MARINE BIOTECHNOLOGY

Molecular Biomarkers and Adaptation to Environmental Stress in Moon Jelly (*Aurelia* spp.)

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Abstract

We describe a strategy that identifies molecular biomarkers and links the study of abiotic stress to evolutionary history. By utilizing the moon jellyfish *Aurelia* spp. as a model, we identified genes differentially regulated in response to the chemical stressor tributyltin by means of complementary DNA subtraction analyses. Expression of 3 out of 25 identified candidate genes, one oxidative stress gene, one heat shock (hsp70) gene, and one GTP-binding gene, was quantified under laboratory conditions and in field tests using semiquantitative reverse transcriptase polymerase chain reaction. Differential expression patterns were found following exposure to tributyltin and temperature treatments. The findings suggest that the identified genes are involved in response to chemical as well as heat-induced stress and may serve as biomarkers for monitoring marine habitats. Gene regulatory patterns combined with phylogenetic inferences of the hsp70 gene support a possible role of ecologically driven divergence within the genus *Aurelia*. We show that added information on genetic variability can raise the predictive power of molecular biomarkers in studies of individual stress response.

Key words: Cnidaria — *Aurelia* — cDNA-subtraction — biomarker — hsp70 — environmental stress

Introduction

Industrial exploitation along seashores and increased sea dumping are the primary causes of the deposition of large numbers of xenobiotics in the ocean. The latter have been affecting marine organisms and ecosystems in a variety of ways (Barrie et al., 1992; Guardans and Gimeno, 1994; Macdonald et al., 2000). Xenobiotics may lead to reproduction disturbances, diseases, or even the extirpation of sensitive organisms (Kime, 1995; Oehlmann and Bettin, 1996; Arkoosh et al., 1998; Moreira et al., 2001). In addition to chemical stress, changing environmental conditions, like temperature shifts, can act as natural stressors (Hoffman and Parsons, 1994). Combined effects of chemical factors and natural factors such as temperature shifts are believed to be responsible for the drastic degradation of sensitive ecosystems, with coral reefs providing a good example over the past decades (Hughes, 1994; Brown, 1997a; Dustan, 1999). Consequently, simultaneous study of both natural and anthropogenic impacts is necessary to understand the risks posed to marine habitats. Because natural stressors act on short as well as evolutionary time scales, the implementation of evolutionary genetic approaches into the study of biological stress response seems promising.

Fluctuations in environmental conditions have produced a broad range of cellular responses and organismal adaptations, including behavioral avoidance and alterations in reproduction (Brown, 1997b). Thus a number of conventional biotests evaluate organismal responses to xenobiotics by utilizing endpoints such as reproductive success, growth, and mortality rates (Römbke and Moltmann, 1996). Current efforts are directed toward establishing sensitive early-warning measurements by means of biomarkers (van Gestel and van Brummelen, 1996; Newman, 1998). Biomarker responses at cellular and molecular levels are increasingly used to supplement traditional endpoint measurements in marine systems (e.g., Cajaraville et al., 2000). It has yet to be established how these markers may be incorporated into the assessment of changes at higher levels of biological organization, such as populations, com-

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munities, and ecosystems. Here, the use of molecular biomarkers (genes) represents an integrative tool: (i) sequence information within a phylogenetic framework can help to elucidate how natural selection has influenced adaptive capacities, (ii) taxonomic units can readily be defined for standardizing experimental systems, and (iii) expression profiling offers a direct measurement of ongoing responses to environmental stress. In such an approach ideal candidate genes are those that are evolutionarily conserved and involved in homeostasis or protective mechanisms like metabolic detoxification and oxidative resistance.

The moon jelly (*Aurelia* spp.) belongs to the phylum Cnidaria (Scyphozoa) and is one of the most common gelatinous coastal zooplankton species worldwide. The life cycle of *Aurelia* consists of a pelagic medusa stage, which is found near-shore, and a sessile polyp stage, which inhabits shallow coastal waters. Its significance for pelagic ecosystems has been well documented in terms of abundance, population densities, feeding ecology, and trophic function (Russel, 1970; Möller, 1984; Olesen et al., 1994; Schneider and Behrends, 1994; Purcell, 1997; Mutlu, 2001). Recently, phylogeographic studies based on morphologic and genetic data have shown that the genus *Aurelia* consists of a number of phylogenetic lineages (species) on a global scale that reflect ecologically driven divergence (Dawson and Jacobs, 2001; Schroth et al., 2002; Dawson, 2003). A biological phenomenon of increasing interest is the rising number of unpredictable jellyfish blooms, including *Aurelia* populations, that cause local ecologic shifts and may have harmful effects on fishery and power plants (summarized by Mills, 2001). Owing to their ecologic and economic significance and their well-studied biology and widespread distribution, *Aurelia* species are highly suitable organisms for biomonitoring. Moreover, the small size of *Aurelia* polyps, combined with their vegetative reproduction, make them ideal invertebrate models for experimental studies in the laboratory.

We have initiated a study to identify genes that are potentially involved in cellular stress responses and can be used as biomarkers in *Aurelia* spp. We constructed complementary DNA libraries enriched for stress-inducible genes from *Aurelia* polyps that were treated with the organic compound tributyltin (TBT). This organotin compound has been extensively used over decades as a toxic paint additive preventing biological material from attaching to vessels' hulls. Because it leaches from paints and has slow biodegradation and strong adhesive capacity to the sediment, it remains one of the most severe pollutants in coastal marine environments (e.g. Fent, 1996). We used TBT because it is generally toxic to aquatic organisms and has endocrine disruption effects, causing imposex, for example, in marine mollusks (Fent, 1996). Stress-inducible genes (biomarkers) were evaluated in laboratory tests by exposures to different TBT concentrations. A field test with *Aurelia* polyps exposed at polluted sites and at more pristine localities in the Adriatic (Mediterranean) Sea was done in order to test the transferability of isolated biomarkers in the field. In an attempt to better understand the gene inducibility pattern from an evolutionary perspective, we chose 2 *Aurelia* lineages that naturally inhabit warm or temperate (UBI lineage) and boreal (BOR lineage) climatic regions (Schroth et al., 2002). The heat shock protein (hsp70) expression response to different elevated temperatures as well as the genetic variation within the gene were compared among these 2 lineages.

Materials and methods

Animals. Specimens of *Aurelia* spp. were collected from different localities in the North Sea, Northern Atlantic, and Mediterranean Sea. Polyps of both the UBI and BOR lineage (Schroth et al., 2002) were cultured year-round for a 5 year period. Polyps representing the UBI lineage were cultured at 17°C year-round. For the cold-adapted BOR lineage, the temperature regimen included a periodically changing temperature between 17°C in summer and 4° to 8°C in winter. Animals were maintained in artificial seawater (35‰ salinity, hw-Meersalz), at a 12-hour-daylight regimen and were fed twice a week with 3- to 4-day old larvae of *Artemia salina*. Laboratory experiments on the BOR specimens were carried out in summer during the 17°C temperature phase.

Preparation of RNA and cDNA. Total RNA was extracted from polyps using an established procedure: tissue was minced in a buffer containing proteinase K (100 mM Tris-HCl, 10 mM EDTA, 0.1 M NaCl, 1% sodium dodecylsulfate [SDS], 50 mM dithiothreitol [DTT], 1% β-mercaptoethanol, 0.5 μg/ml proteinase K) in the presence of a ribonuclease inhibitor (Invitrogen). Following incubation at 55°C for 1 hour, RNA was isolated by acidic phenol–chloroform–isoamyl alcohol (25:24:1) extraction in the presence of 0.2 M sodium acetate (pH 4) and subsequent isopropanol precipitation. RNA pellets were solubilized in RNase-free water and immediately stored at −80°C. RNA samples were treated with RNase-free DNaseI at 37°C for 1 hour followed by phenol-chloroform extraction and precipitation and washing to completely remove genomic DNA. Subsequently, first-strand cDNA was synthesized from 1 μg of purified total RNA in a volume of 20 μl

for 50 minutes at 42°C. The reaction mixture contained 20 pmol of an oligo$(dT)_{20}$VN anchored primer, 200 U of Moloney murine leukemia virus reverse transcriptase (M-MLV; Invitrogen) in 1× first-strand buffer, 10 mM DTT, 0.5 mM each of dNTP, and 5 U of recombinant RNase inhibitor (Invitrogen).

Complementary DNA subtraction library. Subtractive hybridization analyses were done by a combination of SMART cDNA synthesis and PCR-Select technique (Clontech). Five polyps each of the UBI lineage were taken as a reference sample and exposed to TBT for 24 hours at 500 ng/L. This approach aimed to enrich messenger RNAs that are uniquely expressed in the treated sample. First-strand cDNA was synthesized based on 200 ng of total RNA from TBT-treated (experimental) and control samples, respectively. The 5 cDNA specimens were pooled into one reference sample and one experimental sample and amplified according to the manufacturer's recommendations (Clontech) in order to provide sufficient amounts of double-stranded SMART cDNA. Two subtractive hybridization rounds between adapter-ligated experimental cDNA and reference cDNA led to enriched differentially expressed sequences. The latter were used as templates for nested polymerase chain reaction (PCR) with adapter-specific primers (for sequences see Clontech protocol). To generate a cDNA subtraction library, column-purified PCR reactions were ligated into pGEM-T vector (Promega) and cloned in DH5α *Escherichia coli* cells.

To generate probes for subsequent membrane hybridizations, digoxigenin-labeled T7 and Sp6 standard primers were used in bacteria colony PCR for the insert amplification of randomly selected clones. A pairwise dot blot of amplified SMART cDNA from reference and experimental samples (1 μg each) was used as template to verify differential expression of the selected gene probes (virtual Northern blot). Hybridization analyses were done overnight at 42°C by the addition of 10 ng/ml of a subtracted digoxigenin-labeled cDNA probe in hybridization buffer containing 50% formamide, 5 × SSC, 0.1% N-lauroyle sarcosine, 0.02% SDS, and 2% blocking reagent (Roche Diagnostics). Dot blot filters were washed twice for 5 minutes at room temperature (2 × SSC, 0.1% SDS), followed by 2 stringent washing steps of 15 minutes at 68°C (0.2 × SSC, 0.1% SDS). Signals were detected by chemiluminescence using CSPD substrate (Roche Diagnostics) and exposure to x-ray film for 1 to 2 hour. Probes showing differentially expressed signals were reamplified from the respective bacterial clone and directly sequenced on an automatic DNA sequencer (ABI 310; Applied Biosystems).

Gene identification and analysis. Gene sequences related to differentially expressed cDNAs were identified by BLAST search in GenBank. All isolated *Aurelia* sequences have been deposited in GenBank under accession numbers AY836653–AY836667. Biomarker analysis within a phylogenetic framework was done using characterized *Aurelia* lineages UBI (n = 8) and BOR (n = 11). Sequence alignment was carried out with the aid of CLUSTAL X (Thompson et al., 1994). Pairwise sequence divergences and phylogenetic relationships were inferred using reconstruction methods implemented in PAUP* Version 4.0 (Swofford, 2001). A test of purifying selection on gene diversity was done with DAMBE (Xia, 2000).

Laboratory experiments. Experimental approaches consisted of stress induction by TBT and different heat treatments. TBT treatment was performed by means of passive uptake of water-soluble chloride salt (Sigma-Aldrich) from ambient seawater in 100-ml glass containers. Acute toxic effect concentration of TBT was initially determined using 3 phenotypic endpoints: tentacle contraction of polyps, strobilation rate (rate of vegetative production of medusa larvae), and rate of malformed ephyra (larval stage of medusa). On the basis of these range findings, 20 polyps of the UBI lineage were each treated with 5 and 50 ng/L of TBT. Seawater prepared with the defined xenobiotic concentration was changed once a day. All other factors, such as salinity, light regimen, and feeding regimen, were identical for experimental groups and nontreated controls. Variability in time to response following TBT-induced stress induction was accounted for by different exposure times: 3 polyps were taken before exposure (controls) and after 13, 18, 37, and 82 hours of exposure, and RNA was isolated and stored immediately at −80°C. Specimens of the 2 *Aurelia* lineages UBI and BOR were separately exposed to thermal stress of 20°C, 23°C, 26°C, and 28°C seawater temperatures. Three replicates were taken after incubation periods of 1, 8, and 24 hours, and RNA was extracted and stored immediately at −80°C. UBI and BOR specimens maintained at 17°C seawater temperature served as control groups. It is important to note that when the temperature experiments began, BOR specimens had been acclimatized to 17°C for 2 months after switching from winter (4°–8°C) to summer conditions (see section ''Animals'').

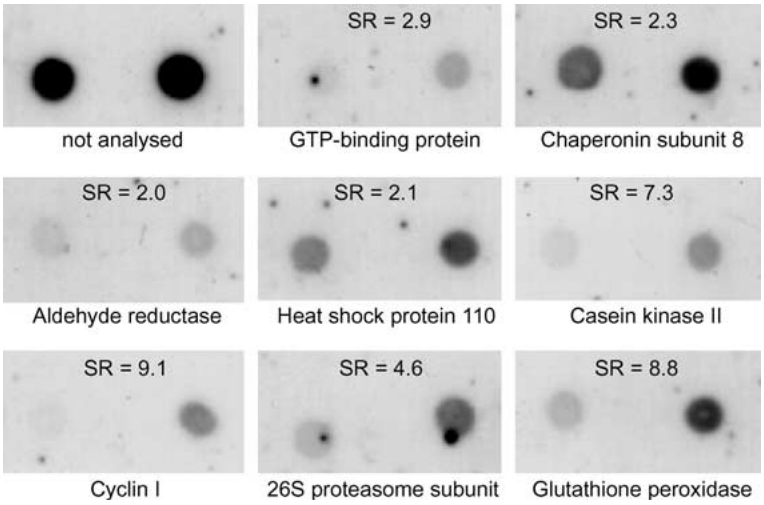

Fig. 1. Example of SMART cDNA Southern dot blots (virtual Northern) of differentially expressed biomarkers in *Aurelia* spp. (UBI lineage). Each pairwise dot blot shows a DIG-labeled subtracted cDNA probe hybridized to control (left) and TBT-treated (right) SMART cDNA. High-stringency hybridization/washing followed by chemiluminescence detection revealed several upregulated signals in the treated sample (SR indicates signal intensity ratio treated/control sample).

Field observations. Selected biomarkers were examined under natural conditions at putative polluted and unpolluted sites at the marina of Ancona (Italy, Adriatic/Mediterranean Sea) and an undisturbed beach outside Ancona (see Figure 3). At both places roughly 20 *Aurelia* polyps from the laboratory (UBI lineage) were exposed in open circuit plastic containers for 5, 6, and 12 days. To avoid artificial oxidative and thermal stress, *Aurelia* polyps were transported from exposure sites to the laboratory in containers with ambient seawater and at temperatures similar to field conditions.

RT-PCR and transcript quantification. PCR assays were designed to normalize biomarker gene expression levels to β-actin transcription rate. We subjected 0.5 to 1 μl of cDNA (from 20 μl of reverse transcription mix) to PCR in 25 μl total volume of 1 × PCR buffer (20 mM Tris-HCL, pH 8.4, 50 mM KCL), 2 mM $MgCl_2$, 0.1 mM each of dNTPs, 5 pmol each of primers, and 0.5 U *Taq* DNA polymerase (Invitrogen). Primers for β-actin and 3 putative biomarkers were as follows: β-actin fw, GGT ATY GCT GAY CGT ATG CA, and rv, GAG ATC CAC ATY TGT TGG AA; hsp*70* fw, GAG ACA CTC ATC TTG GAG GG, and rv, TTG ACT TGC AGA GTT CCT TG; GTP-binding protein fw, AAC TTG ATG CGT TGG AGA GG, and rv, AAT GAC AGA TCG AGC ACG AG; oxidative stress gene fw, TGA CAA TGG AAC AGC AGA GG, and rv, AAT GAG GAG CTG AGT CAA CC. Cycling profile after initial denaturation at 93°C for 3 min was as follows: denaturation at 93°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 40 seconds. The minimum number of PCR cycles to ensure quantification within the exponential phase of amplification was determined to range from 22 to 31 cycles, depending on the gene analyzed. Equal amounts of RT-PCR reactions were loaded on standardized 2% agarose gels containing 0.1 μg/ml ethidium bromide. The gel images were digitalized by a documentation system (Bio-Rad Laboratories). Intensities of bands were measured by means of an imaging computer program (Image J Version 1.26; www.rsb.info.nih.gov/ij/index.html) to calculate the ratio of biomarker mRNA to β-actin mRNA. Band densities of control samples were set to 1.0, and test samples were standardized to the control value such that gene upregulation of the test samples refers to the relative amount of transcripts.

Expression data from experimental TBT exposure and heat shock experiments were tested for time dependency by linear regression and for treatment effects by Kruskal-Wallis and Mann Whitney U test (control vs. treatment replicates). Differences were considered significant at $P \leq 0.05$ (one-sided as only effects of upregulation were measured).

Results

Isolation of candidate genes. The screening of dot-blotted amplified cDNA samples from control and TBT treatments with 300 DIG-labeled subtracted cDNA probes revealed several differentially expressed signals (Figure 1). A subset of 25 upregulated subtracted cDNAs with signal intensities increased at least 2-fold were sequenced. Of these, 21 cDNA sequences showed similarity to known invertebrate and vertebrate genes, and a remaining set of 4 upregulated transcripts could not be classified to known gene families (Table 1). The genes identified by sequence and structural comparisons can be grouped into 5 functional categories of homeostasis, oxygen/radical detoxification, metabolism, signal transduction/cell activation, and degradation/

Table 1. Homology/similarity matches of biomarker genes identified from *Aurelia*

Clone ID	*Accession number*	*Length (nt)*	*Putative match and functional categories*	*Genus*	*Identity (% aa)*
			Cell homeostasis		
009	AY836653	1265	Heat shock protein 70 (Hsp70)	*Xenopus*	81
018	AY836654	355	Heat shock protein 110 (Hsp110)	*Strongylocentrotus*	65
103	AY836658	466	DnaJ homologue (Hsp40)	*Rattus*	52
125	AY836660	693	Tyrosine-rich heat shock protein	*Mus*	58
213	AY836665	539	Chaperonin subunit 8	*Mus*	63
			Oxygen and radical detoxification		
135	AY836662	471	Oxidative stress protein	*Mus*	56
143	AY836663	503	Glutathione peroxidase (GPX)	*Suberites*	67
			Metabolism		
102	AY836657	253	Aldehyde reductase (ALR)	*Mus*	63
034	AY836655	525	Hydroxyacyl-CoA dehydrogenase (HADH)	*Homo*	65
127	AY836661	692	S-adenosylhomocysteine hydrolase (SAHH)	*Caenorhabditis*	74
			Signal transduction/cell activation		
119/126	AY836669	1257	GTP-binding protein	*Mus*	60
228	AY836667	572	Cyclin I	*Mus*	34
101	AY836656	398	Casein kinase II (CK2)	*Danio*	33
			Degradation and subcellular localization		
236	AY836668	782	GM2 activator protein (GM2A)	*Mus*	43
208	AY836664	750	Matrix metalloproteinase 1 (MMP)	*Hydra*	43
215	AY836666	353	26S proteasome subunit p112	*Rattus*	78
108	AY836659	542	Arp2/3 protein subunit p16	*Lumbricus*	50
			Hypothetical proteins		
017		442	Unnamed protein	*Homo*	36
019		333	Hypothetical protein XP_143194	*Mus*	31
136		362	Hypothetical protein XP_108188	*Homo*	68

subcellular localization (Table 1). The homeostasis group consists of 5 clones (*009, 018, 103, 125*, and *213*) that appear to be homologous to conserved heat shock protein and chaperone sequences (hsp70, hsp110, hsp40, tyrosine-rich hsp, and chaperonin subunit 8) of a variety of eukaryotes. The hsp70 fragment was elongated to a total of 1265 nucleotides, which include part of the 5′ untranslated region and the ATP-binding domain of the gene product (370 amino acids).

The functional group of oxygen and radical detoxification consists of a gluthatione peroxidase (*143*) and a gene fragment (*135*) that matches the carboxyl terminus of a protein that is related to oxidative stress in mice macrophages (Ishii et al., 1996). We characterized gene *135* by means of 5′ rapid amplification of cDNA ends and Northern analysis, which revealed a transcript length of approximately 1.8 kb. The gene codes for 419 amino acids and contains different conserved domains including a zinc-finger domain of 36 amino acids and ubiquitin-associated domain at its carboxyl end.

The categories of metabolism and signal transduction/cell activation are represented by 3 metabolic enzymes, aldehyde reductase (*102*), hydroxyacyl–coenzymeA dehydrogenase (*034*), and S-adenosylhomocysteine hydrolase (*127*), as well as cell-cycle activating cyclin I (*228*) and casein kinase II (*101*). Furthermore, we found 2 signals (clones *119* and *126*) that match to the same gene, a guanosine triphosphate-binding protein (GTP or G-protein). The first 80 codons (out of 418 amino acids) of the combined sequence representing the conserved transmembrane domain show identities of 81 to 85% the N-terminal part of G-proteins of *Drosophila*, mouse, *Caenorhabditis*, and human.

The functional group of degradation and subcellular localization genes relates to degradation of deleterious gangliosides in human (GM2 activator protein, *236*), general proteolytic activity (26S proteasome, *215*), and turnover and degradation of extracellular matrix components (matrix metalloproteinase, *208*). Clone *108* shows similarity to the complete 16-kDa subunit coding region of the eukaryotic arp2/3 complex (actin-related protein). Finally, 3 cDNA fragments show amino acid homologies to hypothetical and unnamed proteins from mouse and human (Table 1).

Biomarkers tested in laboratory experiments. Three differentially expressed genes, the oxidative stress gene (*135*), hsp70 (*009*), and the G-protein encoding gene (*119*, *126*), were chosen for testing biomarker capabilities under defined experimental conditions. The chemical stressor used was the organotin compound TBT. We initially deter-

mined the acute toxic effect concentrations using 3 organismal endpoints: tentacle contraction of polyps, strobilation rate, and ephyra deformation. The lowest observed effect concentration (LOEC) of approximately 1 μg/L for TBT were found to be comparable to other invertebrates studied (e.g., EC_{50} 2.5 μg/L TBT in *Daphnia*; Oberdorster et al., 1998a). Exposure concentrations for biomarker testing were therefore reduced by 2 orders of magnitude to 5 to 50 ng/L TBT. A significant 2 to 7-fold transcript upregulation for all 3 genes was seen in response to TBT treatment at both exposure levels, when compared with controls ($P \leq 0.05$, Figure 2). In the 5 ng/L TBT experiment, transcript levels of the oxidative stress gene increased significantly with time ($r = 0.79$, $P \leq 0.05$). Similar, but not significant trends were observed for the G-protein ($r = 0.69$, $P = 0.07$) and for hsp70 ($r = 0.56$, $P = 0.21$). The 10-fold higher TBT concentration (50 ng/L) caused a stronger increase in transcript levels for hsp70 (7-fold at 13 hours) and the oxidative stress protein (8-fold at 82 hours). Differences for time-matched experiments (18, 37, 82 hours) were not significant among both TBT concentrations. Transcriptional induction of the G-protein gene was moderate at both TBT doses.

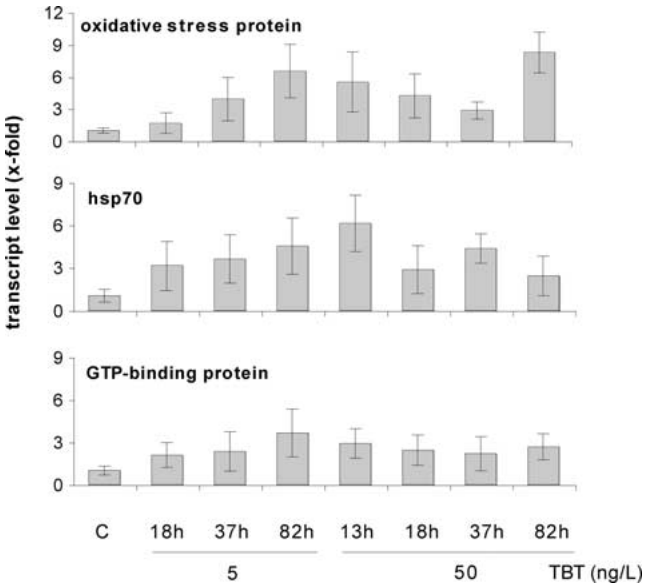

Fig. 2. Relative transcript levels of 3 putative biomarker genes in *Aurelia* specimens after exposure to 5 ng/L and 50 ng/L of TBT. Transcript levels were normalized to β-actin expression, and gene upregulation refers to a dimension of x-fold relative to the control samples (C) which were set to 1.0 (for simplicity, only controls for the 5 ng/L TBT experiment are shown). Shown from top to bottom are the transcript levels (mean and standard deviation of 3 replicates) of the oxidative stress gene, hsp70, and the GTP-binding protein. Variance analyses and pairwise comparisons between treated samples (combined over all time points) and control replicates showed significant differences at both TBT concentrations ($P \leq 0.05$, Mann Whitney U test). At 5 ng/L TBT, a positive correlation between exposure time and transcript levels was observed for the oxidative stress gene ($r = 0.79$, $P \leq 0.05$); similar trends for the G-protein ($r = 0.69$, $P = 0.07$) and for hsp70 ($r = 0.56$, $P = 0.21$) were not significant.

Biomarkers tested in the field. The oxidative stress gene and hsp70 were tested for their suitability to reveal the presence of environmental stress under natural field conditions. Figure 3 shows the RT-PCR products from *Aurelia* polyps exposed at assumed polluted and unpolluted sites in the Adriatic/Mediterranean Sea. An obvious 3 to 6-fold transcript upregulation of both genes occurred at the putatively polluted marina of Ancona compared with the ''unpolluted'' control site at a beach 10 km south of Ancona (FC, Figure 3, a,b). The strongest signal derived from hsp70 after 12 days of exposure in the marina of Ancona. Compared with laboratory control levels (LC, Figure 3, b), transcript levels from field controls were slightly higher, suggesting that putatively undisturbed areas also evoke some stress response at the molecular level.

Hsp70 gene diversity and expression under thermal stress. Phylogenetic reconstruction analysis based on a 415-nucleotide coding fragment of hsp70 revealed 2 well-defined monophyletic groups separating the *Aurelia* lineages UBI and BOR (Figure 4, a). Sequence divergence among both lineages was 4.7% and included 8 synapomorphic nonsynonymous sites. A significant bias toward synonymous substitutions (19%) versus nonsynonymous sites (3.3%) was observed ($P < 0.01$, χ^2 test). We also tested the frequency of the observed nonsynonymous sites distributed on the 3 codon positions against an expected distribution assuming the lack of purifying selection (Li, 1993; Xia, 2000). A significant deviation from expectation was seen at the second codon position (expected 43.8% vs. observed 20%, $P < 0.01$, χ^2 test). To understand the possible functional role of the synapomorphic amino acid changes, we aligned the *Aurelia* sequences to the structurally known bacterial homologue DnaK (subalignment shown in Figure 4, b). One of the substituted amino acids in *Aurelia* (Figure 4, b, arrow) corresponds to an amino acid that promotes hydrophobic interactions within one of 2 helical domains forming the nucleotide binding cleft in the DnaK model (Brehmer et al., 2001). In order to test if lineage-specific hsp70 variation corresponds to phenotypic differences in expression level, we exposed polyps from both *Aurelia* lineages to thermal stress. RT-PCR results from control groups indicate that hsp70 is weakly expressed at 17°C in both lineages (data not shown).

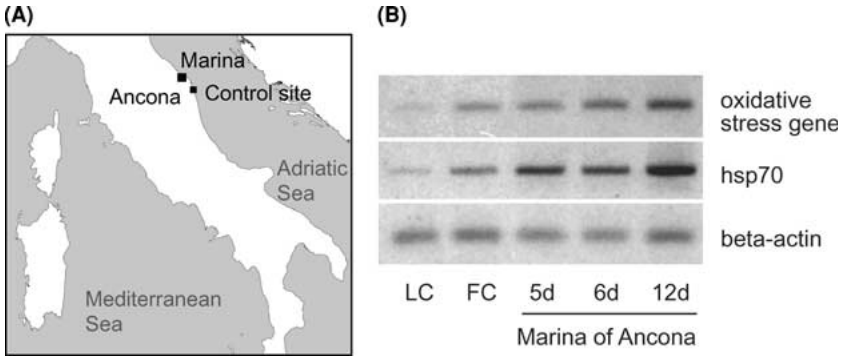

Fig. 3. **A**: Field sites for *Aurelia* polyp exposure in the Adriatic/Mediterranean Sea in Italy, marina of Ancona (putatively polluted) and outside Ancona (putatively unpolluted). **B**: Corresponding RT-PCR banding profiles for the oxidative stress (*135*), hsp70, and β-actin gene are shown for 5, 6, and 12 days of exposure. LC and FC denote laboratory and field control samples, respectively. Each RT-PCR profile represents a cDNA pool of 2 to 10 polyp specimens.

In response to experimental temperature stress, specimens of the BOR lineage raised their hsp70 levels earlier (within 1 to 8 hours) and at lower temperatures (20°C and 23°C) than the UBI lineage ($P \leq 0.05$, Figure 5). In contrast, UBI specimens showed maximal hsp70 expression response after 8 hours at 26°C and 28°C exposure. Temperature-induced transcript upregulations were significant compared with controls ($P \leq 0.05$; except for the 1- and 24 hour exposure at 20°C).

Discussion

Characterization of candidate genes. The experimental approach applied in this study revealed a number of inducible genes that respond to TBT treatment. One major group of upregulated genes represents molecular chaperones. Heat shock proteins and their associated co-proteins are expressed by all known organisms, either constitutively or in response to various stressors, including temperature, oxidative stress, UV irradiation, heavy metals, xenobiotics, and microwaves (Lindquist and Craig, 1988; Sanders, 1993; Vedel and Depledge, 1995; Wiens et al., 1998; de Pomerai et al., 2000). Their function in protein folding and reconstitution during cell stress is well documented (Hightower, 1991; Gething and Sambrook, 1992). Accordingly, the observation of a set of different heat shock genes induced by TBT in *Aurelia* demonstrates that this disruptive compound provokes denaturation and instability of proteins. The protein-denaturating po-

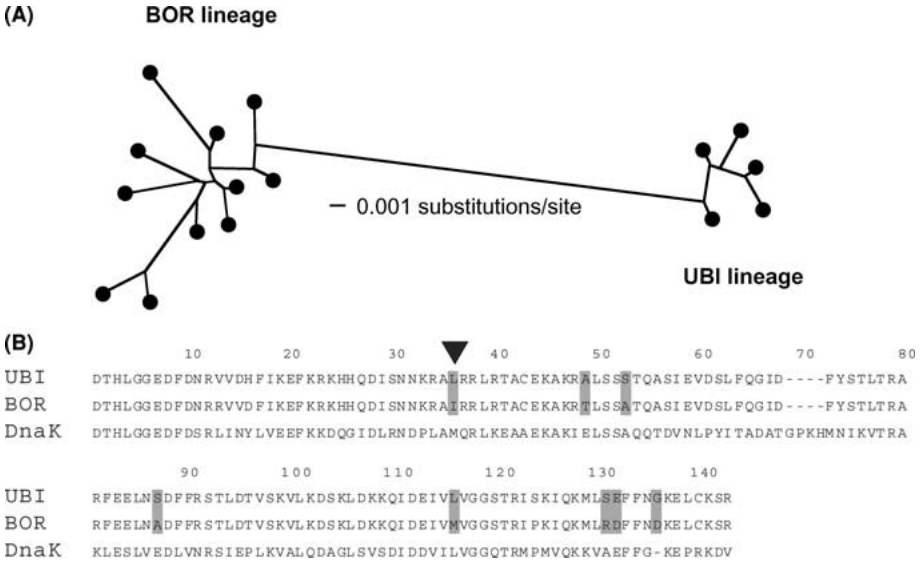

Fig. 4. Phylogenetic analysis based on partial hsp70 fragment (415 nucleotides) encoding 138 amino acids of the ATPase domain. **A**: Phylogenetic relationships among *Aurelia* lineages UBI and BOR based on nucleotide sequences. The unrooted neighbor joining tree depicts Kimura 2 parameter (K2P) distances between 8 (UBI) and 11 (BOR) specimens (only differing haplotypes are shown). **B**: Amino acid alignment of *Aurelia* UBI and BOR lineages and the bacterial homologue DnaK. Shaded amino acids show synapomorphic differences among *Aurelia* lineages. The arrow depicts an amino acid residue that promotes hydrophobic interactions within the helical nucleotide binding cleft of ATPase in the DnaK model, suggesting a possible functional role in *Aurelia*.

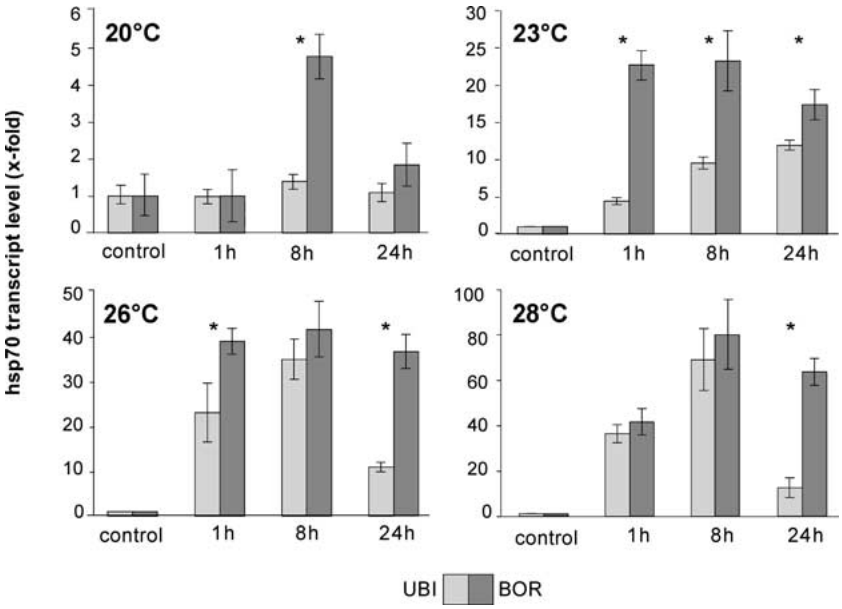

Fig. 5. Semiquantitative analysis of hsp70 gene expression. Time-dependent transcript levels at thermal stress exposures to 20°C, 23°C, 26°C, and 28°C are shown for 2 *Aurelia* lineages (light bars indicate UBI; dark bars, BOR). Controls were maintained at 17°C. Transcript levels are normalized to β-actin expression and standardized relative to the mean of the control replicates (set to 1.0). Mean value and standard deviation of each 3 replicates are shown. Asterisks denote significant differences among the 2 lineages ($P \leq 0.05$, Mann Whitney U test).

tential of TBT has been shown in a number of aquatic test systems (Fent, 1996); moreover, heat shock protein induction by TBT treatment has been reported in blue crabs (Oberdorster et al., 1998b).

A second frequently encountered stress condition is the accumulation of reactive oxygen and radical species. The *Aurelia* cDNA clone *135* shows amino acid similarities of 41% to 50% to an oxidative stress protein from mouse as well as to proteins associated with kinases and viral defense pathways in human and *Drosophila* (Ishii et al., 1996; Wayne et al., 1996; Puls et al., 1997). Hence the *Aurelia* homologue may play a protective role against oxidative stress or it could be involved in stress-related immunity/inflammatory-like signal transduction, which has been shown to exist in lower metazoans such as sponges (Kruse et al. 1999). The presence of multifunctional regulatory domains, including ubiquitin binding and zinc-finger domains, also support a possible role in signaling. We simultaneously observed upregulation of a glutathione peroxidase, which is usually involved in detoxification of oxygen radicals (e.g., Cikryt et al., 1982). It could therefore be argued that a TBT-mediated inhibition of mitochondrial ATP-synthetase causing impaired oxygen consumption (Fent, 1996) could have been responsible for the induction of both genes in *Aurelia*. Accordingly, clone *135* may tentatively be categorized as an oxidative-stress-related signaling molecule, as noted above.

Further biomarker candidates belong to the functional categories metabolism and signal transduction/cell activation. Their human homologues have been reported in the context of various pathologic conditions: hydroxyacyl–coenzyme A dehydrogenase (*034*) has been described in the context of neuronal dysfunction and Alzheimer disease (He et al., 1998); S-adenosylhomocysteine hydrolase (*127*) co-regulates transmethylation and can be viewed as a multipurpose means to regulate cellular processes including biotic and abiotic stress; casein kinase II (*101*) has been reported to regulate enzymes linked to the cell cycle (Allende and Allende, 1995), and more interestingly, by its phosphorylation of tumor suppressor p53 after UV-induced DNA damage (Keller et al., 2001). We further identified a gene encoding a GTP-binding protein that has been described in the context of renal failure (Laping et al., 2001). A TBT induced protein kinase was recently reported to be involved in stress signaling and putative apoptosis induction in sponges (Fafan et al., 2003). It is conceivable that the induced G-protein in *Aurelia* might mirror this process at an early stage during signal transduction.

Finally, we isolated a total of 4 genes representing the functional group of degradation and subcellular localization. Their homologous gene products act as degradative enzymes for deleterious compounds (GM2 activator protein, *236*), for general proteolytic activity (26S proteasome, *215*), or for turnover of

extracellular matrix components (matrix metalloproteinase, *208*). The latter was found to be differentially regulated during head regeneration and morphogenesis in *Hydra* (Yan et al., 2000) and thus can be regarded a marker for either degradative processes (ECM) or regenerative responses following cell damage. In addition, upregulation of protein subunit arp2/3 (*108*) which represents a central regulator of actin dynamics (assembling and cross-linking of actin filaments; May, 2001), was found. Altogether, the presence of inducible genes representing degradative and cell-shaping processes favors the idea that TBT provokes secondary events of stress response at a stage when repair alone is not sufficient to cope with deleterious conditions. This finding is in agreement with several studies showing the strong potential of TBT to induce apoptotic cell degradation (e.g., Lavastre and Girard, 2002).

The experimental strategy used in this study seems promising for identifying biomarkers in general even when dealing with yet unexplored organisms of small size. The obtained cDNA fragments aligned to both the 5′ and 3′ ends of known homologous genes, which indicates that full-length cDNAs were accessible by the cDNA amplification method. The genes found to be upregulated in *Aurelia* represent a broad spectrum of molecular pathways with functions related to disease, oxidative or other stress mediated damage, and cell repair in vertebrates and invertebrates. These findings indicate that TBT or structurally related compounds may be useful test substances to establish new experimental systems in molecular ecotoxicology.

Efficacy of biomarkers in laboratory and field experiments. Three isolated genes, the oxidative stress gene (*135*), hsp70, and the GTP-binding protein, were tested for their use as biomarkers under defined experimental stress conditions. The exposure of *Aurelia* polyps to TBT revealed an upregulation of hsp70 and the oxidative stress gene, even at the low TBT concentration of 5 ng/L. The G-protein transcript was moderately upregulated. Hence the effect concentration at which molecular responses are observed was dramatically reduced by more than 2 orders of magnitude compared with effect concentrations that were measured using organismal endpoints such as rate of malformed ephyra or tentacle shrinking in polyps (1 μg/L). At 5 ng/L TBT, the average level of transcript upregulation of all 3 genes continuously increased with exposure times, suggesting a time-dependent state of physiologic adaptation. This trend was significant only for the oxidative stress gene, but nonetheless supports the idea that the 5 ng/L concentration marks a conditional state at which the organism is challenged to start defense mechanisms.

However, there were no significant differences between time-matched comparisons of low-dose and high-dose TBT exposure, suggesting that the low level of 5 ng/L already provoked the full physiologic responses. This finding is in agreement with studies on marine snails that showed disturbed sex development at TBT concentrations of 5 ng/L (Bryan and Gibbs, 1991). This sensitivity appears sufficient to cover biologically relevant xenobiotic concentrations. At 10-fold higher TBT doses (50 ng/L), expression patterns did not show any time dependency, but caused maximal inductions for hsp70 (13 hours after exposure) and the oxidative stress gene (82 hours after exposure). The opposite gene regulatory patterns seen here makes it plausible that this high xenobiotic concentration exceeds the level at which stress mechanisms act in a physiologically balanced sequence. Protein renaturation and reconstitution by heat shock proteins is an important and general means to rapidly aquire stress tolerance (Gething and Sambrook, 1992; Ryan and Hightower, 1996). Hence its immediate maximal induction at the high exposure concentrations may reflect an ''emergency'' situation, which has also been reported for sponges (Schröder et al., 1999). Different protective pathways may follow in time, such as the elimination of accumulated reactive oxygen species (see Blackstone, 2001), which could be mirrored by the delayed expression peak of the oxidative stress gene in *Aurelia*. The relatively constant and moderate transcript level of the GTP-binding protein at both TBT concentrations could imply that the gene or protein is tightly regulated once the cell is facing extracellular signals; however, its functional role remains unknown. In sum, the 3 genes showed molecular responses that can be used to detect abiotic stress at low and subacute conditions, a situation that is expected for most natural environments. This experimental approach can easily be expanded to include markers that integrate a variety of stress-relevant pathways.

Our field observations at the Adriatic/Mediterranean Sea showed that selected markers, the oxidative stress gene and hsp70, in principle, are suitable biomarkers for the study of environmental impairment. The time-dependent increase in molecular response observed at the marina of Ancona (Italy) is comparable to the experimental results obtained at lower concentrations (5 ng/L) of TBT exposure. This again highlights the sensitivity to detect subacute or chronic stress conditions. It is important to note, however, that hsp70 and possibly

also the oxidative stress gene are considered indicators of physiologic imbalances caused by variety of abiotic stressors. Thus the suitability of biomarker assays in the field depends on a panel of carefully selected biomarkers covering a broad spectrum of physiologic alterations and molecular pathways during stress (gene-chip development), and appropriate control sites with similar microhabitat conditions in order to prevent false conclusions from the test site (in our study we also observed a slight increase in transcript levels within the putative unpolluted field control, which indicates that the control site itself is slightly contaminated).

Heat shock response as a tool to combine gene expression and diversity. We sought to link differential expression of biomarkers to an evolutionary context by adding information on genetic diversity among 2 closely related *Aurelia* lineages, which may provide information on historical events of stress adaptation. The hsp70 gene was chosen because its sequence is highly conserved among eukaryotes. Furthermore, its temperature inducibility makes it ideal to study influences of naturally occurring stress, such as temperature shifts. We found a substantial amount of sequence divergence (4.7%) separating *Aurelia* UBI and BOR lineages, which supports our former hypothesis of past speciation events within the genus (Schroth et al., 2002). The observed bias toward synonymous substitutions, along with the deviation from expected codon distribution under strict neutrality (Li, 1993), indicates that purifying selection acted against mutations, especially at the second codon position of *Aurelia* hsp70. More importantly, the deduced hsp70 protein sequences differ by 8 synapomorphic lineage-specific amino acids. At least for one of the substituted amino acids in *Aurelia*, a functional role is conceivable because its homologous residue in the bacterial hsp70 homologue, DnaK, promotes hydrophobic interactions within one of 2 helical domains forming the nucleotide-binding cleft of the ATPase domain (Brehmer et al., 2001). The pattern of hsp70 gene diversity in *Aurelia* thus favors the idea that positive selective constraints may have been a driving factor for lineage differentiation. In order to test if differing expression patterns in response to thermal stress indirectly support the hypothesis of ecologically driven differentiation, we applied thermal stress to specimens from both lineages. At the control temperature of 17°C, hsp70 was weakly expressed at comparable levels in both lineages (not shown), which indicates that the BOR lineage (shifted from 4°–8°C in winter to 17°C in summer) was already preacclimatized to standard culture conditions. The striking differences among lineages at 20°C and 23°C indicate that the more boreally distributed BOR lineage, inhabiting North Atlantic, North Sea, Baltic Sea, and Black Sea, strongly responds with an immediate hsp70 upregulation at temperatures approaching the maximum value typically observed at its natural habitats (Schroth et al., 2002). In contrast, the UBI lineage which is naturally living in warmer, Mediterrean climates, reacts in a less severe manner at the 20° to 23°C range. It strongly increases the heat shock reaction at 26°C, which is again at the uppermost level seen in its natural habitats (Mediterranean Sea, California/Pacific, Japan/Pacific, and Australia/Indian Ocean).

From our results it is evident that different lineage-specific capacities exist to produce a heat shock response, at least at the gene regulatory level. Further studies will show if this pattern also applies to the protein level. The coincidence of differential gene regulation and genetic divergence among both lineages raises the question whether hsp70 could have played a role in adaptation to changing physical conditions in *Aurelia* (see Schroth et al., 2002). To test if sequence differences at regulatory sites may account for differential expression, we plan to expand our phylogenetic study onto the promotor region of the gene. This analysis will show if variation has accumulated at functional sites, such as heat shock elements and other transcription factor binding sites, and if that variation shows linkage disequilibrium to the lineage-specific alleles found in the present study.

The hsp70 study illustrates how a combined study of gene expression and gene diversity may improve the understanding of species range determinants, such as temperature, and hence raises the applicability of molecular biomarkers to higher taxonomic levels. Effects of global warming, including the shifting of species ranges, pose an increasing threat to biological systems (Forchhammer and Post, 2000; Hughes, 2000). In this context the detection of evolutionary constraints could have prognostic value for the distributional ranges of diverged lineages (congeneric species). Such evolution based marker studies may be useful for the prediction of local extinctions at more vulnerable marine habitats such as shallow coastal regions, and even for the management of nonindigenous species as in the case of unpredictable jellyfish blooms. We will apply the historical versus present-day sequence/expression approach to further *Aurelia* biomarkers, which may contribute to an enhanced understanding of organism-environment interactions in marine ecosystems.

Conclusions

Our study provides a general strategy for the analysis of environmental stress in yet unexplored but ecologically important organisms. By means of cDNA subtraction, we were able to isolate a panel of differentially expressed 'stress' genes, which can be easily expanded for developing gene chip arrays. The choice of the biocide compound TBT as a response-provoking agent has been proven to target different molecular pathways including protein renaturation and unfolding, subcellular localization and proteolytic degradation, oxidative stress protection, and signal transduction/cell activation. Thus it can be regarded as a useful test substance in aquatic study systems. A subset of initially isolated genes was successfully tested for its biomarker potential both in the laboratory and in the field. The implementation of a gene diversity approach in the study of biological stress expands the power of the use of molecular biomarkers to higher taxonomic levels.

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