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MARINE BIOTECHNOLOGY

Development of Expressed Sequence Tags from Eastern Oyster (*Crassostrea virginica*): Lessons Learned from Previous Efforts

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Abstract: Marine organisms respond to the environment by regulating their gene expression. Pollutants may induce changes in a characteristic set of genes to form their genomic expression signature. To identify marine biomarkers a project was initiated to develop expressed sequence tags (ESTs) from the eastern oyster *Crassostrea virginica*. A total of 4572 complementary DNA clones were sequenced, generating 4348 ESTs (95.1% success rate). All these ESTs have been submitted to dbEST. The average EST length was 641 bp. Combined with the existing oyster ESTs, a total of 5206 ESTs are currently available in the dbEST database from *C. virginica*. This work accounted for more than 83% of ESTs available from the eastern oyster. Cluster analysis of the 4348 ESTs indicated the presence of 243 contigs and 1673 singletons, together representing 1916 unique sequences. This was achieved by overgo hybridization subtraction of mitochondrial genes that are expressed abundantly in the gill and gonad tissues from which the ESTs were sequenced. Of the 1916 unique sequences, 875 (46%) were identified as known genes by BLASTX similarity searches and 1041 were unknown gene sequences. This rate of gene identification was low in comparison to vertebrates, but it was reasonably high for oyster ESTs given that relatively low numbers of known genes are available in GenBank from invertebrates, especially the mollusks. The largest number of significant hits was from comparison with *Drosophila* genes. A search of the ESTs using 1395 gene ontology terms found a number of genes involved in stress biology, including several heat shock proteins, stress-induced proteins, and an antimicrobial peptide, defensin B. These ESTs should be useful for studies of environmental pollution and stress biology. Complementary DNA microarrays are being developed for the assessment of differential gene expression under various environmental conditions.

Key words: expressed sequence tag (EST), biomarker, oyster, pollution, gene expression, microarray.

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Introduction

United States coastal zones provide some of the most valuable natural resources, yet these environments are faced with harsh environmental stresses. Expansion of coastal populations and economic activities exert relentless pressure on the coastal ecosystems. These same systems are the ultimate repository for a vast array of substances discharged from human activity inland and carried by rivers and streams to the coast.

The overall gene expression profiles of an organism are affected not only by its development and physiology, but also by the environment in which it grows. Specific gene expression profiles of an organism under a given environment can then be regarded as its genomic expression signatures (GES). Changes in environmental conditions often cause changes in a GES by turning on (upregulating) or turning off (downregulating) gene expression. These induced or suppressed genes may represent active defense, adaptation, or destructive consequences to organisms. A specific pollutant may induce or suppress a characteristic set of genes or GES in an organism exposed to it. Studies of GESs, therefore, can provide biomarkers for monitoring marine environments. However, capturing a GES requires genome resources such as microarrays (Schena et al., 1995; for review, see Ramsay, 1998), which in turn require availability of a large number of unique expressed sequence tags (ESTs) (Adams et al., 1991).

Oysters are found throughout coastal marine ecosystems in the United States. Our study utilized the eastern oyster *Crassostrea virginica*, which is found in coastal ecosystems from Canada to Mexico. Oysters are sedentary and thus are good representations of a particular location. They are filter feeders, feeding directly on particulate organic matter and phytoplankton. They reach sizes practical for analytic purposes in less than a year. Oysters have a longevity of 5 to 7 years, allowing them to serve as effective bioindicators for a relatively long time. Oysters are easy to culture in captivity and can be maintained and labeled in the laboratory and in their natural environment. The ability of oysters to concentrate pollutants such as heavy metals to high concentrations in soft tissues is well known (Vazquez et al., 1993; Al-Madfa et al., 1998). Since we are interested in the potential impact of pollutants on reproduction, particularly sex phase switch and sex ratios, we used oyster gonad tissues to provide accurate molecular information concerning GES. Gill tissues are the first layer of tissues exposed to environment pollutants. In eastern oysters a limited resource of ESTs was available, with the only report of ESTs being from cDNA libraries of hemocytes and embryos (Jenny et al., 2002). The objectives of this project were to develop practical methods for efficient gene discovery in oysters and to expand the EST resources of eastern oysters for the assessment of GES.

Materials and Methods

Complementary DNA Library Construction

Oysters were produced and cultured at the Marine Shellfish Laboratory of Auburn University at Dauphin Island. Gill and gonad tissues were dissected and immediately frozen in liquid nitrogen and stored in a −80°C freezer until extraction of RNA. Total RNA was isolated by the guanidinium thiocynate method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated using the Poly(A)$^+$ Pure kit (Ambion). The cDNA libraries were constructed using pSport-1 plasmid vector as we previously reported (Ju et al., 2000; Cao et al., 2001).

Subtraction with Overgo Probes

Initially 1500 cDNA clones were randomly picked and sequenced to evaluate the quality of the libraries and the redundancy of EST sequencing. Then 19 overgo probes (Table 1) were made against the most abundantly expressed genes, most of which were mitochondrial genes. Colony-lifting hybridization (Sambrook et al., 1989) was conducted using the overgos as probes to reduce the sequencing redundancy of these genes and to evaluate whether or not overgo hybridization is effective for subtraction. After overgo hybridization subtraction, 3072 more clones were selected for sequencing. All clones were sequenced using automatic sequencers.

Oligonucleotides were custom-made by Sigma Genosys. Each pair of oligos composed an overgo probe (Han et al., 2000). Overgos were designed to overlap for 8 bases where the sense and antisense oligos pair, leaving the remaining 5′ overhang for filling in using labeled nucleotides, P^{32}-dATP and P^{32}-dCTP. All the overgos were labeled in a single reaction. Overgo hybridization was conducted at 45°C overnight using conditions as previously reported (Han et al., 2000). The filters were washed using 2× SSC at room temperature 4 times for 15 minutes each. After exposure of x-ray films, bacterial plates were aligned to match

Table 1. Overgo Probes Used for Subtractive Hybridization

Locus	Upper primer	Lower primer (reverse complement overgo)
16S rRNA	CCTTAGCGTGAGGGTGCTAAGGTAGC	CAAAAGGCAAGGAATTTCGCTACCTT
BG624232 (unknown gene)	GCAGCCTTAATATTGCTAAGGATATG	AACAGAATCCTACCTAACCATATCCT
BG624748 (unknown gene)	TGGCACCTCGATGTTGAATCAGGGAT	CTACGCCTTAAAGGTATAATCCCTGA
ATP synthase F0 subunit 6	CGTGGCGGGCCTAATGCCAAGAATGTTCTATAGTG	GATGCATCAAGCTGCATGATATTGACACTATAGAA
Cytochrome *b*	GCCGTAATTAAGCCTGAGTGATACTTTTTGTACGC	GGAATAGACCGTAAAATGGCATAAGCGTACAAAA
Cytochrome *c* oxidase subunit I	GTGGCAGACATGCAGTTCCCTCGATTAAATGC	CCCTGGCAATACTCAGAATCTAAAAGCATTTAATC
Cytochrome *c* oxidase subunit II	GGAGTTACTTTTACTAGAATGCGGGTGCAATG	CAACACAATACATACCAAACGCTCATTGCAC
Cytochrome *c* oxidase subunit III	CCATAGGGCTTTGTCATCTTCTGTTGAGATTGGG	CTAGCCCGACTGGTGGTCAGCAGCACCCAATCTC
NADH dehydrogenase subunit 1	CCTAGGGGTTCTAGTAAGTGTGGCTTACTTTACC	GAGCTTAAACATTTACGCTCTATCAAGGTAAAGT
NADH dehydrogenase subunit 2	GTACTAAGTTTGTGATCCTTCAGTTATCAATAGAG	CACTATTGTGATTGTTGAATCTTGCTCTATTGAT
NADH dehydrogenase subunit 3	CGTTAGGATTTGTTAGCCTTGAAGAAGACTCTTATG	CATAAGGTGATGACTTGTCCCAGTCATAAGAGTC
NADH dehydrogenase subunit 4	CACGTTCAGGCTCCAGTTGGGGGCTCAGTTATTC	GCCTAGCTTAAGAAGAATTCCTGCCAGAATAACTG
NADH dehydrogenase subunit 4L	GGGGCAATACTTAAGAAGTTTTCTAACTTCCTTAG	CGCCCTCTATTAAAATAAGGTATCTAAGGAAGT
NADH dehydrogenase subunit 5	CTAATTTCGGTAAACTTAGAAATATGCCTAGATTG	ACAGAAGCAAAAGTTAAAGATATTCAATCTAGGC
NADH dehydrogenase subunit 6	CTTGCGTTTATAGTTTACATTAGAGGAATTAC	GTAACAAAATACCCAATCACCGCAGTAATTCC
β-Actin	GACGGTCAGGTCATCACCATTGGCAACGAACG	CATGGCCTCTGGGCACCTGAATCGTTCGTTGC
Elongation factor 1α	CTTCAGGATGTCTACAGATTGGAGGTATTGG	CCACTCTGCCGACTGGCACAGTTCCAATACCTC
α-Tublin	GTACGCCAAGCGTGCCTTCGTCCACTGG	CATACCCTCTCCGACGTACCAGTGGACG
Soma ferritin	GGTACTTCATCAGTTTTTCAGCATGTTCCCGTTCTTC	CAAGTTCTTCAAGAATTCATCAGATGAAGAACGGG

Figure 1. X-ray film using overgo probes (dark spots). Negative colonies (light spots) were picked for sequencing.

the patterns of the exposed colonies on the x-ray film. Negative colonies were picked for sequencing (Figure 1).

EST Analysis

EST sequences were analyzed using several different approaches. First, cluster analysis was conducted using Vector NTI (InforMax). The overlap was set at 20 bp, and similarity was left at default (85%). The numbers of contigs and singletons and the average number of clones per contig were calculated and recorded. Previously produced eastern oyster sequences were downloaded from GenBank into Vector NTI local databases before analysis. Second, Basic Local Alignment Search Tool (BLASTX) searches were conducted to determine gene identities. A cut off *P* value of 1×10^{-4} was used for significant hits. Finally, gene ontology analysis was conducted to identify genes involved in specific biological pathways (http://www.geneontology.org; Thomas et al., 2003).

Results and Discussion

Eastern Oyster ESTs

A total of 4572 clones were sequenced and 4348 ESTs were generated from this project (95.1% success rate), of which 2655 ESTs were generated from the gill cDNA library and 1693 ESTs from the gonad cDNA library. The gill and gonad sequences have GenBank accession numbers from CD646372 to CD650719. A summary of the EST sequencing, clustering, and BLAST analysis is shown in Table 2. The ESTs had an average length of 641 bp. Combined with the existing ESTs, a total of 5206 ESTs ar

Table 2. Summary of Oyster ESTs

EST	Number
Total number of ESTs	4348
Gill ESTs	2655
Gonad ESTs	1693
Average EST length (bp)	641
Contigs	243
Singletons	1673
Known genes	875
Unknown genes	1041
Total unique sequences	1916

now available in the dbEST database from *C. virginica*. This work accounted for over 83% of ESTs available from the eastern oyster.

Cluster analysis of the 4348 ESTs indicated the presence of 243 contigs and 1673 singletons, together representing 1916 unique sequences. Sequencing redundancy is a major problem in oysters. Our initial sequencing of 1500 clones indicated that mitochondrial genes accounted for a large fraction of the oyster transcriptome. This initial finding is compatible with previous research in oysters (Shimizu et al., 2002), which found that mitochondrial transcripts accounted for 35% of the 4200 sequenced ESTs in *C. gigas*. In some oyster tissues the transcriptome appears to be less polarized. For instance, sequencing redundancy was lower with hemocytes (Jenny et al., 2002; Gueguen et al., 2003).

Reduction of Sequencing Redundancy by Overgo Hybridization

To reduce sequencing redundancy several options were available, including construction of normalized libraries (Soares et al., 1994). However, funding for oyster EST sequencing was low, essentially ruling out the larger sequencing projects in which normalization is cost-effective. With this in mind we tested overgo hybridization subtraction as a feasible and realistic method for redundancy reduction. Use of only 19 overgos allowed significant reduction in sequencing redundancy (Table 3). Initially, when the library was not sequenced in depth, subtraction of the 19 abundantly expressed genes allowed consistent increase in the numbers of singletons and contigs and reduction in the average number of clones per contig, indicating a rise in unique ESTs (Figure 2). At a greater

Table 3. Comparison of Gene Discovery With or Without Overgo Hybridization Subtraction of 19 Abundantly Expressed Genes at Sequencing Depth of 1000 Clones

Finding	Before overgo subtraction	After overgo subtraction
Clones included in contigs (%)	47	39
Singletons (%)	53	61
Contigs (*n*)	63	78
Singletons (*n*)	538	610
Unique sequences (*n*)	601	688
Redundancy at 600 (%)	28.4	20
Redundancy at 1000 (%)	46.2	39

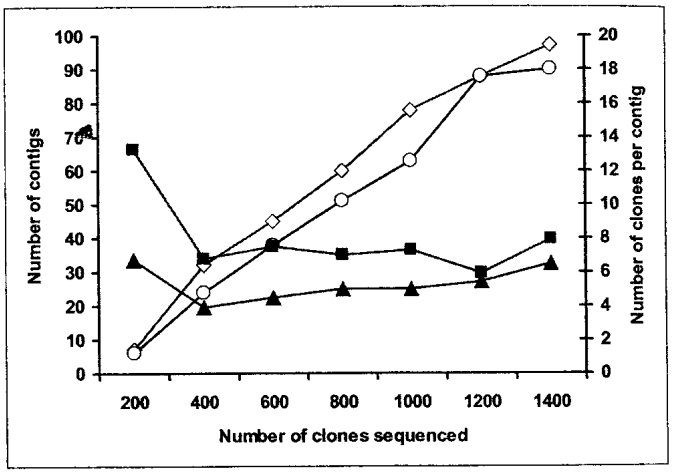

Figure 2. Efficient subtraction with 19 overgos: number of contigs (diamonds indicate subtracted; circles, no subtraction) and average clones per contig (triangles, subtracted; squares, no subtraction).

sequencing depth, this effect was diminished, illustrating the need to once again subtract with newly sequenced, abundant ESTs identified through cluster analysis. Over the course of the project, the average number of clones per contig was reduced by 2 to 3 clones. A reduction of 2 clones per contig in 243 contigs would translate into a reduction of sequencing by 486 clones, reducing sequencing efforts and expense while increasing gene discovery efficiency.

Eastern Oyster Gene Identification

BLASTX similarity searches of the 1916 unique sequences revealed that 875 (46%) were known genes and 1041 were unknown gene sequences. This rate of gene identification is

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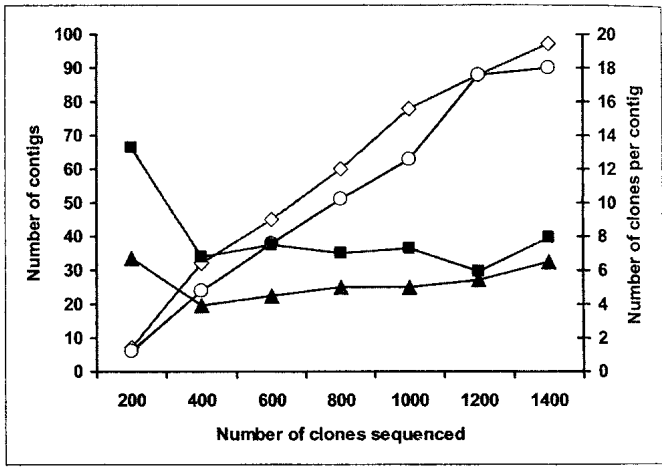

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Table 4. Genes Involved in Stress Biology

Most similar to	Accession number
Heat shock protein 27	CD648461
Heat shock protein 70	CD649237
Heat shock protein 60	CD648752
Heat shock protein 40-3	CD648907 and CD648745
Universal stress protein	CD649124 and CD648514
Stress-induced phosphoprotein 1	CD648711
Hypoxia inducible factor 1α	CD648099
Vitellogenin	CD647526
Aldehyde dehydrogenase 1	CD648520
Ornithine decarboxylase (ODC)	CD646686
Glutathione peroxidase	CD650160
Defensin B	CD650234

low relative to that for vertebrates (Karsi et al., 2002; Kocabas et al., 2002), largely because of the lack of invertebrate genes in GenBank. The vast majority of genes in GenBank are from mammalian species whose sequences share low sequence similarities with oyster sequences. The largest number of significant hits was from comparison with *Drosophila* genes. A BLASTX search against *Drosophila* release 3.1 with e-value E^{-5} using 2926 queries (all clones sequenced after subtraction with 19 overgos) resulted in 845 significant hits with 548 unique hits. However, when the oyster ESTs were searched against dbEST, many more significant hits were obtained, with most of them coming from comparison with mosquito ESTs. Clearly, many genes were evolutionarily conserved, but the identities of most of these genes are yet unknown. Although it is unlikely that funding will be available for oyster research to directly determine the functions of the conserved genes, further efforts among closely related invertebrates will aid greatly in gene identification through comparative genomics.

Oyster Genes Involved in Stress Biology

A search of the ESTs using 1395 gene ontology terms (http://www.geneontology.org) indicated the presence of a number of genes involved in stress biology (Table 4), including heat shock proteins, stress-induced proteins, and an antimicrobial peptide, defensin B (Lowenberger et al., 1999). These ESTs should be useful for studies of environmental pollution and stress biology.

Bioindicators responding to a wide range of environmental stresses have been identified from fish and marine invertebrates, including metallothionein (Roesijadi, 1996), heat shock proteins (Bradley, 1993; Schroeder, 1999), aminolevulinic acid dehydratase (Martin and Black, 1998), catalase (Teisseire et al., 1998), cytochrome P450 oxidases (for review, see Lopez-Barea and Pueyo, 1998), ethoxyresorufin-*o*-deethylase (Kosmala et al., 1998), and vitellogenin (for review, see Palmer et al., 1997). Ideal biomarkers should be pollutant-specific, dose-dependent, and persistent in duration. Many of the existing biomarkers are not pollutant-specific, or they represent irreversible consequences of environmental stresses. Therefore sensitive and reliable biomarkers are needed for environmental biotechnology to provide information concerning gene expression in relation to growth, reproduction, and population structures of organisms in the marine ecosystem. Rapid research progress in this area depends on the development of highly sensitive and efficient technologies such as microarrays for determination of genome-wide differential gene expression under specific environmental stresses. The ESTs developed from this project should be a valuable genome resource for the development of microarray technology.

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