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Potential Indicators of Stress Response Identified by Expressed Sequence Tag Analysis of Hemocytes and Embryos from the American Oyster, *Crassostrea virginica*

Matthew J. Jenny,[1] Amy H. Ringwood,[2] Eric R. Lacy,[3] Alan J. Lewitus,[2,4] Jason W. Kempton,[2] Paul S. Gross,[5] Gregory W. Warr,[5] and Robert W. Chapman[2,*]

[1]*Marine Biomedicine and Environmental Science, Medical University of South Carolina, Charleston, SC 29425, U.S.A.*
[2]*Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, SC 29412, U.S.A.*
[3]*Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, SC 29425, U.S.A.*
[4]*Belle W. Baruch Institute for Coastal Research, University of South Carolina, Georgetown, SC 29442, U.S.A.*
[5]*Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, U.S.A.*

Abstract: A pilot program was initiated to identify genes from the American oyster, *Crassostrea virginica,* that are potentially involved in the stress response for use as bioindicators of exposure to environmental pollutants and to toxic and infectious agents. A PCR-based method was used to construct cDNA libraries from pooled embryos and the hemocytes of a single individual. A total of 998 randomly selected clones (expressed sequence tags, ESTs) were sequenced. Approximately 40% of the ESTs are novel sequences. Several potential biomarkers identified include an antimicrobial peptide, recognition molecules (lectin receptors), proteinases and proteinase inhibitors, and a novel metallothionein. Diversity analysis shows that 363 and 286 unique genes were identified from the hemocyte and embryo libraries, respectively, indicating that full-scale EST collection is a valuable approach for the discovery of new genes of potential significance in the molluscan stress response.

Key words: oyster, biomarker, expressed sequence tags, immunity, development.

INTRODUCTION

Increasing land use and development in coastal zones over the past 50 years have placed additional stresses on resident estuarine organisms. These stressors include such factors as increased nutrient loads, organic and inorganic contaminants, increased runoff, increased sediment loads, and reductions in habitat. The impacts of these stressors on estuarine ecosystems are often not fully realized until long after the damage has been done. Over the past two decades molecular tools have been developed that can provide more sensitive indicators of stress on organism health. While advances have been made in linking particular stressors to individual biochemical pathways, much remains to be learned. This is due to the difficulty of assessing the actions of more than a handful of biochemical traits, the complex interactions between metabolic pathways, the diversity of stressors that may simultaneously assault the organism, and the myriad interactions between species and their environment.

A major recent advance has been "transcript profiling" (Shimkets et al., 1999; Ishii et al., 2000; Nelson et al., 2000), which allows the simultaneous assessment of the levels

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**Corresponding author: telephone 843-762-5402; fax 843-762-5110; e-mail chapmanr@mrd.dnr.state.sc.us*

of expression of many genes, up to and including the whole genome. This approach will greatly expand our understanding of physiology at the molecular level and can revolutionize our understanding of the impacts of anthropogenic modifications on the marine ecosystem. Before transcript profiling can be accomplished, however, an appropriate number of genes must be cloned, sequenced, and identified to permit the assembly of informative suites of genes in microarrays.

In this paper, we present the second in a series of studies (Gross et al., 2001) aimed at creating the necessary tools to evaluate the health of aquatic ecosystems. We have chosen for this investigation the American oyster, *Crassostrea virginica,* because it is an important member of the benthic community in estuarine systems along the Atlantic Coast of the United States and the Gulf of Mexico. Oyster reefs can have a pronounced effect on estuarine ecosystem structure and function by (1) providing physical substrates for colonization and aggregation; (2) filtering particulates from the water, which can impact water quality and plankton composition; and (3) excreting nutrients, which can provide a positive feedback mechanism for supporting plankton production. In addition, *C. virginica* is a commercially important species for aquaculture and harvesting of native populations. The past century has seen a major decline of natural populations for a variety of reasons including overharvesting, loss of habitat, degradation of water quality and increasing susceptibility to disease, including parasitism (Hofmann et al., 1995; Bushek and Allen, 1996; MacKenzie et al., 1997; White et al., 1998; Ford et al., 1999).

Industrial and urbanization activities leading to the contamination of sediment and water with multiple pollutants results in potential threats to human health and the loss of natural resources. Estuarine sediments act as a repository for environmental contaminants. Oysters are particularly susceptible to contaminant exposure due to their association with sediments, sessile nature, and potential for high filtration activity. In addition, oyster bioaccumulation of chemical pollutants, biotoxins, or infectious agents can adversely affect other ecosystem components or humans through trophic transfer.

Harmful algal blooms (HABs), defined as the sudden abundance of toxic or harmful algae within a localized region, can also have detrimental effects on estuarine environments (Hallegraeff, 1993; Anderson, 1995). Because of their filter feeding behavior, bivalves are capable of bioaccumulating harmful toxins by ingestion of these algae. Human consumption of these bivalves can result in paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), and neurotoxic shellfish poisoning (NSP) (Anderson, 1995; Lipp and Rose, 1997; Sierra-Beltran et al., 1998; Steidinger et al., 1998; Poli et al., 2000). In addition, bivalve shellfish are capable of acting as a vector for human pathogens such as *Vibrio* spp., *Salmonella* spp., *Campylobacter jejuni,* hepatitis A, and Norwalk and other enteric viruses (Lipp and Rose, 1997; Linkous and Oliver, 1999; Lee, 2000).

Both cultured and natural populations of oysters are susceptible to infectious agents. Three of the most notable diseases are dermo disease (*Perkinsus marinus*), MSX disease (*Haplosporidium nelsoni*) and juvenile oyster disease (JOD, etiology unknown). *Perkinsus* infections occur in *C. virginica* all along the eastern United States and in the Gulf of Mexico, from south Florida to Mexico (Hofmann et al., 1995). This protozoan is transmitted from oyster to oyster and can contribute to high mortalities throughout its distribution. MSX disease, also responsible for high mortalities along the Atlantic coast, has been reported from Maine to Florida (Burreson and Calvo, 1996; Barber et al., 1997; Ford et al., 1999). Finally, JOD is responsible for significant mortalities in hatchery-reared oysters cultured in the northeastern United States (Lewis et al., 1996). Although the etiological agent is unknown, JOD has been associated with *Vibrio* spp., as well as a novel marine species of α-proteobacteria belonging to the Roseobacter group (Lee et al., 1996; Boettcher et al., 1999, 2000).

C. virginica, because of its close relationship to aquatic sediments, susceptibility to chemical contamination, and ecological and economic relevance, is an important indicator species of estuarine health and a potentially valuable model for evaluating relationships between ecosystem and human health. In addition, the success of future restoration and management practices will rely on a greater understanding of the effects of environmental pressures, both natural and anthropogenic in origin, on organism health. Therefore, a functional genomics approach has been initiated to increase our understanding of stress-related biomarkers associated with oyster responses to chemical pollutants, pathogens, and toxic agents, including those endemic to oysters as well as humans.

Materials and Methods

Animal Handling

Adult *C. virginica* were collected from Lighthouse Creek, Charleston, S.C. and maintained in aerated natural seawater

by the Marine Resources Research Institute, South Carolina Department of Natural Resources (MRRI, SCDNR). Oysters used for hemolymph collection were allowed to depurate for 24 hours in filtered natural seawater before processing. Oysters used to produce embryos were allowed to depurate for 96 hours before collection of gametes and were fed a phytoplankton suspension consisting of *Chaetocerus gracilis* Strain (Bacillariophyceae) and *Isochrysis galbana* Strain (Prymnesiophyceae).

Embryo Collection

Under sterile conditions, gametes were stripped from four female and three male oysters and mixed to allow for fertilization. Successful fertilization and the total number of embryos were determined by light microscopy. Embryos were diluted with sterile natural seawater (NSW) to 50 embryos/ml and incubated 24 hours after fertilization until they reached the shelled D-veliger developmental stage. After the incubation period, replicate samples of embryos were collected and stored on ice. The embryos were pelleted by gentle centrifugation (3 minutes at 800 rpm, 4°C). Seawater was removed by aspiration, and the pellet was resuspended and homogenized in 300 µl of guanidine isothiocyanate buffer containing 10 µl/ml β-mercaptoethanol (RLT buffer, Qiagen, Valencia, Calif.).

Hemolymph Collection

Hemolymph was removed from the adductor muscle of adult oysters (approximately 4 inches in length) using a 1.0-cc tuberculin syringe with a 25-gauge needle preloaded with 100 µl of marine anticoagulant buffer (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) (Söderhall and Smith, 1983). Access was gained by breaking the hinge and creating a slight gap on the dorsal side of the oyster immediately adjacent to the adductor muscle. The average volume of hemolymph recovered was between 1.0 and 1.5 ml. The hemocytes were isolated by centrifugation (3 minutes at 2000 rpm, 4°C). Hemolymph was decanted, and the cells were resuspended and homogenized in 300 µl of RLT buffer (Qiagen).

cDNA Library Construction from Embryo and Hemocyte Tissues

RNeasy Miniprep kits (Qiagen) were used for RNA isolation. Samples for library construction were chosen by the quality of the RNA (high concentration of RNA versus minimal high-molecular-weight DNA) determined by agarose gel (0.8%) electrophoresis run at 60 V. The SMART cDNA Library Construction Kit (Clontech Laboratories, Inc., Palo Alto, Calif.), a PCR-based method, was used for construction of the libraries. The hemocyte library was constructed from a single animal. The embryo library was constructed by pooling together approximately 200,000 embryos for RNA isolation. cDNA was ligated into a λTriplEx2 vector and packaged using the Gigapack Gold λ (Stratagene, LaJolla, Calif.) packaging system. Library construction followed Gross et al. (2001). The hemocyte library contained 2.8×10^6 plaque forming units (pfu), and the embryo library contained 7.7×10^5 pfu. In both libraries, the proportion of vectors without an insert was approximately 3%. Twenty random plaques were chosen from each library for PCR amplification and *Sfi*I digestion. Average insert size was determined by agarose gel (0.8%) electrophoresis run at 80 V with comparison to a standard ladder. The average insert size (mean ± SD) for the embryo library was 768 ± 351 bp, and the average insert size for the hemocyte library was 1172 ± 475 bp.

Sequencing and Sequence Analysis

The λTriplEx2 vector was converted to the pTriplEx2 vector with *E. coli* strain BM25.8, which utilizes the Cre-loxP recombination system. Plasmids were isolated using Qiagen Turboprep 96 kits on the Qiagen Biorobot 9600, according to the manufacturer's instructions. All plasmid samples were sequenced by the Biotechnology Resource Laboratory, MUSC, using an ABI prism, Model 377 sequencer, and Big Dye terminator reactions. Samples were sequenced from the 5′ end using the Clontech sequencing primer (5′-AGCTCCGAGATCTGGACGAGC-3′) for the pTriplEx2 plasmid. The expressed sequence tags (ESTs) were converted to FASTA format and deposited in the NCBI dbEST database. EST sequences were submitted to the NCBI nonredundant database for BLASTN and BLASTX searches for matches to known sequences in GenBank. Database searches were limited to ESTs > 150 bp in length and matches with values less than 5.0×10^{-2} were considered significant.

Analysis of Library Diversity

In an effort to determine the diversity of expressed genes from each library, subsets of the EST sequences were constructed by sampling 50, 100, 150, 200, 250, 300, 350, and 400 sequences from each library. ESTs were sampled in the order of sequencing and categorized as products of either unique or redundant expressed genes. To account for the

Table 1. Discovery of Novel Genes and Analysis of Diversity in cDNA Libraries of *C. virginica*

	Hemocyte	Embryo
Total number of cDNAs sequenced	487	511
Average EST length*	708	606
Total number of cDNAs compared w/GenBank†	471	481
Matched ESTs‡	224	307
Nonredundant unmatched ESTs§	241(97.6%)	175 (94.1%)
Number of unique genes‖	363	286
H¶	5.44	4.51
H_{max}#	6.19	6.24

*Length of sequence used for comparison after editing.
†Vectors with inserts <150 bp were excluded.
‡e values $<5.0 \times 10^{-2}$.
§Parentheses indicate the percentage of unmatched ESTs that are unique.
‖Number of unique sequences including matched and novel sequences.
¶Shannon's diversity index.
#Maximum value of the Shannon diversity index if each EST was unique.

large percentage of novel sequences, a similarity comparison was performed between these sequences. To take into consideration the inaccuracies of the EST approach, a conservative cutoff of >80% sequence identity was used to classify ESTs as the product of the same gene. These data were then subjected to linear regression in order to determine the rate of new gene discovery. The Shannon diversity index (Shannon and Weaver, 1949) was calculated for each library.

Results

The embryo and hemocyte sequences have GenBank accession numbers between BG624106 and BG624523 and BG624524 and BG624961, respectively. The number of clones sequenced and the numbers of (putatively) identified ESTs and novel ESTs are given in Table 1. The similarity comparisons among the novel sequences indicated most of these ESTs from the hemocyte and embryo libraries were distinct, 97.6% and 94.1%, respectively (Table 1). ESTs from the hemocyte and embryo libraries represent 363 and 286 unique genes, respectively. In addition, the number of unique sequences from each library was plotted as a function of the total number of clones sequenced (Figure 1). After sequencing approximately 500 ESTs from both libraries, there is no indication of a plateau effect in the rate of new gene discovery. In addition, the Shannon-Weaver diversity indices for the hemocyte and embryo libraries are high, at 5.44 and 4.51, respectively, compared to the H_{max} values of 6.19 and 6.24 (Table 1).

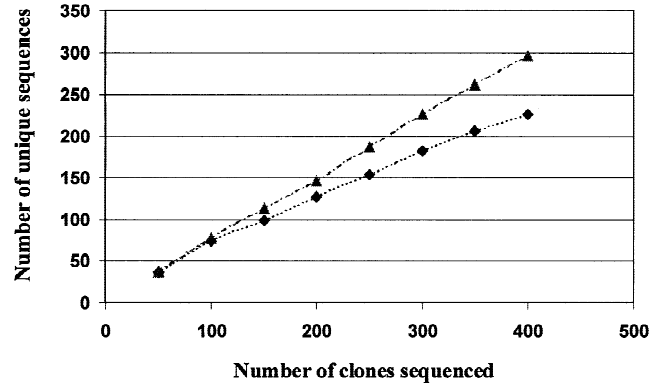

Figure 1. Number of unique sequences plotted as a function of the total number of clones sequenced from the hemocyte (▲) and embryo (◆) cDNA libraries.

Based on comparisons with the NCBI nonredundant database, the ESTs were clustered into ten categories (Figure 2). These were: (1) novel sequences that yield no matches or had poor matches (e-value $<5.0 \times 10^{-2}$) against the NCBI database; (2) ESTs with significant homology to genes of unknown function; (3) genes involved in DNA replication, repair, transcription, and translation, including ribosomal RNAs; (4) genes of known or potential immune function; (5) genes involved in metabolism and homeostasis; (6) cell shape, motility, and extracellular matrix genes; (7) membrane structure and channel protein genes; (8) genes with putative receptor, signal transduction, or hormonal function; (9) lysosomal and proteosomal genes; and (10) although identified, ESTs that did not fit appropriately into the above listed categories. Both the embryo and hemocyte libraries were dominated by sequences falling into the novel sequence or DNA replication categories. In each library, the sum of these two categories was greater than 70%. Novel sequences ranged from 36.2% to 52.4% in the embryo and hemocyte libraries, respectively. The embryo library was dominated by the DNA replication category, which made up 43.5% of the sequences compared to 20.2% in the hemocyte library. Surprisingly, both libraries had a minimal percentage of genes falling into the known or potential immune function category (2.7% and 3.8% for the embryo and hemocyte libraries, respectively).

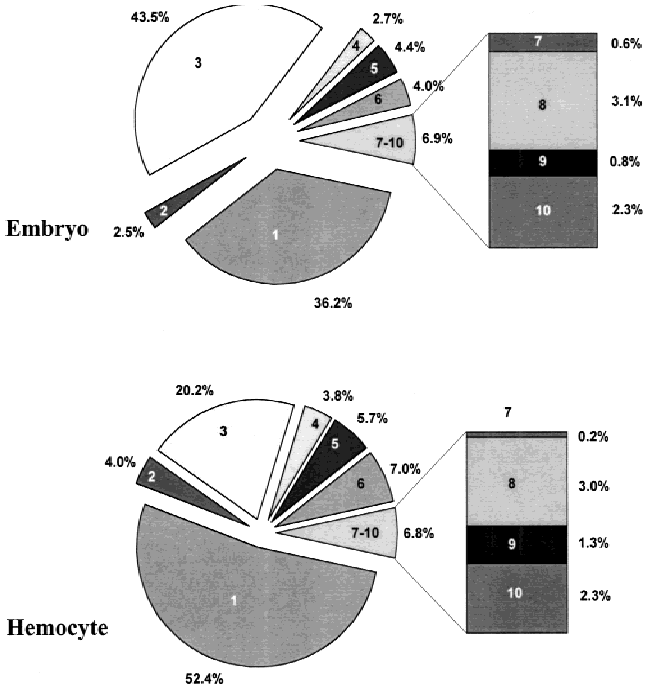

Figure 2. Distribution of ESTs by functional classes. Functional classes include: (1) novel sequences; (2) ESTs with significant homology to genes of unknown function; (3) DNA replication, repair, transcription, and translation, which includes ribosomal RNAs and mitochondrial DNA; (4) known or potential immune function; (5) metabolism and homeostasis; (6) cell shape, motility and extracellular matrix genes; (7) membrane structure and channel protein genes; (8) putative receptor, signal transduction or hormonal function; (9) lysosomal and proteosomal genes; and (10) no fit with other listed categories.

Table 2. Distribution of Expressed Genes Excluding Those Involved in DNA Replication, Transcription, or Translation.

Functional categories	Hemocyte library (%)	Embryo library (%)
Novel sequence	65.7	64.0
Matched to gene of unknown function	5.1	4.4
Known or potential immune function	4.8	4.8
Metabolism/homeostasis	7.2	7.7
Cell shape/motility/extracellular matrix proteins	8.8	7.0
Membrane structure/channel protein	0.3	1.1
Receptor/signal transduction/ hormonal function	3.7	5.5
Lysosomal/proteosomal	1.6	1.5
No fit with listed categories	2.9	4.0

Discussion

Overall Library Comparisons

Both libraries show similar patterns of gene expression, with the exception that the DNA replication category dominated sequences from the embryo library. Because the embryo library represents an early developmental stage, it is not surprising that such a high percentage of expressed genes would fall into this category. In fact, removal of this category from both libraries and comparison of the remaining sequences shows nearly identical expression patterns (Table 2). Notable observations from our libraries include the high frequency of novel sequences and the apparent lack of immune-related messages. It is not unreasonable to expect to find immune-related messages in the embryo library. *Mytilus edulis* larvae are capable of producing enzymes associated with hemocytes from adult mussels, and hemocytes have been demonstrated in trochophore and veliger larvae of these mussels (Dyrynda et al., 1995, 1997). In addition, because hemocytes play a primary role in host defense, a large percentage of expressed genes in this category was expected. Our results are in marked contrast to those obtained from a similar approach to immune-related gene discovery with penaeoid shrimp (Gross et al., 2001). Hemocyte libraries constructed from *Litopenaeus setiferus* and *L. vannamei* showed a strong representation of immune-related messages (27.6% and 21.2%, respectively). Because the *C. virginica* hemocyte library represents a single individual, it is important not to extrapolate too widely. However, the large percentage of novel sequences within both oyster libraries suggests the possibility of new gene discovery with respect to innate immune function in these molluscs.

Analysis of Library Diversity

The linear relationship between the number of clones sequenced and the number of unique genes (Figure 1) indicates the richness and diversity of both libraries. These observations are further supported by the Shannon-Weaver diversity indices (Table 1). The Shannon diversity index is an overall measure of diversity, which combines aspects of richness and abundance. For the purpose of these analyses,

richness represents the number of expressed genes and abundance denotes the number of copies of each expressed gene. The maximum diversity index (H_{max}), determined by the sample size, is used for comparison with the calculated index. The large diversity of the libraries is indicated by the close fit of our calculated values to the H_{max}. However, it is important to note certain inaccuracies that are inherent with an EST approach, in that individual ESTs could represent either different regions within the same protein or closely related proteins. This reservation aside, the continued sequencing of these libraries is likely to discover new genes at about the same rates as that shown in Figure 1. Subtractive hybridizations or other methods to eliminate high frequency transcripts are not warranted at the present time.

Molluscan Immunity and Potential Biomarkers

Molluscan host defense mechanisms are comprised of various innate, nonadaptive mechanisms. The primary cell responsible for immunoregulation is the hemocyte, which participates in a variety of functions, such as digestion and nutrient transport, wound healing, shell repair, excretion, and internal defense (Cheng, 1981). Hemocyte defense activities include encapsulation, nacrezation, and phagocytic activity with subsequent production of cytotoxic reactive oxygen intermediates (ROIs) (Cheng, 1981). Humoral components of the immune response include the prophenoloxidase (pPO) cascade, antimicrobial peptides, and secretion of hemolymph constituents, such as lectins and lysosomal enzymes (Dyrynda et al., 1995; Charlet et al., 1996; Asokan et al., 1997, 1998; Glinski and Jarosz, 1997; Roch, 1999).

Although techniques are well established for determining the levels of pathogens and contaminants within the environment, biomarker tools for addressing pathogenicity, toxicity, bioavailability, and the significance of the stress caused by these factors are needed. Environmental pollutants have the potential of acting as immunosuppressants by targeting particular tissues, thereby affecting the function of specific cell types and possibly disrupting host resistance. Compared to vertebrate systems, our understanding of the molluscan immune response is very limited. The development of bivalve immunomarkers will assist in the prediction of disease impacts on fisheries and can be used as predictors of public health risks from ingesting contaminated bivalve products (Oliver and Fisher, 1999). Established parameters (reviewed in Oliver and Fisher, 1999) useful in assessing the cell-mediated immunocompetence of oysters include: phagocytic activity, production of ROIs, hemocyte density and population ratios, and lysosomal stability. In addition, humoral factors, such as lysosomal hydrolases or agglutinins, are measurable parameters of immune response. The development of molecular biomarkers offers an efficient means of assessing cellular and molecular recognition and effector processes in immunity. Several potential biomarkers identified in our EST screening are summarized in Table 3.

Recognition Proteins

Three ESTs (GenBank accession numbers BG624594, BG624932, and BG624809) in the hemocyte library were similar to various C-type lectin receptors. Lectins are specialized proteins that bind to specific sugar moieties and cause agglutination of cells, promote cell–cell adhesion, and mediate the innate immune response (Drickamer, 1999). They have been demonstrated in the hemolymph and on the plasma membrane of circulating phagocytic cells of oysters (Cheng et al., 1984; Vasta et al., 1984; Olafsen, 1995). Two of the lectin sequences (BG624594 and BG624932) showed similarity to homologs of natural killer (NK) cell receptors. Both of these receptors have inhibitory functions that enable NK cells to discriminate self from nonself and prevent autoreactivity (Carlyle et al., 1999; Kung et al., 1999; Lohwasser et al., 1999). The third sequence (BG624809) matched to a probable mannose-binding C-type lectin (CD-SIGNR), expressed in a subset of dendritic cells, which shares conserved regions with the mammalian CD23 antigen (Soilleux et al., 2000).

A related EST (GenBank accession number BG624783) from the hemocyte library showed similarity to a scavenger receptor, expressed by human endothelial cells, which mediates the binding and degradation of acetylated low-density lipoprotein (LDL) (Adachi et al., 1997). This EST appears to contain a cysteine-rich region that is homologous to the scavenger receptor cysteine-rich (SRCR) domains characteristic of the SRCR superfamily. In addition to binding modified LDL, these receptors can also bind bacterial endotoxins as well as intact gram-negative bacteria (Dunne et al., 1994; Resnick et al., 1994).

Acute-Phase Response

One EST (GenBank accession number BG624815) from the hemocyte library matched a precerebellin-like protein that

Table 3. Potential Biomarkers Identified from EST Screenings of *C. virginica* Libraries

Accession number*	Putative match†	Genus match‡	e value§	Accession number‖
BG624524	Big Defensin	*Tachypleus*	3e-04	P80957
BG624594	NKR-P1B (NK receptor)	*Rattus*	1e-3	AAB01986
BG624932	Killer Cell lectin-like receptor	*Mus*	1.8e-2	NP_034782
BG624809	Probable mannose binding C-type lectin	*Homo*	3e-5	AAG13848
BG624783	Scavenger Receptor (SREC)	*Homo*	5e-15	NP_003684
BG624815	Precerebellin-like protein	*Oncorhynchus*	1e-4	AAF04305
BG624706	Cathepsin Y	*Rattus*	1e-37	BAA82844
BG624471	Cathepsin B-like proteinase	*Sarcophaga*	1e-16	S38939
BG624476	Cathepsin B	*Mus*	3e-17	NP_031824
BG624322	Thiol protease	*Phaedon*	1e-7	CAA76927
BG624574	Cystatin B	*Homo*	4e-11	XP_009791
BG624590	Serine Carboxypeptidase 1 precursor	*Homo*	1e-27	NP_067639
BG624852	Squamous cell carcinoma antigen 2	*Mus*	8e-4	NP_033152
BG624635	Bacterial-induced peroxidase	*Gossypium*	9e-5	AF155124
BG624190	Bacterial-induced peroxidase	*Gossypium*	2e-8	AF155124
BG624166	Sesquiterpene cyclase	*Artemisia*	6e-6	AJ271792
BG624427	Sesquiterpene cyclase	*Artemisia*	8e-5	AJ271792
BG624734	Thymosin beta	*Oncorhynchus*	1e-11	BAA85772
BG624884	Thymosin beta-4 precursor	*Rattus*	5e-13	I52084
BG624386	Thioredoxin	*Schizosaccharomyces*	4e-22	AAF05765
BG624216	Nucleoredoxin	*Mus*	2e-25	NP_032776
BG624250	Nucleoredoxin	*Mus*	7e-25	NP_032776
BG624332	Nucleoredoxin	*Mus*	3e-26	NP_032776
BG624486	Metallothionein	*Arianta*	3e-10	P55946
BG624579	Transcription Factor AP-1	*Gallus*	6e-16	P18870
BG624651	Transcription Factor AP-1	*Gallus*	2e-7	P18870
BG624554	c-jun protein	*Xenopus*	2e-10	CAB51636
BG624249	Vitellogenin B	*Locusta*	8e-3	AF115317
BG624136	Heat Shock Factor binding protein 1	*Homo*	9e-20	NP_001528

*GenBank accession number of submitted ESTs from the hemocyte and embryo libraries.
†Putative protein determined by comparison of EST with the nonredundant database (GenBank).
‡Genus of best match.
§The e value determined by the BLAST search.
‖Accession number of the matched entry in GenBank.

was originally isolated and further characterized from the rainbow trout (*Oncorhynchus mykiss*) by inducing an acute-phase response (APR) with *Vibrio* bacterin (Gerwick et al., 2000). Interestingly, precerebellin has strong similarities to the globular (non-collagen-like) region of the B chain of human complement component C1q (Urade et al., 1991; Kishore and Reid, 1999). Similar to the rainbow trout, this EST appears to have an open reading frame that encodes a 143-amino acid protein lacking the characteristic Gly-X-Y repeats expected with the collagen region of C1q. Collectins, a group of calcium-dependent lectins to which C1q belongs, mediate a variety of immune functions, including complement activation, opsonization, and modulation of humoral immune responses (Sastry and Ezekowitz, 1993; Vasta et al., 1999; Kishore and Reid, 2000).

Proteinases and Proteinase Inhibitors

With respect to invertebrate immune function, the role of proteinases and proteinase inhibitors has been well charac-

terized in the regulation of melanization, mediated by the prophenoloxidase system, and hemolymph coagulation (Söderhall and Cerenius, 1998; Kanost, 1999). In support of their protective role in molluscan immunity, antibodies against various cathepsins, cysteine proteinases, have demonstrated the presence of these proteins in the granules of hemocytes from *Mytilus edulis* (Pipe, 1990). In addition, proteinase inhibitors support host defense mechanisms by affording protection from a variety of proteinases associated with pathogenic virulence (Kanost, 1999). A comparative study demonstrated the presence of proteinase inhibitors in the hemolymph of *Crassostrea* spp. (Faisal et al., 1998). In addition to inhibiting a variety of activities attributable to the mechanistic classes of proteinases, plasma from *Crassostrea* spp. inhibited extracellular proteinases produced by *Perkinsus marinus* and *Vibrio vulnificus* (Faisal et al., 1998). The production of extracellular serine proteinases by *Perkinsus marinus* is an important factor in the virulence of this parasite. Interestingly, *C. gigas,* which is resistant to *Perkinsus,* appears to possess proteinase inhibitors with greater activity compared to those in *C. virginica* (Faisal et al., 1998, 1999).

In support of these previous observations, one serine proteinase and several cysteine proteinases were identified from our cDNA libraries. A single EST (GenBank accession number BG624590) in the hemocyte library showed strong similarity to a novel serine carboxypeptidase I precursor protein. A single EST from the hemocyte library showed similarity to a novel cysteine proteinase (GenBank accession number BG624706), cathepsin Y. In addition, two copies (BG624471 and BG624476) of cathepsin B and a single copy (BG624322) of a thiol proteinase were identified from the embryo library. Finally, a copy (BG624574) of cystatin B (cysteine proteinase inhibitor) and a putative protein (BG624852) containing a serpin (serine proteinase inhibitor) domain were identified in the hemocyte library. Serpins and cystatins, along with their respective proteinases, have been demonstrated to play a role in coagulation and complement activation (Iwanaga et al., 1998; Smith et al., 1999; Vasta et al., 1999).

Antimicrobial Peptides

The presence of antibacterial or antifungal peptides and their role in the innate immune response were first characterized in bivalves with the isolation of antimicrobial peptides from the hemolymph of *Mytilus edulis* (Charlet et al., 1996). To date, four families of antimicrobial peptides, including the defensins, mytilins, myticins, and mytimycin, have been identified in *Mytilus* spp. (reviewed in Mitta et al., 2000). Molluscan defensin and myticins appear to be more active against gram-positive bacteria compared to gram-negative bacteria or fungi. The mytilin isoforms B, C, and D are active against both gram-negative and gram-positive bacteria, whereas the G1 isoform displays activity only against gram-positive bacteria. Mytimycin is strictly active against fungi.

Surprisingly, only one EST (GenBank accession number BG624524) from our hemocyte library appears to encode an antimicrobial peptide. This EST shows similarity to big defensin, an antimicrobial peptide originally found in the granules of hemocytes from the horseshoe crab, *Tachypleus tridentatus* (Saito et al., 1995). In horseshoe crabs, northern blot analysis demonstrated the presence of big defensin message in hemocytes and additional tissues (Kawabata et al., 1997). The open reading frame of big defensin encodes 117 amino acid residues of the preprotein. Cleavage sites for a signal peptidase and a processing protease yield the mature 79-residue big defensin. The 37 residues found in the COOH-terminal domain are similar in sequence to mammalian defensins and potent against gram-negative bacteria. However, big defensin has an extended hydrophobic N-terminal domain with a greater potency for gram-positive bacteria (Saito et al., 1995).

Other Potential Immune Effectors

The hemocyte library contained two messages (GenBank accession numbers BG624734 and BG624884) with strong homology to β-thymosin proteins, which are small proteins involved in the vertebrate host immune response and cell differentiation (Naylor and Goldstein, 1988; Tzehoval et al., 1989; Bonnet et al., 1996). Thymosin β 4 has also been implicated in wound healing (Malinda et al., 1999). Although we can only speculate about the potential role of these proteins, it seems reasonable that these factors may play a role in hemocyte differentiation.

Two messages (GenBank accession numbers BG624166 and BG624427) matching a sesquiterpene cyclase from *Artemisia annua* were identified in the embryo library. In addition, ESTs (BG624635 and BG624190) with similarity to a bacterial-induced peroxidase expressed in *Gossypium hirsutum* were identified in both libraries. Sesquiterpene cyclases mediate the production of antimicrobial isoprenoids in a variety of plant species. *Artemisia annua,* an annual herbaceous plant, has received recognition as a medicinal herb

with antimalarial properties. Artemisinin, a sesquiterpene lactone endoperoxide, and two of its derivatives, artemether and artesunate, have been effective against the malarial parasite, *Plasmodium falciparum* (Klayman, 1985).

Related Biomarkers

Multiple thiol-based antioxidants were identified in the embryo library including one copy (GenBank accession number BG624386) of thioredoxin and three copies (BG624216, BG624259, and BG624332) of nucleoredoxin. Thiol-based antioxidants have been implicated in the transcriptional control of an important immune-related transcription factor, NF-κB. Reduced thioredoxin enhances the DNA binding of activated NF-κB by maintaining the cysteine residue in the Rel homology domain in a reduced state (Sen, 2000). Multiple ESTs (BG624579, BG624651, and BG624554) with similarity to the Jun protein were found in the hemocyte library. The AP-1 protein, a regulatory transcription factor, is formed by a combination of Jun and Fos proteins. This protein complex has been implicated in the expression of genes involved in cellular response to oxidative stress (Guyton et al., 1996; Muller et al., 1997; Mietus-Snyder et al., 1998). Thioredoxin, nucleoredoxin, and AP-1 are potential indicators of oxidative stress in oysters.

An EST (GenBank accession number BG624486), which appears to encode a novel metallothionein (MT) distinct from the previously identified Cd-MT (Roesijadi et al., 1989), was identified in the embryo library. Metallothioneins are low-molecular-weight proteins with a high cysteine content (up to 30%), characterized by repeating cysteine motifs, C-X-C or C-X-X-C. They function in metal metabolism and homeostasis as well as detoxification. In the past, metallothionein expression has proven to be a useful biomarker in molluscan and other estuarine organisms (Hylland et al., 1996; Pedersen et al., 1997; Ringwood et al., 1999; Cajaraville et al., 2000; Cosson, 2000). In addition to binding and sequestering metals, MTs are oxyradical scavengers implicated in immunoregulatory pathways in oyster hemocytes as a result of their ability to scavenge antimicrobial ROIs (Anderson et al., 1999). MTs have also been implicated in NF-κB activation (Abdel-Mageed and Agrawal, 1998; Sakurai et al., 1999).

Heat-shock proteins are molecular chaperones that protect the cell and maintain homeostasis under stressful conditions. Several studies support the validity of their use as biomarkers for a variety of cellular stressors (Heikkila et al., 1982; Cochrane et al., 1991; Bauman et al., 1993; Yamada and Koizumi, 1993; Scofield et al., 1999; Matranga et al., 2000). A single message (GenBank accession number BC624136) for heat-shock factor binding protein 1, which negatively regulates the classical heat-shock response by inhibiting the DNA binding of heat-shock factor 1, was identified in the embryo library (Satyal et al., 1998).

Finally, one EST (GenBank accession number 624249) showed strong similarity to vitellogenin B. Vitellogenins are lipophosphoglycoproteins produced under female hormonal control as a result of estrogen-induced synthesis. They have been used as biomarkers of environmental estrogens, natural or man-made chemicals possessing estrogenic effects, in a variety of vertebrate species (Stancel et al., 1995; Palmer and Palmer, 1995; Allner et al., 1999; Yadetie et al., 1999).

Conclusion

The current study has identified a number of transcripts that should be valuable to studies of the impacts of environmental effects on oysters. However, the observation that most of the messages (ca. 70%) encode proteins that are either completely novel or are related to genes of unknown function may be the most interesting finding. If taken at face value, it suggests that a large proportion of metabolic energy is utilized, in oyster embryos and hemocytes, to generate proteins whose functions are unknown. Clearly much remains to be learned about oyster metabolism; however, studies of the expression of their genes should not only provide clues to their function, but should also offer a new approach to the evaluation of marine environmental health.

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