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Note

Preliminary EST analysis of immune-relevant genes from the liver of LPS-stimulated rock bream *Oplegnathus fasciatus*

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We constructed a rock bream (*Oplegnathus fasciatus*) liver cDNA library and a total of 1533 expressed sequence tag (EST) clones were generated. Gene annotation procedures and homology searches of the sequenced ESTs were analyzed using BLASTX. Of the 1533 EST clones, 1165 different ESTs showed significant homology to previously described genes while 368 ESTs were unidentified, hypothetical, or unnamed proteins. Encoding 106 different sequences were identified as putative bio-defense genes or genes associated with immune response.

Key words : Expressed sequence tags, ESTs, Rock Bream, Liver

Rock bream, *Oplegnathus fasciatus,* belongs to the family Oplegnathidae and inhabits the coastal rocky-shores of Korea, Japan, Taiwan, and Hawaii (Nakabo, 2002). This species has attracted great interest among Korean fish farmers due to its high market value and consumer demand. The total production of this species, however, is not satisfactory compared to other commercially important fishes in Korea. The RSIV disease has been the major culprit for the mass mortality of rock bream in Korea (Sohn *et al.,* 2000; Jung and Oh, 2000).

The liver is a dynamic organ playing an important role in carbohydrate, lipid, steroid, amino acid, and prostaglandin metabolism. It is also responsible for the detoxification of foreign substances and production of seroprotein and biliary acid. With an estimated 130 million cells per gram of liver, the hepatocyte fulfills the majority of the organ's functions including the bulk production of plasm a proteins (e.g. proteins involved in binding and transport, blood clotting, and immune responses), detoxification, and ATP production (Feldmann, 1994).

Many molecular tools are available for characterizing the immune systems of mammals, especially human being and mice. With regard to fish, however, information on immune-related molecules is still limited. Recently, remarkable progress has been made in genetic technology with the discovery of rapid expressed sequence tag (EST) analysis, which allows the acquisition of massive DNA sequence information of many organisms, including several fish species, in a short time period (Zeng and Gong, 2002; Clark *et al.,* 2003; Rise *et al.,* 2004). Large scale EST analysis is also an efficient way for identification of genes and for analysis of their expression by means of expression profiling (Franco *et al*., 1995; Azam *et al*., 1996; Lee *et al*., 2000). It offers a rapid and valuable first look

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at genes expressed in specific tissue types, under specific physiological conditions or during specific developmental stages. Currently, the number of fish-related ESTs in public databases is still small compared to mammalian sequences and there are relatively few tissue-specific cDNA libraries (Ton *et al*., 2000). There has been an increasing public interest in this topic, but relatively little information is available about rock bream genes (Cho *et al.,* 2006). The lack of information may be one of the obstacle to the effective use of genetics in aiding both rock bream aquaculture and conservation activities. In this study, EST sequenced to screen for bio-defense or immune related genes in rock bream liver after treatment with LPS.

Materials and methods

Animals

Rock bream (*O. fasciatus*), with an average weight of 150 grams were obtained from the Genetics and Breeding Research Center (Geoje). Forty-eight rock bream were injected intra-peritoneally (IP) with LPS (6 ㎎/㎏). The fish were maintained in a tank with running artificial seawater at 23-25℃. At each sampling time (days 1, 3, 5, and 7), a total of six fish from each experimental group were sacrificed, liver were dissected out and quickly frozen in liquid nitrogen and stored at -80℃.

DNA library construction

The cDNA library was constructed using mRNA prepared from LPS stimulated liver tissue of rock bream. The purified mRNAs taken from four different time periods were pooled to ensure complete coverage of expressed genes in the allotted timeframe and were used to construct a cDNA library. Libraries were constructed by using a modification of Maruyama and Sugano (1994). The synthesis of the first-strand cDNA from the purified mRNA and cDNA amplification were performed as described by Maruyama and Sugano (1994). The amplified PCR products were then digested with *Sfi*I, and cDNAs longer than 400 bp were ligated into *Dra*III-digested pCNS-D2 in an orientation-defined manner. The pCNS-D2 vector contains 5' *Eco*RI-*Dra*III-*Eco*RV-*Dra*III sites at multi cloning sites, which was achieved by modifying the pCNS vector (GenBank Accession no. AF416744). The ligated cDNA was then transformed into *E. coli* Top10F' (Invitrogen) by electroporation (Gene Pulser II, BioRad).

Single-pass sequencing of the 5'-termini of 1533 selected rock bream liver cDNA clones in plasmid form was performed using the ABI 3700 automatic DNA sequencer (PE Applied Biosystems) and the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

EST sequencing and analysis

Bioinformatic analysis was conducted to determine gene identities using Genetyx ver. 8.0 software (SDC software, Japan). Briefly, vector sequences were then removed and database search were limited to ESTs >400 bp in length. The sequence of each cDNA was compared with sequences in the peptide sequence databases at the National Center for Biotechnology Information (NCBI) using the BLAST network service. Nucleotide sequence

comparisons were carried out using the program BLASTX (Gish and David, 1993).

Results and Discussion

cDNA library construction

A cDNA library of 8×10^6 clones were constructed from the polyadenylated fraction of mRNA purified from rock bream liver injected with LPS.

The number of clones in the constructed cDNA library was deemed sufficient to cover the predominantly expressed mRNAs in rock bream liver stimulated with LPS.

A total of 1533 randomly selected clones were single-pass sequence from the 5' end, resulting in the characterization of cDNA clones that were longer than 400 bp after elimination of vector sequence. The number of clones sequenced from the cDNA library, the average size of inserts, and the redundancy of the obtained sequences are given in Table 1.

Table 1. Summary of sequences and clones represented

Numbers of ESTs (n)	1533
Putatively identified clones (n)	1165 (76%)
Hypothetical protein and unnamed protein	180
Ribosomal protein	65
Immune related genes (n)	530
Other genes	390
Unidentified clones (n)	368 (24%)
Putatively identified different genes (n)	319
Total nucleotides (bp)	941,530
Average of sequenced length (bp)	614

EST sequencing and analysis

We performed single pass sequencing on 1533 randomly selected clones from a rock bream liver cDNA library targeting the 5'-terminus of each insert.

Of the 1533 clones, 1165 (76%) were identified as orthologs of known genes from rock bream or other organisms.

We performed a BLASTX search on all the sequences. 368 of the 1533 sequences (24%) resulted to unmatched, i.e., did not show any significant similarity to the sequences present in the public databases based on nucleotides or translated peptides.

Among the 1165 identified EST clones, 530 clones were immune related genes identified as homologous to the previously reported genes from rock bream or other organisms. 319 different rock bream genes were identified including the 65 genes for ribosomal proteins that we have obtained (Table 1).

Gene annotation procedures and homology searches of the sequenced ESTs have been analyzed using BLASTX. The ESTs with significant similarities to known proteins were evaluated to determine if these remarkable similarities were caused by simple amino acid matches (Table 2).

The most redundant clones (Table 2) in immune related genes were found in those coding for apolipoprotein A-1, a high-density lipoprotein peculiar to fish which transcripts have been observed in liver and brain of fugu (Kondo *et al*., 2005).

The second most redundant clones (Table 2) in immune related genes were observed in those coding for fibrinogen, a plasma protein that can be transformed into an insoluble fibrin network during blood

coagulation. Because of its potential biotechnological significance, fish fibrinogen has been purified and characterized (Manseth *et al*., 2004).

We have sequenced clones for the C-type lectin and for several complement components (Table 2). The C-type lectin superfamily is comprised of functional proteins which is important to glycoprotein metabolism, mechanisms of multi- cellular integration, and immunity (Zelensky and Gready, 2004). The study of the complement proteins in teleosts is important not only to better understand the evolution of the innate immune system in vertebrates, but also for the development of therapeutics in aquaculture (Holland and Lambris, 2002).

The discovery of novel teleost genes related to the immune response has been accelerated by high-throughput sequencing techniques combined with searches for homologous sequences in public databases. It also indicates that the range of rock bream liver ESTs identified in this study covers the known liver functions and therefore should be useful to monitor liver gene expression under different physiological conditions.

In conclusion, this study reports a 1533 EST based gene sequences derived from LPS stimulated rock bream liver cDNA library. These EST analyses would be useful for the construction of cDNA microarray and recombinant proteins. The application of cDNA microarrays may facilitate the research attempting to answer questions concerning about the immune and other protective responses of rock bream upon infection of pathogens.

Table 2. Rock bream LPS stimulated liver ESTs encoding for immune related genes.

Clone no.	Putative identification	Accession no.	Closest species	E-value	I[a]	bp	F[b]
05-G02	alpha-2-macroglobulin	AAR06589.1	*Sparus aurata*	5.00E-62	89	721	2
14-F12	ankyrin-repeat and fibronectin type III domain containing 1, partial	XP_001345271.2	*Danio rerio*	2.00E-05	59	858	1
13-G11	apobec1 complementation factor isoform 2	XP_707799.1	*Danio rerio*	5.00E-50	69	718	1
15-A12	apolipoprotein 14kDa	ACF21984.1	*Oplegnathus fasciatus*	1.31E-67	94	845	27
13-A04	apolipoprotein A-I	ACF21981.1	*Oplegnathus fasciatus*	1.00E-128	100	980	209
14-A12	apolipoprotein B	ACO82023.1	*Perca flavescens*	1.00E-125	91	911	5
07-H10	apolipoprotein C-II	ACF21983.1	*Oplegnathus fasciatus*	4.00E-36	84	881	16
10-B12	apolipoprotein E	ACF21982.1	*Oplegnathus fasciatus*	1.00E-143	100	922	4
20-F06	B2-microglobulin	ABB60035.1	*Pseudosciaena crocea*	1.30E-30	81	850	1
05-F07	beta-2 microglobulin	AAW65850.1	*Stizostedion vitreum*	2.00E-39	84	762	1
11-E03	C1q-like adipose specific protein	AAM73701.1	*Salvelinus fontinalis*	5.00E-55	69	808	3
20-F01	C1R/C1S subunit of Ca2+-dependent complex	NP_001117852.1|	*Oncorhynchus mykiss*	1.00E116	84	807	1
05-C01	Ca2+-dependent complex C1R/C1S subunit	ABU63968.1	*Perca flavescens*	1.00E-132	87	888	8
14-B04	calcium binding hemolysin protein, putative	YP_002685011.1	*Rhodobacteraceae bacterium KLH11*	2.00E-15	59	850	1
17-B07	cathepsin F	ACC86111.1	*Paralichthys olivaceus*	5.89E-20	91	726	1
02-C02	cathepsin L-like protein	ACO82386.1	*Lutjanus argentimaculatus*	2.00E-29	98	533	1
09-E04	CCAAT/enhancer-binding protein beta 2	ACL98106.1	*Epinephelus coioides*	1.00E-101	86	809	1
03-H12	CD209 antigen-like protein A	ACO14261.1	*Esox lucius*	3.00E-08	61	685	1
01-C03	CD48 antigen precursor	ACQ58805.1	*Anoplopoma fimbria*	4.00E-31	59	890	1
14-H04	CD59 glycoprotein precursor	ACI68532.1	*Salmo salar*	4.00E-14	62	656	1
11-G11	CD9antigen	ACQ58340.1	*Anoplopoma fimbria*	5.00E-36	78	378	1
02-D03	Chain A, Crystal Structure Of A F-Lectin	ABB29989.1	*Morone saxatilis*	1.00E-07	100	658	1
13-D11	chemotaxin	CAM96031.1	*Larimichthys crocea*	8.00E-75	94	811	4
21-B07	chitinase 3	BAD15061.1	*Paralichthys olivaceus*	2.36E-102	73	894	2
17-B12	coagulation factor VII	AAK74192.1	*Danio rerio*	4.21E-91	85	852	1
01-B02	coagulin factor II	ACA30405.1	*Larimichthys crocea*	1.00E-138	84	919	6
06-E10	Complement C1q-like protein 4 precursor	ACM08719.1	*Salmo salar*	2.00E-40	71	525	1

01-B04	complement component c3	AAW72004.1	Hippoglossus hippoglossus	1.00E-137	90	914	18
02-B12	complement component C 3-1	NP_001098552.1	Oryzias latipes	7.00E-85	81	647	1
09-B09	complement component C4	CAD45003.1	Takifugu rubripes	4.00E-42	79	716	1
18-G12	complement component C8 alpha chain	NP_001118096.1	Oncorhynchus mykiss	1.00E103	76	885	1
20-H09	complement component C9	BAA86878.1	*Paralichthys olivaceus*	1.41E-101	81	851	1
14-C08	complement control protein factor I-A	BAB88920.1|	*Cyprinus carpio*	4.00E-56	56	817	1
10-G10	complement control protein factor I-B	BAB88921.1	*Cyprinus carpio*	5.00E-09	61	460	2
13-A06	complement factor B	NP_001098275.1	*Oryzias latipes*	9.00E-11	82	477	1
13-C11	complement factor H precursor	NP_001117882.1	*Oncorhynchus mykiss*	2.00E-18	47	777	1
20-B04	complement factor H-related 1	NP_056595	*Mus musculus*	3.00E-04	37	840	1
09-G10	complement regulatory plasma protein	AAA92556.1	*Paralabrax nebulifer*	2.00E-16	79	852	8
15-C04	C-type lectin	AAU50548.1	*Fundulus heteroclitus*	1.42E-65	72	616	3
15-C12	c-type lysozyme	Q9DD65.1	*Paralichthys olivaceus*	9.08E-60	89	937	5
19-E05	Cu/Zn superoxide dismutase	AAT36615.1	*Oplegnathus fasciatus*	4.24E-83	100	776	1
10-B11	cytochrome b	YP_001218742.1	*Parajulis poecilepterus*	1.00E-100	82	744	10
13-D05	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial precursor	ACQ58024.1	*Anoplopoma fimbria*	2.00E-84	94	761	1
04-E03	Cytochrome c oxidase subunit 6C-1	P80977.2	*Thunnus obesus*	6.00E-31	90	460	1
19-E01	cytochrome c oxidase subunit I	YP_001974620.1	*Oplegnathus fasciatus*	3.26E-115	76	909	10
03-E12	cytochrome c oxidase subunit II	YP_001218733.1	*Parajulis poecilepterus*	3.00E-40	96	615	11
18-B03	cytochrome c oxidase subunit III	YP_001974624.1	*Oplegnathus fasciatus*	2.22E-111	85	788	16
19-E09	cytochrome c oxidase subunit Vb precursor	AAQ14280.1	*Scombridae gen. sp.*	5.45E-54	89	610	1
17-A02	Cytochrome c oxidase subunit VIb isoform 1	ACO10158.1	*Osmerus mordax*	2.24E-44	100	548	1
18-H04	cytochrome P450 1A	CAB63650.1	*Dicentrarchus labrax*	8.55E-96	90	640	1
16-F07	cytochrome P450 2N14	ABH05127.1	*Micropterus salmoides*	7.73E-112	83	797	2
21-F11	cytochrome P450 3A80	XP_414782.1	*Gallus gallus*	1.11E-13	75	885	1
01-C01	cytokine induced apoptosis inhibitor 1	ACH70653.1	*Salmo salar*	1.00E103	77	896	1
13-G03	Ddx5 protein	AAH67585.1	*Danio rerio*	1.00E-123	92	889	1

07-A01	Endothelial differentiation-related factor 1 homolog	ACI67525.1	*Salmo salar*	3.00E-71	95	884	1
20-G08	FBP32 precursor	ABB29989.1	*Morone saxatilis*	8.05E-31	89	666	2
04-H11	FBP32II precursor	ABB29991.1	*Morone saxatilis*	1.00E-74	87	772	1
22-H01	Ferritin, heavy subunit	ACQ59065.1	*Anoplopoma fimbria*	1.00E-42	100	599	5
04-D03	Ferritin, middle subunit	ACQ57862.1	*Anoplopoma fimbria*	4.00E-69	97	547	1
19-B08	fibrinogen alpha chain	NP_001002039.1	*Danio rerio*	2.37E-54	79	888	3
01-C11	fibrinogen beta chain precursor	ABQ41317.1	*Paralichthys olivaceus*	1.00E-101	96	945	16
09-F01	fibrinogen gamma polypeptide	NP_998219.1	*Danio rerio*	1.00E111	76	902	11
09-G06	Fibronectin	ACN60244.1	*Salmo salar*	1.00E-138	88	848	2
07-G03	goosefish kalliklectin	BAG66037.1	*Lophiomus setigerus*	4.00E-96	73	887	6
07-H04	heat shock protein 90 beta	AAP20179.1	*Pagrus major*	2.00E-79	80	783	1
19-E04	Heat-shock protein 90	BAF57908.1	*Lepomis macrochirus*	8.12E-96	81	817	1
18-G09	heme oxygenase 1	ABL74501.1	*Dicentrarchus labrax*	3.90E-55	93	797	3
20-D05	hepcidin antimicrobial peptide 3	BAH03287.1	*Pagrus auriga*	1.14E-35	89	684	2
01-D11	hepcidin isoform 2	ACF49395.1	*Oplegnathus fasciatus*	8.00E-23	100	894	2
14-B11	hepcidin isoform 3	ACF49396.1	*Oplegnathus fasciatus*	5.00E-27	100	631	1
16-E03	hepcidin-1	ACD13023.1	*Micropterus salmoides*	2.02E-44	96	842	2
10-E04	HGF activator like protein	XP_001513341.1	*Ornithorhynchus anatinus*	8.00E-54	61	815	1
21-F10	Interferon regulatory factor 3	ACN11005.1	*Salmo salar*	1.00E-38	81	856	1
13-E04	interferon stimulated gene15	BAG72218.1	*Sebastes schlegelii*	5.00E-51	82	713	1
01-B12	interleukin 1 beta receptor type 1 soluble form, partial	XP_001339589.2	*Danio rerio*	9.00E-26	46	737	1
16-B08	leucine-richalpha-2-glyco protein	AAW71998.1	*Ctenopharyngodon idella*	3.00E-31	44	939	2
15-F11	leukocyte immune-type receptor TS32.15L1.1a	XP_692412.3	*Danio rerio*	3.46E-10	55	331	1
08-H10	lipocalin	ACO82027.1	*Perca flavescens*	1.00E-24	74	741	2
04-A05	Lipocalin precursor	ACQ58872.1	*Anoplopoma fimbria*	4.00E-76	89	549	1
01-E08	macrophage stimulating 1 (hepatocyte growth factor-like)	XP_541884.2	*Danio rerio*	1.00E125	90	843	1
05-H04	mature parasite-infected erythrocyte surface antigen	XP_724665.1	*Plasmodium yoelii yoelii str. 17XNL*	2.00E-04	51	372	1
07-E01	MHCII invariant chain	AAS77256.1	*Siniperca chuatsi*	1.00E-118	89	864	1
01-B01	microfibril-associated 4	ACM41864.1	*Epinephelus coioides*	2.00E-75	85	909	2
19-A11	Microfibril-associated glycoprotein 4 precursor	ACO13477.1	*Esox lucius*	2.28E-88	79	841	1

05-C03	mitogen-activated protein kinase 6	NP_001039017.1	*Danio rerio*	1.00E-117	83	826	1
15-E05	pancreatic progenitor cell differentiation and proliferation factor b	NP_956302.1	*Danio rerio*	1.22E-25	69	840	9
19-H09	Peroxiredoxin-6	ACI67571.1	*Salmo salar*	1.02E-101	93	720	1
17-H12	plasminogen	NP_001117863.1|	*Oncorhynchus mykiss*	1.00E145	87	898	1
08-H12	properdin P factor complement 2 precursor	CAJ55494.1	*Oncorhynchus mykiss*	9.00E-47	46	812	1
03-B03	Proteasome (prosome, macropain) 26S subunit, ATPase, 1a	AAI54337.1	*Danio rerio*	3.00E-50	100	553	1
01-E05	Protein AMBP; Contains: Alpha-1-microglobulin	P36992.1	*Pleuronectes platessa*	1.00E-100	84	858	1
19-G12	putative complement factor Bf/C2	CAD21938.1	*Tetraodon nigroviridis*	1.00E-98	78	840	4
14-C02	putative hepatocyte growth factor activator/GRAAL	AAG30031.1	*Oncorhynchus mykiss*	8.00E-09	91	370	1
04-G12	receptor for activated protein kinaseC	AAQ91574.1	*Oreochromis mossambicus*	1.00E-134	97	725	1
02-F12	retinal G protein coupled receptor	NP_001017877.1	*Danio rerio*	5.00E-95	96	615	1
16-H09	RING finger protein 170	NP_001134278.1	*Salmo salar*	2.55E-80	81	675	1
18-D08	serum lectin isoform 3	BAF34210.1	*Verasper variegatus*	1.05E-49	73	903	1
14-C03	Stat 3	BAH47263.1	*Danio rerio*	1.00E-157	99	864	1
17-H07	techylectin	AAY79281.1	*Siniperca chuatsi*	1.74E-14	98	865	1
10-C03	Thioredoxin-interacting protein	ACN10667.1	*Salmo salar*	1.00E-145	95	851	1
16-D11	Thymosin beta-12	P33248.2	*Lateolabrax japonicus*	4.36E-13	98	495	1
18-H09	TNF receptor-associated protein 1	NP_001107097.1	*Danio rerio*	4.33E-117	95	882	1
02-H02	TP53-regulated inhibitor of apoptosis 1	ACO09417.1	*Osmerus mordax*	5.00E-26	89	610	1
17-F07	transferrin	ACN80997.1	*Dicentrarchus labrax*	4.68E-143	91	894	5
16-E08	transferrin receptor	ABD61719.1	*Scophthalmus maximus*	2.74E-40	87	620	1
21-H01	translationally-controlled tumor protein	ACO82289.1	*Oryzias latipes*	2.68E-64	84	928	9

a Identity (%).

b Frequency (time).

Bold are putative bio-defense and immune related genes.

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