 DOI: 10.4067/s0301-732X2014000300013

ORIGINAL ARTICLE

Analysis of differentially expressed gene fragments in the head kidney of lipopolysaccharide-stimulated Malabar grouper (*Epinephelus malabaricus)*

Análisis de fragmentos de gen expresados diferencialmente en el riñón cefálico del mero malabárico (*Epinephelus malabaricus*) estimulado con lipopolisacáridos

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RESUMEN

Se estudiaron los efectos inmunológicos y el mecanismo molecular preliminar de lipopolisacáridos (LPS) en el mero malabárico (*Epinephelus malabaricus*). El mero se inyectó intraperitonealmente dos veces (con 7 días de diferencia) con 4 mg/kg^{-1} peso corporal de LPS provenientes de *Escherichia coli* (grupo de prueba, probadores) o solución de tampón fosfato pH 7,2 (grupo control, conductores). Veintiocho días después, la actividad media de antibacterianos, lisozima y polifenol oxidasa del suero mostraron valores de probador de 0,228, 0,032 y 21,8 U/ml x min, respectivamente, mientras que los valores de controlador fueron 0,200, 0,015 y 15,5 U/ml x min. Se elaboró una librería subtractiva de cDNA del riñón cefálico estimulado con LPS de mero malabárico utilizando hibridación substractiva por supresión. Se seleccionaron y secuenciaron 376 clones de fragmentos de genes expresados de probadores específicos, y se obtuvieron 326 EST calificados. Después de una búsqueda con los programas BLASTn y BLASTx, 312 ESTs mostraron gran similitud para 13 fragmentos de genes (14 ESTs no se asemejaron a ninguno de los genes en el GenBank). De los 13 fragmentos, dos (15,4%) estaban relacionados con inmunodefensa (factor 2 regulador de interferón-proteína de unión 2-A y complejo T de proteínas 1-theta); cinco (38,5%) estuvieron relacionados con la transcripción o la traducción; uno (7,7%) estuvo involucrado en el metabolismo (proteína CT054); dos (15,4%) codificarían homologoproteína de diferenciación, leucemia mieloide y la proteína 3 que contiene el dominio parche G; y tres (23,1%) eran genes de transporte de oxígeno que codifican la cadena α de hemoglobina, la cadena β de la hemoglobina y de la cadena pesada de ferritina. Los resultados mostraron que LPS podrían mejorar significativamente la inmunidad innata y regular la expresión de genes relacionados con la inmunidad, la producción de energía celular, el crecimiento, metabolismo o la resistencia al estrés en mero malabárico. Además, LPS pueden ser utilizados como un inmunoestimulante eficaz para esta especie, proporcionando una visión de los mecanismos de los efectos de LPS en animales acuáticos a niveles serológicos y moleculares.

Palabras clave: *Epinephelus malabaricus*, lipopolisacárido (LPS), hibridación subtractiva, inmunoestimulate.

SUMMARY

The immunological effect and preliminary molecular mechanism of lipopolysaccharide (LPS) on Malabar grouper (*Epinephelus malabaricus*) were studied. The grouper was injected intraperitoneally twice (7 days apart) with 4 mg kg^{-1} body weight of LPS from *Escherichia coli* (test group; testers) or pH 7.2 phosphate buffer solution (control group; drivers). Twenty-eight days later, the mean antibacterial, lysozyme and polyphenol oxidase activities of the serum showed tester values of 0.228, 0.032 and 21.8 U/ml×min respectively, and driver values of 0.200, 0.015 and 15.5 U/ml×min. A subtracted cDNA library from the head kidney of LPS-stimulated Malabar grouper was constructed using suppression subtractive hybridization. A total of 376 clones from tester-specific expressed gene fragments were selected and sequenced, and 326 qualified expressed sequence tags (ESTs) were obtained. After searching with the BLASTn and BLASTx programs, 312 ESTs showed great similarity for 13 gene fragments (14 ESTs did not match any genes in the Genbank). Out of these 13 fragments two (15.4%) were related to immune defense (interferon regulatory factor 2-binding protein 2-A and T-complex protein 1-theta); five (38.5%) were related to transcription or translation; one (7.7%) was involved in metabolism (CT054 protein); two (15.4%) might encode myeloid leukemia differentiation protein homologue and G patch domain-containing protein 3; and three (23.1%) were oxygen transport genes that encode haemoglobin α chain, haemoglobin β chain and ferritin heavy chain. Results showed that LPS could significantly improve innate immunity and regulate the expression of genes related to immunity, cellular energy production, growth, metabolism and/or stress-resistance in Malabar grouper. Also, LPS could be used as an effective immunostimulant for this species and provided an insight to the mechanisms of the effects of LPS on aquatic animals at serological and molecular levels.

Key words: *Epinephelus malabaricus*, lipopolysaccharide (LPS), subtractive hybridization, immunostimulant.

INTRODUCTION

Groupers are regarded as high-quality seafood and cultured widely in Southeast Asia. Malabar grouper, *Epinephelus malabaricus* (Bloch and Schneider 1801), is one of the most cultured groupers in China due to its desirable taste and high economic value. The rapid development of Malabar grouper culture meant that diseases caused by bacteria, viruses and parasites became becoming increasingly problematic. However, previous studies on Malabar grouper focused mainly on its culture technique, nutrition and physiology (Lin and Shiau 2005, Tuan and

Accepted: 08.05.2014.

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Williams 2007), and little information about its immunology or molecular immunology is available.

Lipopolysaccharide (LPS), an essential component of the cell wall of Gram-negative bacteria, can effectively improve the non-specific immunity of aquatic and terrestrial animals and is widely used as an immunostimulant to control their infectious diseases (Nya and Austin 2010). Jian *et al* (2004) reported that, after injection of LPS for 28 days, the immunity of many aquatic animals was significantly improved. Savan and Sakai (2002) and Wang and Wu (2007) detected the differentially expressed genes in yellow grouper (*Epinephelus awoara*) and common carp (*Cyprinus carpio*) after stimulation with LPS. In addition, it also could induce the differential expression of immunoregulatory genes in murine macrophages and human monocytes (Barber *et al* 1995) and the expression of cytokines and phase proteins in fish cells *in vitro* (Neumann *et al* 1995, MacKenzie *et al* 2003).

Several polymerase chain reaction (PCR)-based methods, including differential display-PCR, representational difference analysis, RNA fingerprinting by random primed PCR, cDNA-amplified fragment length polymorphism and suppression subtractive hybridization (SSH), have been used to analyse differential gene expression (Liang and Pardee 1992, Lisitsyn and Wigler 1993, Perucho *et al* 1995, Diatchenko *et al* 1996, Gabriëls *et al* 2006). Of all these methods, SSH is considered an ideal for obtaining low abundant differentially expressed cDNAs because it has the advantages of high level enrichment, low background noise and normalised abundance of cDNA in the subtracted mixture. To assess the immunological effects of LPS on Malabar grouper and study the molecular mechanisms underlying the stimulation process, three immunological indices of serum were assayed and differentially expressed genes from the head kidney of Malabar grouper were identified after being stimulated with LPS in this study.

MATERIAL AND METHODS

ANIMAL MAINTENANCE AND SAMPLE PREPARATION

Malabar groupers weighing 50±10 g were obtained from a fish hatchery in Wenchang City, Hainan Province, China. Prior to the experiments, fish were maintained for a week at 28 °C in tanks with a circulating seawater system and fed daily with artificial pellets. Ten fish from the test groups (testers) were injected intraperitoneally with 4 mg kg^{-1} body weight of LPS (Xu *et al* 2010) from *Escherichia coli* (Sigma, USA), while ten fish from the control group were injected with phosphate buffer solution (PBS; pH7.2) (drivers). To improve the effect of immunization, one week after the first injection all groupers were injected again with the same solution as before. Twenty eight days after the second injection, fish were anesthetised. Blood was extracted with a sterile syringe from the caudal vein, placed into 1.5 mL centrifugation tubes and kept at –4 °C overnight. Then, the clot was centrifuged at 112 ×g for 10 min. Serum was collected and stored in sterile 1.5 mL tubes at -20 °C. Meanwhile, head kidneys were separated and frozen in liquid nitrogen, then stored at -80 °C for further use. All animal challenges were carried out following IACUC approved protocols of Hainan University.

ASSAY OF ANTIBACTERIAL ACTIVITY

To detect the antibacterial activity (Ua) of the serum from Malabar groupers, a modified turbidimetric growth assay method was performed according to Noga *et al* (1994). After being incubated overnight in trypticase soy broth (TSB) supplemented with 2% NaCl, *Vibrio alginolyticus* ATCC 33787 was washed and diluted with PBS (pH 7.2; supplemented with 2% NaCl) to an absorbance at 570 nm of 0.3-0.5. Then, in separate test tubes 2 µL of each serum was added to 3 mL of *V. alginolyticus* suspension. One milliliter of each mixture was immediately transferred into 1.0 cm path-length cuvettes and the absorbance at 570 nm was measured (A_0). The test tubes were placed in a water bath at 25±1 °C for 30 min, before being transferred to an ice-cold water bath to stop the reactions. Absorbance values (A_{end}) were recorded and Ua was calculated for each sample using the following formula: $Ua = [(A_0-A_{end})/A_{end}]^{1/2}$. Mean Ua of the test group and control group were calculated and a Student's t test was performed to evaluate their difference significant using the Origin 7.5 software (OriginLab, USA). Differences were considered to be statistically significant when $P < 0.05$.

ASSAY OF LYSOZYME ACTIVITY

Lysozyme activity (Ul) was determined using a method modified from Parry *et al* (1965) and Zhang *et al* (2010). *Micrococcus lysodeikticus* (Sigma, USA) was added to PBS (pH6.4) to obtain an absorbance at 570 nm of 0.3. Three milliliters of *M. lysodeikticus* suspension was aliquoted into separate test tubes in an ice-cold water bath, and 50 µL of each serum sample was added. Each suspension was mixed and A_0 at 570 nm was tested immediately. Test tubes were placed in a water bath at 25±1 °C for 30 min, before being transferred to an ice-cold water bath to stop the reactions. The A_{end} was assayed and Ul was calculated according to the following formula: $Ul = (A_0 - A_{end})/A_{end}$. Mean Ul of the test group and control group were calculated and their difference significant was evaluated as above.

ASSAY OF POLYPHENOL OXIDASE ACTIVITY

Polyphenol oxidase (PPO) activity was determined using a modified spectrophotometric method based on the initial rate of increase in absorbance at 490 nm (Ashida 1971, Gundogmaz *et al* 2003). One hundred microlitter of 0.01 mol L^{-1} L-dihydroxyphenyl-alanine (L-DOPA; Sigma, USA) and 2.8 mL of 0.1 mol L^{-1} PBS (pH 6.0) was added to 100 µL of each serum sample. The suspensions were mixed in 4-mL quartz cuvettes and the absorbances at 490 nm were recorded at 25±1 °C at 30 s intervals for 10 min using a UV-visible spectrophotometer (Shimadiu

UV2450, Japan). The instrument was zeroed using the mixture without the serum. One unit of PPO activity was defined as the amount of enzyme in 1 mL of serum that caused an increase in absorbance at 490 nm of 0.001 per min at 25 °C in the linear portion of the curve. Mean PPO activity of the test group and control group were calculated and their significant difference was evaluated as above.

CONSTRUCTION OF A SSH LIBRARY AND ANALYSIS OF SUBTRACTION EFFICIENCY

Total RNA was extracted from the head kidneys of the drivers and testers using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA integrity was checked using 1% formaldehyde agarose gel. Total mRNA was purified by PolyATtract® mRNA Isolation System III (Promega, USA) according to the protocol of the manufacturer. cDNA synthesis and SSH were performed using the PCR-Select^{TM} cDNA Subtraction kit (Clontech, USA) according to the manufacturer's instructions. The tester cDNA for control subtraction was constructed by mixing the control skeletal muscle cDNA with φX174/Hae III DNA. The resulting subtracted cDNA fragments were amplified, ligated into the pMD-19 vector (Takara, Japan) and transformed into *E. coli* strain JM109 (Takara, Japan). Positive clones were selected by blue/white spot screening and colony PCR amplifications, which were performed with nested PCR primers 1 (5'-DTCGAGCGGCCGCCCGGGCAGGTD-3') and 2R (5'-DAGCGTGGTCGCGGCCGAGGTD-3').

To evaluate the efficiency of adaptor ligation, the relative amount of the constitutively expressed housekeeping gene coding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was compared in subtracted and unsubtracted cDNA after 18, 23, 28 and 33 cycles of PCR. PCR reactions were carried out using GAPDH gene-specific primers (forward primer: 5'-ACCTGATGCTCCAATGTTT-3'; reverse primer: 5'-AGCAACTGGCACCCTGAA-3').

Southern dot blotting was performed with DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany). The DIG-labeled driver DNA probes were generated with DIG-High Prime according to the random primed labeling technique. The PCR products from positive clones were hybridised into the probes. The transfer of DNA to the nylon membrane (Promega, USA) and hybridisation conditions were performed with standard methods. The clones of negative blots were considered to represent the subtractive clones that contained the unique tester fragments, and these were sequenced using M13 universal primers at Shenzhen Huada Gene Institute (Shenzhen, Guangdong Province, China).

DATABASE ANALYSIS

Vector contamination sequences were removed using the Cross match program. Then, sequences were compared against the non-redundant database at the National Center for Biotechnology Information[1] using the BLASTn and BLASTx programs (threshold E-value ≤ 1E-5).

REAL-TIME PCR

Real-time PCR was performed in a total volume of 20 µl containing 0.5 µl 10µM each primer, 2 µl diluted cDNA and 9 µl 2X SYBR Green Master Mix Reagent (Bio-Rad). Reactions were run using the following cycling parameters: 95 °C for 2 min, 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 68 °C for 30 s. The final extension was performed at 72 °C for 5 min. Primer sequences (table 1) were designed with Primer-Premier 5 software based on the sequences of the selected clones.

STATISTICAL ANALYSIS

Arithmetic mean ± standard error (SE) were used to represent the values of each measured parameter. The software programme SPSS (v. 16.0) for Windows was used for the analysis.

RESULTS

ANTIBACTERIAL, LYSOZYME AND POLYPHENOL OXIDASE ACTIVITY

Mean Ua, Ul and PPO activities in the serum of fish in the test group were 0.228±0.0104, 0.032±0.0036 and 21.8±0.9539 U/ml×min, respectively, while the values in the control group were 0.200±0.0072, 0.015±0.0030, 15.5±0.5196 U/ml×min (figure 1). All the Ua, Ul and PPO activities were significantly different ($P < 0.01$) between the test group and the control group.

CONSTRUCTION OF A SSH CDNA LIBRARY AND ANALYSIS OF SUBTRACTION EFFICIENCY

Products of the second PCR amplification were separated by electrophoresis and stained with ethidium bromide (figure 2). After the second PCR amplification, the smear of the subtracted cDNA was lighter than that of the unsubtracted products, which suggested fewer cDNA types in the subtracted cDNA than in the control (figure 2, lanes 4 and 5). The PCR products of the subtracted cDNA occurred as a smear consisting of a series of fine bands ranging from 0.5-1 kb (figure 2, lane 4). The PCR control subtracted cDNA (figure 2, lane 3) provided a positive PCR control consisting of the skeletal muscle sample and contained a successful subtracted mixture of *Hae* III-digested φX174 DNA. The secondary PCR products of the subtracted tester cDNA for control subtraction (figure 2, lane 1) mostly contained DNA fragments corresponding to the φX174/*Hae* III digest, which was the same pattern

1 www.ncbi.nlm.nih.gov

Table 1. Primers used for real-time RT-PCR.
Partidores usados para RT-PCR en tiempo real.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
Interferon regulatory factor 2-binding protein	CTGGTCGGCTCCAATGTG	GGGCTTGCTGGGTGATTT	203
T-Complex protein 1-theta	TAAATCACCCAGCAAGCC	AGCAAACAGTGCCATCCA	306
Haemoglobin beta chain	ATGTGGTCTACAATGCCTCC	ATGCTGACTTCTTCTGGGT	213
Haemoglobin alpha chain	ATCGCCCTTCCCAACAGT	CGTATCCAGAGCCTTCGT	290
Ferritin heavy chain	ACCCTAATAATCGCTCTG	TCCTGGTGGTAGTTCTGT	221
Eukaryotic translation elongation factor-2	ATTAGACGCCACAAGCAA	GAATGAGGCAAGAGCAAA	139
Procollagen C-endopeptidase enhancer 1 precursor	GCAGAGGTGCCTACTACAT	CAGGGTTGGTTCTGGATA	133
Ribosomal protein S28	CAGGTCCGTGTTGAGT	GAGGTCTTCTGCGTCA	178
Ribosomal protein L3	GGCAGAAGAAGTCTCACC	GCCAGGCACCAATACA	252
40S ribosomal protein S8	GTGGTCTACAATGCCTCC	TCTTCTGGGTCCTCTTCG	202
CT054 protein	AGGGTCAGGACAAACAGG	TGGCTTGGGTCAATGCTC	310
Myeloid leukemia differentiation protein homologue	AGAGGGACTGGCTGGTTA	ACGTGGAGGAATTGTTGC	218
G patch domain containing 3	TCGTGTTTGGTTGCCGTCTC	GGGCTGGAAGGATGGTGAA-	230

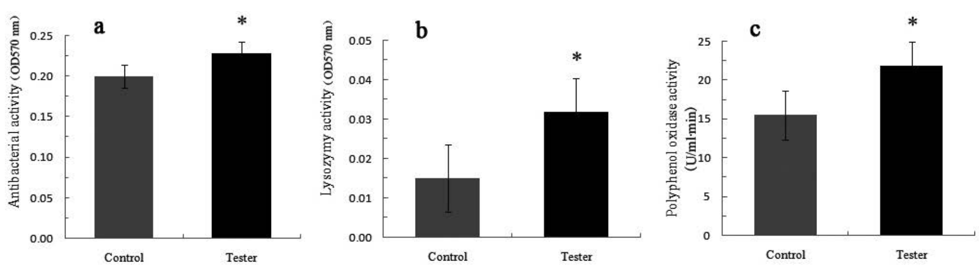

Figure 1. Effects of *E. coli* LPS on the antibacterial activity (a), lysozyme activity (b) and polyphenol oxidase activity (c) of Malabar grouper serum. Values are mean ± S.D.; n = 10.* P < 0.01.

Efectos de LPS de *E. coli* sobre la activiadad antibacterial (a), actividad de lisosoma (b) y actividad de polifenol oxidasa del serum de mero malabárico. Valores corresponden al promedio ± D.S.; n = 10.* P < 0,01.

as the control subtraction provided in the kit (figure 2, lane 3). It confirmed that the experimental subtracted processes had worked efficiently.

The results of blue/white spot screening showed that approximately 96% of the transformants contained inserts. Totally 2664 positive clones were obtained, and subsequent colony PCR revealed that the sizes of the inserts ranged from 200-1000 bp. PCR products from 376 clones gave negative spots in the dot blotting. Thus, a putative LPS-stimulated subtracted cDNA library from the head kidney of Malabar grouper was successfully constructed and it consisted of 376 clones containing tester-specific expressed gene fragments.

ESTS ANALYSIS

A total of 376 tester-specific cDNA clones from the head kidney of LPS-stimulated Malabar grouper were sequenced. After removing the vector sequence and trimming the poor-quality sequences, 326 qualified ESTs were obtained (table 2). In these qualified ESTs, 312 ESTs showed great similarity to 13 previously reported gene fragments, but the remaining 14 ESTs did not share any matches in the gene database. A summary of the identified genes and Genbank accession numbers for the ESTs are shown in table 2. Two genes related to immune defense, namely interferon regulatory factor 2-binding protein 2-A and T-Complex protein 1-theta, were identified. Three genes associated with oxygen transport were cloned (haemoglobin α chain, haemoglobin β chain and ferritin heavy chain). Five genes related to transcription and translation were identified. One clone corresponded to the CT054 protein, a metabolism related protein. Finally, two genes that might encode myeloid leukemia differentiation protein homologue and G patch domain-containing protein 3 were also identified. The most frequently identified clones corresponded to haemoglobin β chain (n = 268).

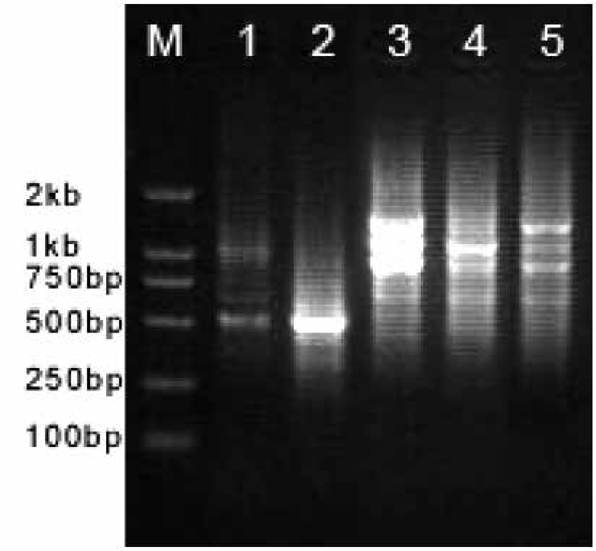

Figure 2. Secondary PCR products on 2% agarose gel. Lane M, DL-2000 marks; lane 1, subtracted tester cDNA for control subtraction; lane 2, unsubtracted tester cDNA for control subtraction; lane 3, control subtracted cDNA (provided by the kit); lane 4, subtracted cDNA; lane 5, unsubtracted cDNA.

Productos secundarios en gel de agarosa 2%. Carril M, , marcas DL-2000; carril 1, cADN de probador sustraído para sustracción control; carril 2, cADN de probador sustraído para sustracción control; carril 3, cADN de control sustraído (proporcionado en el kit); carril 4, cADN sustraído; carril 5, cADN no sustraído.

REAL-TIME PCR ANALYSIS

To analyse the expression profiles of the SSH identified genes within the 4-week period of stimulation with LPS, real-time RT-PCR analysis was performed in the head kidney. The specificity of the RT-PCR products was documented using high-resolution gel electrophoresis. All genes resulted in a single product with the desired length.

DISCUSSION

Antibacterial, lysozyme and phenoloxidase activities were significantly increased when crab *Scylla paramamosain* was challenged with bacterial LPS (Nya and Austin 2010, Gopalakrishnan *et al* 2011). The results of this study showed that LPS had a similar effect on Malabar grouper and could be a potential effective immunostimulant for it.

Interferon regulatory factors (IRFs) can effectively combine with the promoter of the interferon (IFN) gene and thus induce and/or regulate IFN gene expression[2] (Mamane *et al* 1999). Although IRF-2 can activate the expression of many genes, it is usually considered an inhibitory factor

[2] Lehtonen A. 2005. Expression and activation of STAT and IRF family transcription factors in mononuclear leukocytes. Doctoral Dissertation, http://ethesis.helsinki.fi/julkaisut/bio/bioja/vk/lehtonen/, University of Helsinki, Finland.

Table 2. Differentially expressed genes detected in the head kidney cDNA of LPS-stimulated *Epinephelus malabaricus.*

Genes expresados diferencialmente detectados en el cADN de riñón cefálico en *Epinephelus malabaricus* estimulado con LPS.

Clone number	Putative identification [Closest species]	Accession number	Accession number of closest species	E-Value	Identity	Frequency
	Genes related to immune / defense					
C5	Interferon regulatory factor 2-binding protein 2-A [*Salmo salar*]	GT067256	ref|NP_001133325.1 gb|AY823273.1|	3e-15	94%	4
D153	T-complex protein 1-theta [*Notothenia coriiceps*]	GT067257		2e-101	88%	1
	Genes related to oxygen transport					
C130	Haemoglobin beta chain [*Epinephelus coioides*] Haemoglobin alpha chain [*Thunnus thynnus*]	GT067259	gb|AAK38736.1|	5e-72	97%	268
D34	Ferritin heavy chain [*Psetta maxima*]	GT067260	gb|AAK38736.1|	2e-53	72%	9
E45		GT067261	gb|ADI24353.1|	1e-05	96%	1
	Genes related to transcription and translation					
C34	Eukaryotic translation elongation factor 2 [*Danio rerio*]	GT067262	gb|ABJ98666.1|	4e-59	84%	4
F39	Procollagen C-endopeptidase enhancer precursor [*Salmo salar*]	GT067263	gb|ACQ58156.1|	8e-66	85%	8
F22	Ribosomal protein S8 [Solea senegalensis] Ribosomal protein L3 [*Solea senegalensis*]	GT067276	dbj|BAF45896.1|	7e-48	100%	1
C136	40S ribosomal protein S28 [*Danio rerio*]	GT067268	dbj|BAF98579.1|	3e-135	93%	4
E26		GT067269	ref|NP_998199.1|	3e-21	94%	1
	Genes related to metabolism					
G129	CT054 protein [*Salmo salar*]	GT067264	ref|NP_001133955.1|	2e-76	82%	1
	Other genes					
E112	Myeloid leukemia differentiation protein homologue [*Salmo salar*]	GT067265	ref|NP_001117034.1| ref|NP_001074099.1|	9e-22	79%	8
E108	G patch domain-containing protein 3 [*Danio rerio*]	GT067267		5e-64	75%	2
Unknown						14

of IFN. IRF-2 could regulate an abnormal IFN response, maintain a constant IFN level *in vivo* in animals, and avoid the inflammatory response caused by excessive expression of IFN (Tanaka *et al* 1993, Vaughan *et al* 1995, Chung and Kawamoto 2004). Barber *et al* (1995) demonstrated that IRF-related gene expression might represent a molecular pathway involved in the response to LPS. Clone C5 (GT067256) in our SSH library had 94% similarity to interferon regulatory factor 2-binding protein 2-A of *Salmo salar*. Therefore, it is presumed that an appropriate dose of LPS could induce the expression of IRF-binding protein in Malabar grouper, which might be related to maintaining a constant IFN level. Another immune-related gene, the T-complex protein 1-theta gene (GT067257) (Yin *et al* 1999, Izzotti *et al* 2003), was also obtained in this study. The results provided direct molecular evidence to explain why Malabar grouper showed strong innate immunity 28 days after LPS stimulation.

Haemoglobin β-chain gene, haemoglobin α-chain gene, and the ferritin heavy chain (*fhc*) gene were reported to contribute to oxidative stress resistance and apoptosis in vertebrates and invertebrates (Connie and Hsia 1998, Orino *et al* 2001, Larade and Storey 2004, Aung *et al* 2007). They were cloned in the SSH library of chickens infected with an avian leukosis virus subgroup (Zhao *et al* 2010). Moreover, Parish *et al* (2001) proved that haemoglobin (the intact tetramer of α-chain or/and β-chain) had the roles of oxygen transportation and was an important multi-defense agent against a wide range of microorganisms in snake, alligator, horse and human. In this study, haemoglobin β-chain gene, haemoglobin α-chain gene, and the ferritin heavy chain (*fhc*) gene were also cloned in the SSH library of Malabar grouper. It elucidated that these three genes could be differentially expressed after the animal was stimulated by a special foreign substance, and LPS might enhance the non-specific immunity of Malabar grouper by up-regulation of haemoglobins and *fhc*.

The α-ketoglutarate dehydrogenase is responsible for the conversion of α-ketoglutarate to succinyl coenzyme A, an important energy-producing step in citric acid (TCA) cycle. The TCA cycle-related gene *sucA* (CT054) can down-regulate α-ketoglutarate dehydrogenase activity (Belland *et al* 2003, Nicholson *et al* 2004). In this SSH library of Malabar grouper, one clone was the homological sequence (GT067264) to CT054, and five gene fragments related to transcription and translation (GT067262, GT067263, GT067276, GT067268, and GT067269) were also found. Interestingly, similar results were reported in some recent papers. For example, Micheluccia *et al*[3] revealed that LPS stimulation can up-regulate itaconic acid(from the TCA cycle intermediate cisaconitate) synthesis in macrophages. These results showed that LPS might regulate metabolism and gene expression at transcriptional and/or translational levels. However, more experiments should be performed to confirm these immunity-related changes.

Procollagen C-endopeptidase enhancer (*pcpe*) can regulate the level of high-density lipoprotein (HDL) particles *in vivo* (Zhu *et al* 2009). HDL particles have the function of anti-inflammatory, anti-aggregative and antioxidant properties (Stein and Stein 1999, Fredenrich and Bayer 2003). Walker *et al* (2003) proved that HDL particles played an important role on crustacean immune recognition mechanisms. Pajkrt *et al* (1996) also found that HDL particles could neutralise LPS stimulated activity *in vitro* and *in vivo*. The fact that the *pcpe* gene fragment (GT067263) was obtained in this study indicated that *pcpe* gene expression was an adaptive response of Malabar grouper to LPS.

The myeloid leukemia differentiation protein homologue is relevent to human leukemia and cell cycle regulation (Bürger *et al* 1994, Sen *et al* 1997). Gariglio *et al* (1998) reported that LPS could induce cells of the myeloid/monocyte lineage to constitutively express p202 and p204 proteins, which belong to myeloid leukemia differentiation protein homologue group, and thus could slow down cell proliferation and regulate cell growth and differentiation. In this study, a gene fragment encoding myeloid leukemia differentiation protein homologue (GT067265) was obtained, which indicated that LPS might affect growth rate to some extent in Malabar grouper.

Haemoglobin β-chain gene was the most frequently identified clones (10.05% of total clones) in this SSH library of Malabar groupe. This is probably because of the incomplete subtraction of Haemoglobin β-chain gene cDNA in the tester, which was due to the multifold amount of mRNA in the tester compared with the driver. However, this would not affect the differential display of the specific expressed genes in the tester. On the contrary, the up-regulation of the genes was better illustrated.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundations of China (Nos.30660144, 30760190, 31060360 and 31260644), the National Marine Public Welfare Research Project of China (No. 201205025), Specialized Research Fund for the Doctoral Program of Higher Education(No. 20124601110006) and the Project of Fok Ying Tong Education Foundation (No. 121030).

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Barber SA, MJ Fultz, CA Salkowski, SN Vogel. 1995. Differential expression of interferon regulatory factor 1 (*irf*-1), *irf*-2, and interferon

[3] Micheluccia *et al* 2013. Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. Proc Natl Acad Sci USA Early Edition, www.pnas.org/cgi/doi/10.1073/pnas.1218599110.

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