by Gene Ontology (http://www.godatabase.org/).

RESULTS

Construction of abalone cDNA library

In order to analyze gene expressed in LPS-stimulated abalone hemocytes and to facilitate functional analysis of cDNA clones, a SMART cDNA library was constructed. Total RNA with high quality is shown in Figure 1. The primary titer of the library was 2×10^8 pfu/ml, and the recombinant phage in the library was 90%. The quality of cDNA library was examined by sequencing of the inserted cDNA clones (Julio and Miguel, 2002; Wakimoto, 2000). The insert size was distributed in a range from 200 to 2000 bp which reflected the size distribution of the first-strand cDNA was convergence (Figure 2).

Sequence analysis

From cloned PCR products, 300 randomly-selected clones containing inserts were sequenced. After re-moving the vector sequence and the poor-quality sequence (with trim cutoff 0.05), 180 qualified ESTs were grouped into 24 contigs, and another 119 singlets with an average length of approximately 400 bp. They were submitted to GenBank database with accession numbers HS040369-HS040662. Tentative annotations were performed by using BLASTX and results were manually validated. These sequences were used to analyze gene expression profile from the LPS -stimulated abalone hemocytes.

Fifty seven (57) sequences were assigned to defined annotations, whereas 32 sequences showed similarity with unclassified genes and 54 sequences remained unknown. These sequences according to the functions were involved in immune response, protein biosynthesis, metabolism, biological process and cellular component, while the remainder belonged to none of the categories mentioned above (Table 1).

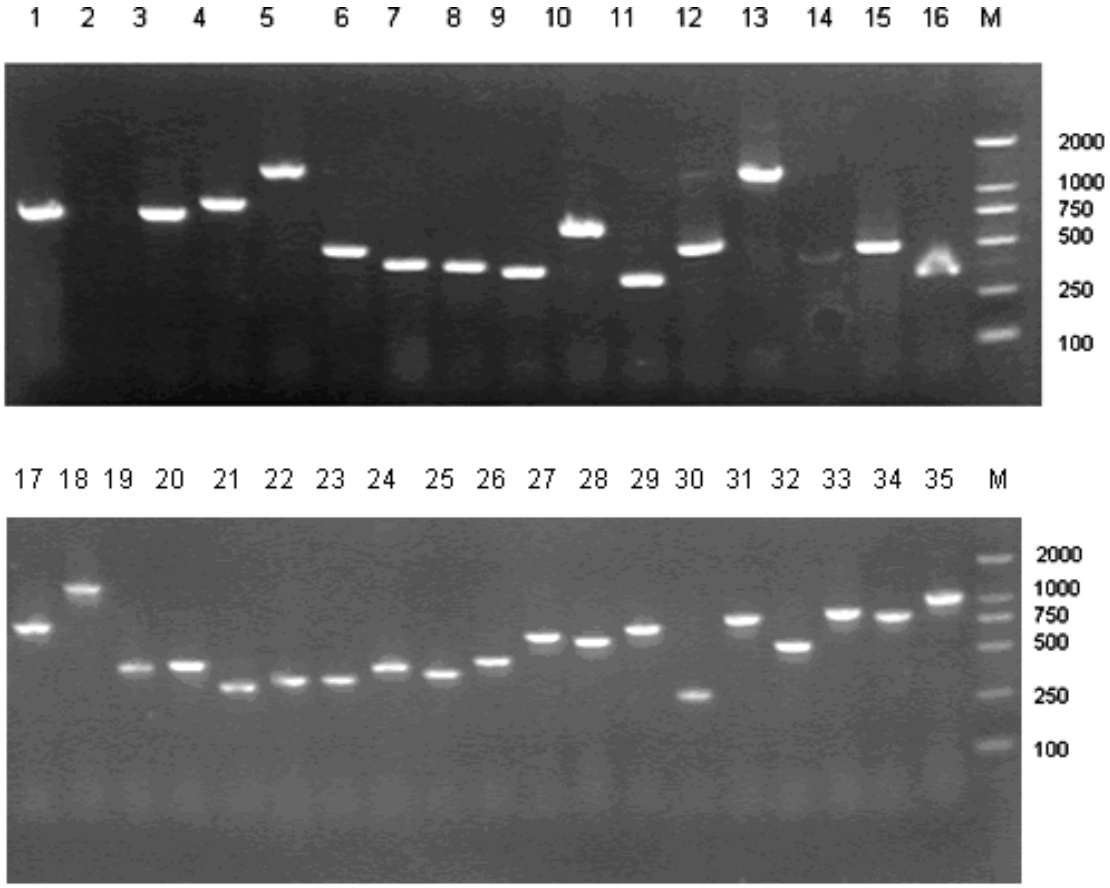

Figure 2. PCR identification of randomly selected clones from cDNA library. Lane1-35: randomly selected clones; M: marker DL2000.

DISCUSSION

To date, there were several methods used for constructing cDNA library, for example; Oligo-Capping (Maruyama and Sugano, 1994), SMART (Chenchik et al., 1996), Cap-Select (Schmidt et al., 1999) and CAP-jumping (Fimove et al., 2001). Among these methods, the SMART^{TM} cDNA Library Construction Kit provides a way for producing high-quality, full-length cDNA libraries from nanograms of total or poly A+ RNA. All commonly used cDNA synthesis methods rely on the ability of reverse transcriptase to transcribe mRNA into single-stranded (ss) DNA in the first strand reaction. In some cases, RT terminates before transcribing the complete mRNA sequence. This is particularly true for long mRNAs, especially if the first strand synthesis is primed with oligo (dT) primers only or if the mRNA contains abundant secondary structures. In addition, conventional cDNA cloning procedures use the T4 DNA polymerase to generate blunt cDNA ends after second strand synthesis. As a result, under-represented 5' ends of genes in cDNA populations tend to be 5–30 nucleotides shorter than the original mRNA.

The SMART protocols are designed to preferentially enrich for full-length cDNAs, while eliminating T4 DNA polymerase and adaptor ligation. SMART libraries are proven to contain a higher percentage of full-length clones than libraries constructed by conventional methods or other full-length cDNA synthesis protocols (Sughura et al., 2001; Kato et al., 1994). Thus, clones isolated from SMART cDNA libraries contain sequences corresponding to the complete 5' untranslated region of the mRNA. SMART cDNA libraries can facilitate preliminary mapping of transcription start sites due to the high percentage of full length clones. However, SMART cDNA libraries may not be suitable for immunoscreening for certain proteins.

The major characteristic of cDNA construction by the SMART method could improve the ratio of full length cDNA sequences. It is important to identify a new gene by constructing a cDNA library and EST sequencing to get full-length cDNA from a special tissue, even if that EST is a fragment of one gene. The SMART method has been used in aquatic organism, such as *Tegillarca granosa* (Bao and Lin, 2010) and *Miichthys miiuy* (Xu et al., 2010). Using this approach, we identified 24 contigs and 119 singlets finally. All of the genes are reported for the first time from gastropod abalone, which are involved

Table 1. Key unigenes shared significant similarities with known genes of other species.

Length	Score	E value	Identity	Subject Name	Annotation
149	303	3.00E-80	100	gb|ABW90692.1|	cytidine deaminase
151	187	2.00E-45	68	ref|XP_002066344.1|	GK18243
231	149	6.00E-68	81	gb|ABY87393.1|	ATP synthase F0 subunit 6
142	154	2.00E-35	52	gb|ABY87349.1|	profilin
128	161	2.00E-37	69	ref|NP_001080724.1|	NHP2 non-histone chromosome protein 2-like 1
239	169	5.00E-40	41	ref|XP_002588730.1|	hypothetical protein BRAFLDRAFT_100190
206	281	9.00E-74	72	gb|ABY87388.1|	calmodulin-dependent protein kinase
171	344	1.00E-92	100	gb|ABY87353.1|	ferritin
1040	221	4.00E-56	44	ref|XP_002719999.1|	connector enhancer of kinase suppressor of Ras 2 isoform 1
147	208	1.00E-51	68	ref|XP_002407055.1|	nonmuscle myosin essential light chain
189	304	1.00E-80	85	gb|ABO26639.1|	transgelin
142	290	3.00E-76	97	gb|ABY87349.1|	profilin
205	441	1.00E-121	100	gb|ABY87355.1|	stanniocalcin-like protein
76	77.8	3.00E-12	56	emb|CAX70431.1|	LIM, zinc-binding,domain-containing protein
257	491	1.00E-137	99	gb|ABY87386.1|	60S acidic ribosomal protein P0
246	388	1.00E-106	74	ref|XP_002736568.1|	PREDICTED: exosome component 4-like
535	208	7.00E-52	65	ref|NP_001122125.1|	cortactin
106	116	4.00E-24	52	ref|XP_001969325.1|	GG10043 [Drosophila erecta]
72	87.4	3.00E-15	100	gb|ABY87379.1|	omega class glutathione S-transferase
326	70.9	2.00E-22	100	ref|ZP_06718036.1|	hypothetical protein CUS_0263
127	135	6.00E-39	100	gb|ABV25015.1|	beta-galactosidase a-peptide
103	171	2.00E-40	86	ref|ZP_04858619.1|	ribosomal protein S10
126	120	3.00E-25	53	ref|XP_784178.1|	PREDICTED: hypothetical protein isoform
580	478	1.00E-133	80	gb|ACB73226.1|	NADH dehydrogenase subunit 5
140	114	2.00E-23	45	ref|XP_970004.1|	similar to mitochondrial NADH
600	103	6.00E-20	25	ref|YP_519561.1|	hypothetical protein DSY3328
426	277	2.00E-72	83	ref|ZP_05633094.1|	adenylosuccinate synthetase
147	296	2.00E-78	91	ref|XP_001120139.1|	similar to Ubiquitin-conjugating enzyme E2-17
513	354	1.00E-95	93	gb|ACL99801.1|	cytochrome c oxidase subunit I
533	79	4.00E-13	56	ref|XP_002734661.1|	PREDICTED: sialin-like
174	172	9.00E-41	53	ref|XP_002424313.1|	Nucleoside diphosphate kinase, putative
174	172	9.00E-41	53	ref|XP_002424313.1|	Nucleoside diphosphate kinase, putative
192	350	2.00E-94	91	gb|ABY87354.1|	calmodulin 2
449	212	8.00E-53	83	gb|AAT44354.1|	isocitrate dehydrogenase
483	204	2.00E-50	62	ref|XP_001517928.1|	PREDICTED: similar to PLC alpha, partial
122	190	3.00E-51	88	gb|ABY87409.1|	CD63 antigen-like protein

Table 1. continues

176	226	4.00E-57	70	ref|NP_001090206.1|	ribosomal protein L18a
832	48.5	4.00E-15	43	ref|XP_002933957.1|	PREDICTED: hypothetical protein
107	49.3	3.00E-10	56	ref|ZP_02207458.1|	hypothetical protein COPEUT_02273
140	234	1.00E-59	98	gb|ABV44723.1|	60S ribosomal protein L23-like protein
314	500	1.00E-139	84	gb|ACL99797.1|	NADH dehydrogenase subunit 1
486	99.4	8.00E-19	50	gb|AAF91388.1|	AF263243_1 SocE
520	511	1.00E-143	79	ref|XP_001603746.1|	similar to nucleolar KKE/D repeat protein
1222	70.1	5.00E-10	38	gb|ABN58714.1|	pol-like protein
176	311	9.00E-83	83	emb|CAD91425.1|	actin related protein 2/3 complex,
257	397	1.00E-108	77	gb|ABW23163.1|	ribosomal protein rpl7a
192	57.4	3.00E-06	100	gb|ABY87354.1|	calmodulin 2
786	144	2.00E-32	40	ref|XP_002733949.1|	PREDICTED: CG1104-like
307	145	7.00E-33	39	ref|XP_002931362.1|	PREDICTED: GTPase IMAP family member 4-like
341	81.3	2.00E-13	79	ref|XP_002639536.1|	C. briggsae CBR-FBP-1 protein
326	69.3	8.00E-22	100	ref|ZP_06718036.1|	hypothetical protein CUS_0263
245	165	1.00E-38	38	ref|NP_001133826.1|	Lysosomal-associated transmembrane protein 4A
235	464	1.00E-128	99	gb|ABO26645.1|	proteasome alpha type 2
341	80.5	4.00E-13	34	ref|XP_002738783.1|	PREDICTED: transcription factor AP-4
113	218	8.00E-55	85	gb|EFA01469.1|	hypothetical protein TcasGA2_TC007016
340	145	1.00E-32	42	gb|AAG61258.1|	CCAAT/enhancer binding protein
220	389	1.00E-106	88	gb|ABO26631.1|	Ran-1-prov protein
163	106	5.00E-21	50	ref|XP_002734957.1|	PREDICTED: C. briggsae CBR-GLRX-10 protein-like

in immune reaction, biological process, metabolism, protein binding and so on. Of the 57 known genes, 6 immune-relevant genes were identified and isolated: cytidine deaminase, ferritin, nonmuscle myosin essential light chain, cytochrome c oxidase subunit I, CD63 antigen-like protein, and lysosomal-associated transmembrane protein. These immune-relevant genes should be useful for analyzing gene function during disease defense and for developing molecular markers related to disease-resistance. Possible reasons for the low number of immune-related genes uncovered in this study were as follows: the need to obtain more transcripts from fully differentiated abalone hemocytes treated with different dose of LPS and sampled from other times, and the need for additional sequencing of the library.

In summary, a cDNA Library was constructed to isolate and identify expressed genes in LPS-treated gastropod abalone hemocytes. 24 contigs and 119 singlets were obtained finally; 6 of which appeared to be important immune-relevant genes. Further investigations will be required to determine expression mechanisms and functions of these genes and processes involved in the defense mechanism of the gastropod abalone - *H. diversicolor supertexta.*

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