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Response of the Pacific oyster *Crassostrea gigas* to hydrocarbon contamination under experimental conditions

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Abstract

Hydrocarbon contamination perturbs the metabolism of the marine bivalve *Crassostrea gigas*. To understand the response of this organism to hydrocarbon exposure, a suppression subtractive hybridisation method was employed to characterise up- and down-regulated genes during hydrocarbon exposure. The number of differentially expressed gene sequences obtained via this method was 258. The expression of genes involved in hydrocarbon detoxification (cytochrome P4501A1-like protein, cytochrome b_5, flavin-containing monooxygenase 2 and glutathione *S*-transferase omega class), protection against oxidative stress (copper/zinc superoxide dismutase) and cell protection (heat shock protein 70 family) was analysed by RT-PCR. An increase in the mRNA level of all genes studied was observed. A quantification of HSP70 by Enzyme Linked Immunosorbent Assay (ELISA) showed a significant increase of this protein during exposure. This study provides a basis for studying hydrocarbon detoxification processes in marine bivalves, especially *C. gigas*.

Keywords: Suppression subtractive hybridisation libraries; Hydrocarbons; Gene expression; Detoxification; *Crassostrea gigas*

1. Introduction

For the last several decades, coastal ecosystems have been subjected to increased hydrocarbon contamination from compounds such as polycyclic aromatic hydrocarbons (PAH) and polychlorinated biphenyl (PCB). Hydrocarbon exposure is considered a major stress for marine organisms. Biotransformation and detoxification of these compounds are vital mechanisms for these organisms. A variety of enzymes and other proteins are produced in response to xenobiotics exposure, such as cytochrome P450 (Cyp450), flavin-containing monooxygenase (FMO), monoamine oxidase (MAO), glutathione-*S* transferase (GST), catalase, etc. (see Stegeman and Hahn, 1994 for a review).

Proteins involved in biotransformation processes in eukaryotic cells may be divided according to its two phases. The first phase is characterised by the oxygenation of xenobiotics and endogenous substrates by the inducible cytochrome P450-dependent microsomal monooxygenase. Oxygenation of xenobiotic compounds by Cyp450 results either in directly excreted polar metabolites or into more reactive molecules which are used as substrates by phase II enzymes (Lüdeking and Köhler, 2002). Phase II enzymes catalyse the conjugation of the xenobiotic to endogenous compounds. GSTs are described as the major, widely distributed phase II enzymes that conjugate glutathione and electrophilic substrates (Lüdeking and Köhler, 2002). Recently, a third phase in the xenobiotic biotransformation mechanism, characterised by the induction of multi-drug resistance and multi-xenobiotic resistance, has been described in marine invertebrates such as the oysters *Crassostrea gigas* and *Crassostrea virginica* (Keppler and Ringwood, 2001; Minier et al., 2002) These proteins act as a pumps involved in the export of xenobiotics out of the cell.

Abbreviations: Cu/Zn SOD, copper/zinc superoxide dismutase; Cyp450, cytochrome P450; ELISA, enzyme linked immunosorbent assay; FMO, flavin containing monooxygenase; GST, glutathione *S*-transferase; HSP70, heat shock protein 70; MAO, monoamine oxidase; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; ROS, reactive oxygen species; SSH, suppression subtractive hybridisation.

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The effects to organisms due to exposure to PAH and PCB have been extensively studied, especially in marine animals in monitoring programs related to human health (Mondon et al., 2001). Organisms exposed to hydrocarbons produce reactive oxygen species (ROS) that, in turn, lead to DNA damage, enzyme inactivation, and possibly cell death (Regoli et al., 2002). ROS production also entailed lipid peroxidation and lipid metabolism disruption (Ferreira and Vale, 1998), and reproductive irregularities if germ cells are affected or the hormonal balance is perturbed (Armstrong, 1990). The correlation of DNA damage with cyp450 induction is well described in marine molluscs, like the mussels *Mytilus galloprovincialis* and *Mytilus edulis* (Canova et al., 1998; Shaw et al., 2002). These studies were conducted using heterolog mRNA probes and antibodies developed in fishes (Shaw et al., 2002). To our knowledge, no sequence for either cDNA or gene encoding Cyp450 in molluscan species was in the available databases. Many studies have been conducted with Mytilid bivalves but only a few deal with the response in oyster species. Some authors have shown disruption of lipid metabolism due to PCB accumulation in lipid-containing tissues in the oysters *Crassostrea angulata* and *C. virginica* exposed to PCB (Ferreira and Vale, 1998; Chu et al., 2000). Some developmental inhibition in Pacific oyster, *C. gigas,* larvae exposed to PAH (Lyons et al., 2002) was also described. In their paper, Schlenk and Buhler (1989) investigated the xenobiotic metabolism in *C. gigas* and concluded that these organisms displayed a lack of Cyp450 activity and an important FMO activity. To our knowledge nothing has been done to identify mechanisms involved in the hydrocarbon response of *C. gigas*.

We report, for the first time, genes involved in the stress response induced by hydrocarbons in *C. gigas*. As a first step, we determined the inhibited and induced genes after 7 and 21 days of hydrocarbon exposure using a suppression subtractive hybridisation (SSH) method. In the second step, we used RT-PCR to analyse the expression of four genes known to be involved in hydrocarbon biotransformation, CYP1A1-like protein, cytochrome b_5, FMO-2 and GST omega class (GSTO). We also characterised cDNA and genomic sequences of an oxidative stress protection enzyme, the copper/zinc superoxide dismutase (Cu/Zn SOD) and analysed its mRNA expression. Finally, the protein quantification and mRNA expression of molecular chaperones, from the heat shock protein 70 family (HSP70), were studied using an ELISA developed in our laboratory (Boutet et al., 2003) and RT-PCR, respectively.

2. Materials and methods

2.1. Oyster conditioning and treatment

Adult oysters, *C. gigas*, were collected from La Pointe du Château (Brittany, France). After an acclimatisation period of 7 days in aerated 0.22 μm-filtered seawater at constant temperature and salinity (15 °C and 34‰, respectively), oysters were challenged as follows. Groups of 25 oysters were exposed to 0.1% of hydrocarbon mixture (consisting of the water-soluble fraction of domestic fuel homogenised for 3 days in filtered seawater according to Snyder et al., 2001), for 3 weeks and another group of 25 oysters was maintained in seawater, without contaminant, as a control. Hydrocarbon concentration was estimated in oyster dry tissues (pools of five oysters) at the beginning and end of the experiment by gas chromatography coupled to mass spectrometry (Hewlett Packard 5890) according to protocol described by Baumard et al. (1997). No mortality was observed either in the control or in the exposed oysters.

2.2. Suppression subtractive hybridisation

Total RNA was extracted from the digestive gland of control and exposed oysters after 7 and 21 days of contamination using RNAble (Eurobio, les Ulis, France) according to the manufacturer's instructions. Poly(A+) mRNA was isolated from total RNA using the PolyATtract®mRNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Forward and reverse subtracted libraries were made. The forward libraries contained partial transcripts of genes up-regulated in the digestive glands of the hydrocarbon-challenged oysters, while the reverse libraries contained those that were down-regulated. The libraries were made by subtracting out the cDNA common to both the hydrocarbon-challenged and uncontaminated digestive gland tissue, leaving the differentially expressed partial transcripts from the contaminated (uncontaminated) oysters in the case of the forward (reverse) library to be amplified. First and second strand cDNA synthesis, *Rsa*I endonuclease enzyme digestion, adapter ligation, hybridisation, and PCR amplification were performed as described in the PCR-select cDNA subtraction manual (Clontech, Palo Alto, CA, USA). The differentially expressed PCR products were cloned into pGEM-T vector (Promega). Two hundred white colonies per library were cloned, the vector extracted using an alkaline lysis plasmid minipreparation and screened by size after digestion. One hundred clones per library were sequenced using a Li-COR IR^2 (Sciencetech) and Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Bioscience, Uppsala, Sweden). All sequences were subjected to an homology search through the BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3. Hydrocarbon detoxification gene expression analysis by RT-PCR

Total RNA was extracted from the digestive gland of control and exposed oysters after 0, 3, 7, 11, 15 and 21

days of exposure using a method based on extraction in guanidium isothiocyanate. Ten micrograms of RNA was submitted to reverse transcription using oligo dT anchor primer (5′-GAC-CACGCGTATCGATGTCGACT$_{(16)}$V-3′) and M-MLV reverse transcriptase (Promega). The amplifications of CYP1A1-like protein, cytochrome b_5, FMO-2 and GSTO were performed in 2 mM $MgCl_2$ and 10 pmol of each primers (Table 1). 28S ribosomal DNA was used as a PCR internal control under the same conditions with primer combination described in Table 1. The resulting PCR products were electrophoresed in a 0.5X TBE/1.5% agarose gel, and visualised with U/V light after BET coloration. Quantification of band intensities was measured by using Gene Profiler 4.03 Software (Scanalytics).

2.4. Cloning and sequencing of 5′ and 3′ flanking regions of Cu/Zn-SOD cDNA

The procedures for the generation of Cu/Zn SOD cDNA 5′ and 3′ untranslated regions (UTR) were carried out according to the commercial 5′/3′ rapid amplification of cDNA ends protocol (5′/3′ RACE Kit, Roche, Mannheim, Germany) using specific primers antisense (5′-TGTGGAGTGATCG-GTATCACCAAG-3′) and sense (5′-ATGTCATCTGCTCTGAAGGCCGT-3′) for the 5′ and the 3′ UTR, respectively. Primers were designed in Cu/Zn SOD fragment isolated from the SSH libraries. The 3′ UTR was amplified as follows: 200 ng of reverse transcription product was submitted to amplification using one cycle at 94 °C for 5 min, 58 °C for 2 min, 72 °C for 1 min 30, then 40 cycles at 94 °C for 15 s, 58 °C for 30 s, 72 °C for 1 min and a final step at 72 °C for 10 min with 2 mM $MgCl_2$ and 10 pmol each of PCR anchor primer (5′ -GACCACGCGTATCGATGTCGAC-3′) and specific primer. Amplification of the 5′ UTR was carried out according to the following procedure: denaturation at 94 °C for 2 min, then 10 cycles at 94 °C for 15 s, 58 °C for 30 s, 72 °C for 1 min, then 30 cycles at 94 °C for 15 s, 58 °C for 30 s, 72 °C for 1 min with an increase of 20 s per cycle for the elongation time, and a final step at 72 °C for 15 min with 2 mM $MgCl_2$ and 10 pmol each of oligo dT anchor primer and specific primer. The resulting 500- and 850-base pair cDNA fragments corresponding to the 5′ and 3′ UTRs, respectively, were cloned and sequenced using the procedure described in SSH library section.

Table 1
Combinations of primers used in RT-PCR expression analysis

Genes	Primer sequences
Cytochrome P4501A1-like protein	sense GTGCATCAAAGAATTTTGGATAC antisense TGCAATAATTTTTGAAGCCCCGG
Cytochrome b_5	sense AAAAAGATTTATAGAGAGTCTGAGGT antisense GACATGAAGTATCTGTAGACAAATGC
Flavin-containing monooxygenase 2	sense GGAGAAGACATCTCTACGCATGT antisense TATCCGGTGCAGAATATAACGGC
Glutathione *S*-tranferase omega class	sense TATTTGGACCAGGTGTATCCCGA antisense AGAATACGTTCAAACCATGGCCA
Superoxide dismutase copper/zinc	sense CGAGTAAATTTCGCGCCGGTGAA antisense GCCTCCACTCGGTCCACTGTT
Heat shock protein 70	sense GGAATAGATCTTGGAACCACATA antisense TTGCCAAGATATGCTTCTGCAGT
28S ribosomal DNA	sense AAGGGCAGGAAAAGAAACTAAC antisense TTTCCCTCTAAGTGGTTTCAC

2.5. Cloning and sequencing of Cu/Zn-SOD gene

Total genomic DNA was isolated from oyster gills using phenol/chloroform/isoamyl alcohol (25:24:1). The resulting preparation was amplified with primers (5′-CGAGTAAATTTCGCGCCGGTGAA-3′ and 5′-GCCTCCACTCGGTCCACTGTT-3′) designed in the full-length cDNA sequence previously characterized using Extrapol III DNA polymerase (Eurobio, les Ulis, France). Two hundred nanograms of DNA was submitted to amplification using denaturation at 94 °C for 5 min, then 15 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 3 min, then 15 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 3 min with an increase of 10 s per cycle for the elongation time, and a final step at 72 °C for 30 min with 2 mM $MgCl_2$, and 10 pmol of each primer.

The 5′ and 3′ UTRs were obtained by PCR amplification of a *C. gigas* gills genomic library (Stratagene, La Jolla, CA, USA) using T7 universal primer (5′-TAATACGACTCA-CTATAGGG-3′) and the specific primers, antisense and sense, used to amplify the 5′ and 3′ UTRs of the corresponding cDNA. Amplification, cloning and sequencing were performed according to the procedure described above.

2.6. Cu/Zn SOD expression study by RT-PCR

PCR was performed using one cycle consisting of denaturation at 94 °C for 2 min, then 30 cycles at 94 °C for 30 s, 58 °C for 1 min, 72 °C for 30 min, and a final step at 72 °C for 7 min with 2 mM $MgCl_2$ and 10 pmol each primers (Table 1). The resulting PCR products were electrophoresed in a 0.5X TBE/1.5% agarose gel, and visualised with U/V light after BET coloration. Quantification of band intensities was measured by using Gene Profiler 4.03 Software (Scanalytics).

2.7. HSP70 expression analysis by RT-PCR

PCR was performed using one cycle consisting of denaturation at 94 °C for 2 min, then 30 cycles at 94 °C for 30 s, 58 °C for 1 min, 72 °C for 30 min, and a

final step at 72 °C for 7 min with 2 mM $MgCl_2$ and 10 pmol each primers (Table 1). The resulting PCR product was analysed as described in the Cu/Zn SOD expression study section. Quantification of band intensities was measured by using Gene Profiler 4.03 Software (Scanalytics).

2.8. Protein extraction and quantification of HSP70 by ELISA

On days 0, 7, 15, and 21, the digestive glands from exposed and control oysters ($n = 10$ for each sample) were collected, homogenised in protein extraction buffer (150 mM NaCl, 10 mM NaH_2PO_4, 1 mM phenylmethanesulfonyl fluoride, pH = 7.2) and centrifuged. Protein concentration was estimated with a D_c Protein Assay kit (Bio-Rad Laboratories, Hercules, USA) using bovine serum albumin as the standard. Optical density was measured at 620 nm using a micro-plate reader. Microtitre plates were coated with 20 μg per well of total proteins and incubated for one night at 4 °C. HSP70 concentrations were estimated by ELISA using rabbit anti-CgHsc72 polyclonal antibody and recombinant CgHsc72 as standards, according to the procedure described by Boutet et al. (2003).

2.9. Statistical analysis

The variations in Hsp level during the experiment were analysed by analysis of covariance ($\alpha = 0.05$) using CSS Statistica (Statsoft).

3. Results

3.1. Quantification of hydrocarbons in oyster tissues

Quantification of hydrocarbon accumulation in oyster tissues by gas chromatography coupled to mass spectrometry showed an increased from 1.4 mg/kg dry weight tissue in the control oysters to 34.6 mg/kg dry weight tissue in the exposed oysters.

3.2. Identification of hydrocarbon-regulated genes

SSH libraries were made from pooled digestive glands of *C. gigas* after 7 and 21 days of exposure. The search for homology using the BLASTX program revealed 258 different sequences, with 117 sequences (about 45%) unidentified. Four tables list the sequences obtained from the various SSH libraries: 7 days up-regulated (Table 2), 21 days up-regulated (Table 3), 7 days down-regulated (Table 4) and 21 days down-regulated (Table 5). These results indicate that hydrocarbon exposure up- and down-regulated nien major cellular physiological functions during the contamination experiment: hydrocarbon detoxification, stress proteins, protein regulation (including protein synthesis, transport and degradation), nucleic acid regulation (including tran-

Table 2
Identified SSH up-regulated clones (after 7 days of exposure) with significant database matches

Homolog (protein); blastx value	GenBank accession no
Hydrocarbon detoxification (4.8%)	
Cytochrome b_5; 3e-34	CB617384
Cytochrome P450 pHPah1; 6e-09	CB617404
Flavin-containing monooxygenase FMO-2; 2e-16	CB617386
Glutathione *S*-transferase omega-class; 4e-22	CB617406
Stress proteins (2.4%)	
Glutamine synthetase; 5e-10	CB617403
Heat Shock Protein 70; 4e-74	AJ305315
Protein regulation (7.2%)	
Cathepsin 1; 1e-05	CB617378
α-2-macroglobulin; 3e-15	CB617381
Myosinase-I; 1e-36	CB617388
Procathepsin L; 1e-12	CB617396
Ubiquitin activating enzyme; 4e-53	CB617402
NASCENT polypeptide-associated complex α polypeptide; 2e-42	CB617385
Nucleic acid regulation (7.2%)	
Arginyl-tRNA synthetase; 4e-71	CB617392
Geminin; 1e-14	CB617390
Nuclear RNA helicase; e-114	CB617405
5′ nucleotidase precursor; 6e-25	CB617398
Poly (ADP-ribose) polymerase; 6e-10	CB617394
RNA Adenosin deaminase; 5e-56	CB617393
Respiratory chain (4.8%)	
Cytochrome b; e-102	AF177226
NADH dehydrogenase subunit 4; 2e-41	AF177226
NADH dehydrogenase subunit 5; 2e-49	AF177226
Mitochondrial ATP-synthase γ-subunit; 2e-43	CB617389
Lipid metabolism (2.4%)	
2,4-dienoyl CoA reductase 2; 5e-18	CB617391
Triacylglycerol lipase precursor; 2e-39	CB617387
Cell communication, membrane receptor, immune system (8.3%)	
EGF-like protein; 2e-17	CB617395
Fibrinogen-like protein; 4e-31	CB617382
G protein-coupled receptor 48; 8e-15	CB617400
Lipoprotein receptor-related protein 5; 2e-24	CB617399
Mammalian ependymin related protein 1; 3e-06	CB617379
Peritrophin; 2e-05	CB617383
RAB32; 2e-20	CB617401
Cytoskeleton (1.2%)	
β-thymosin; 2e-07	CB617380
Ribosomal proteins (14.4%)	CB617366 – CB617377
Unidentified sequences[a] *(47.6%)*	CB617326 – CB617365

[a] Sequences presented no significant alignment (< 0.01) or significant alignment with an unknown protein.

Table 3
Identified SSH up-regulated clones (after 21 days of exposure) with significant database matches

Homolog (protein); blastx value	GenBank accession no
Hydrocarbon detoxification (4.8%)	
Cytochrome P450 nifedipine oxidase; 1e-28	CB617459
Glutathione S-Transferase; 2e-21	CB617447
Monoamine oxidase A; 1e-57	CB617454
Stress proteins (3.2%)	
Heat Shock Protein 90α; 2e-66	CB617443
Superoxide dismutase [Cu–Zn]; 3e-59	AJ496219
Protein regulation (16%)	
Cathepsin L like protease precursor; 2e-33	CB617457
α-2-macroglobulin; 4e-39	CB617381
Chaperonine containing TCP1 6B-subunit; 4e-25	CB617445
Coatomer protein complex β-subunit; e-115	CB617439
Mitochondrial carrier protein; 5e-35	CB617452
Mitochondrial processing peptidase β chain; 4e-77	CB617453
Myosinase I; 7e-22	CB617388
Solute carrier family 3 member 1; 2e-12	CB617440
Valosin-containing protein; 7e-86	CB617456
Zinc metalloproteinase STE24 homolog; 2e-35	CB617436
Nucleic acid regulation (4.8%)	
Cell cycle regulator p21 protein; 4e-19	CB617437
myc homolog; 5e-12	CB617451
Translation elongation factor 1-α; 3e-97	CB617441
Respiratory chain (11.3%)	
Cytochrome c oxidase subunit I; e-143	AF177226
Cytochrome c oxidase subunit III; 2e-96	AF177226
H^+ -transporting ATPase; 6e-36	CB617449
NADH dehydrogenase subunit 4; 2e-41	AF177226
NADH dehydrogenase subunit 1; 3e-46	AF177226
Nit protein 2; 2e-30	CB617446
Sulfite oxidase; 8e-78	CB617450
Lipid metabolism (1.6%)	
2,4-dienoyl CoA reductase 2; 2e-56	CB617391
Cell communication, membrane receptor, immune system (6.4%)	
Immunolectin-B; 7e-04	CB617455
Ganglioside GM2 activator precursor, 6e-07	CB617434
Lipopolysaccharide and β-1,3-glucan; 8e-49	CB617438
β-N-acetylhexosaminidase; 6e-26	CB617435
Cytoskeleton (3.2%)	
Actin; e-134	AF026063
Tubulin β5; 2^e-66	CB617442
Energetic metabolism (4.8%)	
Aspartate Amino Transferase; 1^e71	AJ496218
Phosphoenolpyruvate carboxykinase; 3^e56	CB617458
Retinal short chain dehydrogenase/reductase; 1^e52	CB617444
Ribosomal proteins (8.1%)	CB617428 – CB617433
Unidentified sequences [a] *(35.5%)*	CB617354; CB617407 – CB617427

[a] Sequences presented no significant alignment (<0.01) or significant alignment with an unknown proteins.

scription, cell cycle regulation and metabolism of nucleic acid components), respiratory chain, lipid metabolism, cell communication (including immune system and membrane receptors), cytoskeleton and energetic metabolism (including digestive enzymes).

Table 4
Identified SSH down regulated clones (after 7 days of exposure) with significant database matches

Homolog (protein); blastx value	GenBank accession no
Hydrocarbon detoxification (3.4%)	
Glutathione *S*-transferase; 8^e-17	CB617512
Probable glutathione *S*-transferase protein; 2^e-08	CB617513
Protein regulation (10.3%)	
Apolipophorin precursor; 2^e-12	CB617501
Cathepsin 8; 1^e-07	CB617504
GRAAL 2 protein; 5^e-05	CB617493
Importin α; 3^e-30	CB617497
Lysozyme; 8^e-19	CB617495
Trypsin; 8^e-09	CB617494
Nucleic acid regulation (5.2%)	
Similar to ADP-ribosylation factor 2; 1^e-09	CB617505
Similar to homeobox domain; 0.009	CB617515
Myc homolog; 1^e-05	CB617451
Respiratory chain (6.8%)	
ATP-gated ion channel subunit P2X4; 3^e-30	CB617507
ATP synthase, H^+ *transproting,* mitochondrial FO complex subunit b, isoform 1; 2^e-11	CB617499
NADH-ubiquinone oxidoreductase 20kDa subunit, mitochondrial precursor; 9^e-57	CB617510
Oxidoreductase UCPA; 3^e-19	CB617509
Lipid metabolism (3.4%)	
CDP-diacylglycerol synthase 2; 9^e-43	CB617500
Δ9 desaturase; 3^e-69	CB617498
Cell communication, membrane receptor, immune system (13.6%)	
Calcium/calmoduline-dependent protein kinase IV; 1^e-57	CB617508
Similar to CDC-like kinase 2; 7^e-54	CB617496
Similar to C1q-related factor precursor; 4^e-11	CB617506
Hypertension-related calcium-regulated gene; 6^e-37	CB617516
Microfibril-associated glycoprotein 4; 2^e-20	CB617514
Similar to Transmembrane 4 superfamily; 2^e-35	CB617502
Transmembrane protein HEM-2; 2^e-17	CB617503
Transmembrane protein NRF-6; 1^e-04	CB6175511
Ribosomal proteins (5.2%)	CB617369; CB617370; CB617372; CB617375; CB617491; CB617492
Unidentified sequences[a] *(46.5%)*	CB617326; CB617464 – CB617490

[a] Sequences presented no significant alignment (<0.01) or significant alignment with an unknown protein.

Table 5
Identified SSH down-regulated clones (after 21 days of exposure) with significant database matches

Homolog (protein); blastx value	GenBank accession no.
Hydrocarbon detoxification (1.7%)	
Sulfotransferase 1C1; 1^{e}-16	CB617550
Stress protein (3.4%)	
Melanogenic peroxidase; 2^{e}-27	CB617557
Soma ferritin; 2^{e}-25	CB617552
Protein regulation (6.8%)	
Betain-homocystein methyltransferase 2; 4^{e}-30	CB617563
GRAAL 2 protein; 5^{e}-05	CB617493
Serine (or cysteine) proteinase inhibitor; 3^{e}-26	CB617554
Pre-pro-TPE4A protein; 2^{e}-09	CB617553
Nucleic acid regulation (6.8%)	
Chromatin assembly factor 1 p55 subunit; e-124	CB617555
SWI/SNF complex 170 kDa subunit; 9^{e}-15	CB617547
U2 small nuclear ribonucleoprotein auxiliary factor (U2AF); 5^{e}-34	CB617564
Elongation factor 2; 3^{e}-62	CB617558
Respiratory chain (3.4%)	
NADH dehydrogenase subunit 3; 3^{e}-13	AF177226
NADH-ubiquinone oxidoreductase 42 kDa subunit; 2^{e}-06	CB617549
Lipid metabolism (1.7%)	
Δ9-desaturase; 3^{e}-71	CB617498
Cell communication, membrane receptor, immune system (10.4%)	
β-amyloid binding protein; 6^{e}-48	CB617559
EBP-α; 4^{e}-15	CB617562
Hepatic lectin; 1^{e}-12	CB617560
Mammalian ependymin related protein-1 precursor; 3^{e}-09	CB617379
Putative cell adhesion protein sym32; 2^{e}-20	CB617561
Rab20-like protein; 1^{e}-27	AF288677
Energetic metabolism (5.2%)	
Alkaline phosphatase; 4e-20	CB617556
β-1,3-glucanase; 2e-28	CB617548
Malate dehydrogenase; 1e-09	CB617551
Ribosomal proteins (12.1%)	CB617366; CB617371; CB617377; CB617543 – CB617546; CB617565
Unidentified sequences[a] *(48.3%)*	CB617517 – CB617542

[a] Sequences presented no significant alignment (< 0.01) or significant alignment with an unknown protein.

3.3. Expression of hydrocarbon detoxification genes

The time-dependent expression of phase I and phase II detoxification enzymes, CYP1A1, cytochrome b_5, FMO-2 and GSTO were analysed by RT-PCR using digestive glands of oysters after 0, 3, 7, 11, 15 and 21 days of hydrocarbon exposure (Fig. 1). The expression of CYP1A1 reached a maximum value between 7 and 15 days (8.8-fold compared to control) before decreasing to a value of two-fold of the control value (Fig. 1A). The level of FMO-2 mRNA presented an elevated value between 7 and 11 days of exposure (2.2-fold compared to control), before dropping to the level observed in control oysters (Fig. 1B). The expression of cytochrome b_5 was constitutive, but RT-PCR analysis showed a two-fold increase at 11 days and a mean value of 1.2-fold of the control value until the end of the experiment (Fig. 1C). GSTO showed a strong induction between 3 and 11 days of exposure 2.4-fold compared to the control), then decreased and remained constant until the end of the experiment (Fig. 1D). No variations were observed in 28S rDNA expression (Fig. 1E).

3.4. Molecular characterisation and expression of Cu/Zn SOD

The sequences of the Cu/Zn SOD cDNA and gene show a 471 bp coding region and 5′ and 3′ UTRs of 29 and 361 bp, respectively (Fig. 2). The alignment of the corresponding amino acid sequence, determined by the ALIGNP program, revealed an identity of 62.2% with other species. The Cu/Zn SOD genomic sequence of *C. gigas* did not contain introns. Expression analysis showed a two-fold SOD mRNA increase at 7 days, decreased, then increased again at the end of the exposure (Fig. 3).

3.5. Expression of HSP70

Both RT-PCR analysis and quantification by ELISA showed a strong expression of HSP70 at 7 days until the end of the experiment (Fig. 4A and B). HSP70 concentration reached a maximum value between 7 and 15 days of exposure (87.6 ± 4.8 mg HSP70/g proteins) before decreasing at 21 days of exposure to the level observed in the control (75.7 ± 4.0 mg HSP70/g proteins). At this period, there was a 2.2-fold increase in HSP70 mRNA expression compared to control oysters.

4. Discussion

In this paper, we characterised for the first time the response to hydrocarbon exposure under experimental conditions of a marine mollusc, the oyster *C. gigas*. Using a suppression subtractive hybridisation method, we obtained 258 different partial sequences of cDNA, encoding proteins involved in the stress response induced by hydrocarbons in oysters. Application of this method has previously identified 242 genes in zebra mussel, *Dreissena polymorpha*, treated with various contaminants (Bultelle et al., 2002).

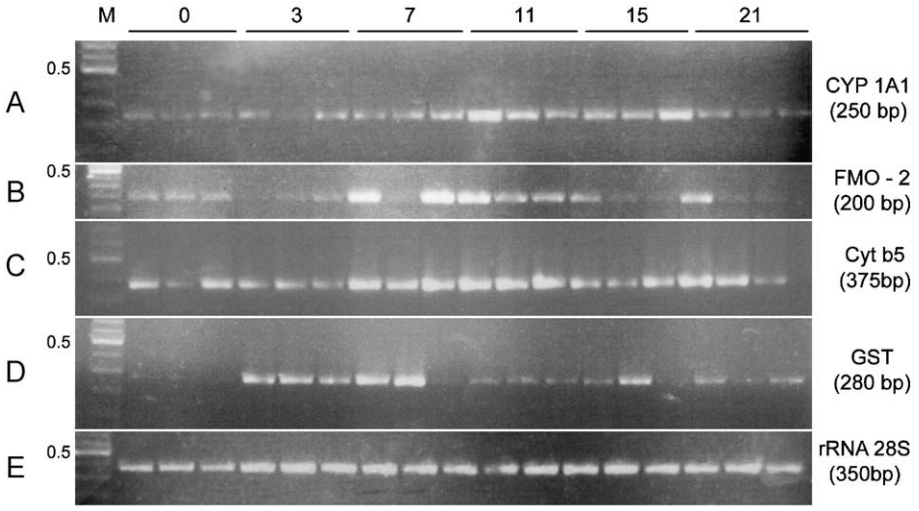

Fig. 1. Expression analysis of hydrocarbon detoxification genes by RT-PCR in digestive gland of *C. gigas*. Three individuals were analysed for each sample day. Cyt P4501A1 (A), FMO-2 (B), cytochrome b_5 (C), and GST omega class (D) were amplified using 1 μl of the same reverse transcriptase diluted products (1/2 for cyt P4501A1, FMO-2 and cytochrome b_5 and 1/20 for GST omega class) for all genes. 28S ribosomal DNA (E) was amplified in the same conditions. Molecular weight marker (M) is 100 bp DNA ladder (Promega, Madison, WI, USA).

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gaaacgtgtattttaaggaaaattaaatc                                  29
ATGTCATCTGCTCTGAAGGCCGTCTGTGTATTGAAGGGTGACAGCAATGTCACAGGAACC  89
M  S  S  A  L  K  A  V  C  V  L  K  G  D  S  N  V  T  G  T    20

GTCCAATTTAGTCAAGAGGCTCCTGGAACACCTGTGACATTGTCCGGAGAAATTAAAGGA  149
V  Q  F  S  H  E  A  P  G  T  P  V  T  L  S  G  E  I  K  G    40

TTAACACCAGGACAGCATGGATTCCACGTCCATCTGTTCGGCGACAATACTAATGGCTGT  209
L  T  P  G  Q  H  G  F  H  V  H  L  F  G  D  N  T  N  G  C    60

ACCAGTGCTGGACGGCACTTTAACCCCTTCAACAAAGAGCATGGCGTCCCAGAGGATCAC  269
T  S  A  G  R  H  F  N  P  F  N  K  E  H  G  V  P  E  D  H    80

GAGAGGCACGTGGGGGACCTTGGTAATGTCACCGCTGGCGAAGACGGTGTCGCCAAAATC  329
E  R  H  V  G  D  L  G  N  V  T  A  G  E  D  G  V  A  K  I    100

AGTATCACAGACAAAATGATTGACTTGGCCGGCCCTCAATCCATCATTGGAAGAACTGTG  389
S  I  T  D  K  M  I  D  L  A  G  P  Q  S  I  I  G  R  T  V    120

GTTATTCATGGCGATGTTGATGATCTTGGAAAAGGAGGTCATGAACTCAGTAAGACGACC  449
V  I  H  G  D  V  D  D  L  G  K  G  G  H  E  L  S  K  T  T    140

GGAAACGCTGGCGGACGATTGGCTTGTGGAGTGATCGGTATCACCAAGTAG           500
G  N  A  G  G  R  L  A  C  G  V  I  G  I  T  K  *             156

actccactagcctccactcggtccactgttatctattgtcctcttgttttctcacttttgtg  563
gatatacagcaaaaaagctacaagtctgataacttgtcacatttatcagattgtgtgataac  626
aataataatttgtaaaagatggttcaggaaaatcagaccacctatccatatatcatgtacct  689
atatgtgactcgatctcagtatctaatgccctaatggcaatgacagtttctttaaaactaaa  752
tacgctctacaatacatttaccaaatcaatgacactttatttatgtcttatagatctatac  815
atgtgatatggtaataaatatatgtaatatggaaaagaaaaaaaaaaaaa             861
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Fig. 2. The nucleotide sequence and derived amino acid sequence of copper/zinc superoxide dismutase gene. 3′ and 5′ UTR regions are in lower case. Primers used in RT-PCR analysis are double underlined. The stop codon is marked by an asterisk. The polyadenylation signal is underlined.

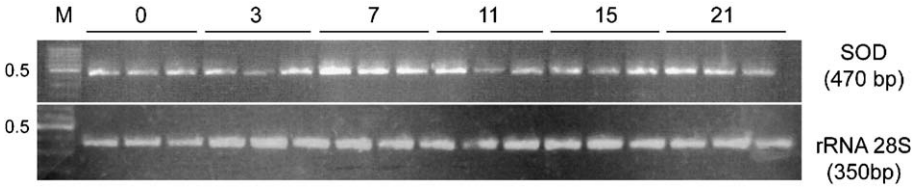

Fig. 3. Expression analysis of copper/zinc superoxide dismutase by RT-PCR. RT-PCR were performed using the digestive gland from three individuals per sample day using 1 μl of reverse transcriptase diluted products (1/20). Molecular weight marker (M) is 100 bp DNA ladder (Promega, Madison, WI).

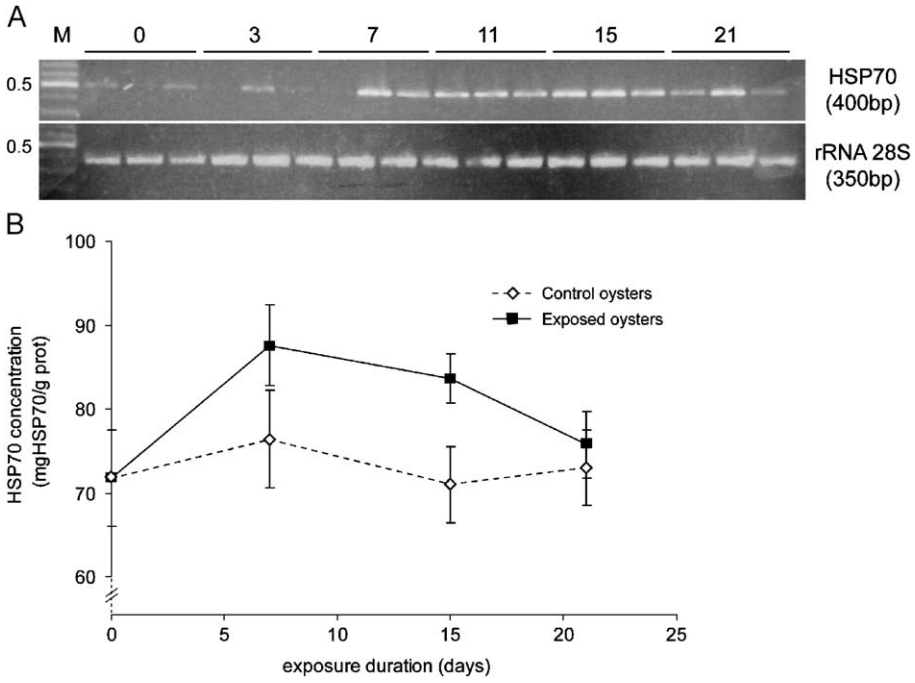

Fig. 4. Expression analysis by RT-PCR and ELISA of Heat Shock Protein 70. (A) RT-PCR was performed using the digestive gland from three individuals per sample day using 1 μl of reverse transcriptase diluted products (1/2). Molecular weight marker (M) is 100 bp DNA ladder (Promega, Madison, WI). (B) Immunochemical quantification using anti-CgHsc72 as probe was performed using the digestive gland from ten individuals per sample day (mean ± SE).

In this study, we analysed the mRNA expression of 4 genes involved in hydrocarbon detoxification, three from phase I (CYP1A1, FMO-2 and cytochrome b_5) and one from phase II (GSTO). Elevation of CYP1A1 protein levels due to hydrocarbon exposure is preceded by an increase in CYP1A1 mRNA levels (Stegeman and Hahn, 1994). A two-phase cyt P450 induction was observed in PCB-exposed rainbow trout: the first phase induction consisted of activation of existing enzymes, while the second phase included de novo enzyme synthesis. It is generally assumed that de novo protein synthesis is the most important enzyme induction process (Stegeman and Hahn, 1994). To date, the measurement of CYP1A1-like protein in molluscs has been conducted using heterolog probes and antibodies; no specific molecular and antibody tools have been available for analysis in molluscan tissues. Analysis of CYP1A1 mRNA expression in our experiments is in agreement with P450 activity in other molluscs in previous studies (Weinstein, 1995; Livingstone, 1998), but in contradiction with another study conducted on *C. gigas* (Schlenk and Buhler, 1989) and *M. galloprovincialis* (Akcha et al., 2000) that showed an unexpected lack of Cyp450 activity and no significant variations, respectively. Another cyp450 gene, cyp450 nifedipine oxidase (CYP3A4), was shown to be expressed in *C. gigas* digestive gland after 21 days of exposure. The expression of this enzyme is transcriptionally activated by many natural and xenobiotic compounds (Takeshita et al., 2002).

Cyp450-mediated biotransformation requires the involvement of cytochrome b_5 by donation of an electron from NADH via cytochrome b_5 reductase. An increase of cytochrome b_5 mRNA level was observed in the digestive gland of exposed *C. gigas*. Previous studies showed the same induction in fishes exposed to crude oils or PCB (White et al., 1997).

The last phase I enzyme analysed by RT-PCR in this work was FMO. This enzyme is a family of monooxygenases involved in the oxidation of many sulfur-containing compounds. Exposure to hydrocarbons resulted in the induction of FMO-2 activity in *C. gigas* (Schlenk and Buhler, 1989) in agreement with our results showing a mRNA level increase during hydrocarbon exposure. Detection of FMO activity in the digestive gland of bivalves suggests that this system plays a key role in hydrocarbon detoxification. Most of the interest in oxidative metabolism of xenobiotics has been devoted to the role of the Cyp450 system. In addition to Cyp450, oxidation of xenobiotics can also be mediated by non-P450 enzymes, among the most significant of which are FMO, MAO, alcohol dehydrogenase, aldehyde dehydrogenase and xanthine oxidase (Beedham, 1997). Xenobiotics oxidation catalysed by enzymes such as FMO and MAO may often produce the same metabolites as those generated by P450 (Beedham, 1997). Our SSH libraries revealed the presence of 3 major class members of phase I enzymes (CYP1A1, CYP3A4, FMO and MAO) which are induced by hydrocarbon exposure in *C. gigas*. These results could serve as a basis of an hypothesis indicating conservation of the phase I detoxification mechanism among phyla.

The second phase of hydrocarbon detoxification was studied through GSTO mRNA expression. We observed a

strong increase in GSTO mRNA levels after three days of exposure. Members of GSTO have been reported to be involved in protection against oxidative stress (Dulhunty et al., 2001). Following an oxidative stress, a number of cellular proteins form S-thiol adducts with gluthatione and cysteine, the formation of these adducts being able to inactivate the enzymatic functions of affected polypeptides (Ravichandran et al., 1994). A conceivable function for GSTOs is to reduce this type of S-thiol adduct and restore enzymatic function. Three other GSTs were identified, one in the up-regulated SSH library after 21 days of exposure and two in the down-regulated SSH library after 7 days of exposure. Generally, GSTs play a critical role in conjugation of electrophilic compounds (phase I metabolites) on one hand, and in the defence against oxidative damage and peroxidative products of DNA and lipids (Van der Oost et al., 2003) on the other hand. An increase of GST activity has been reported in several studies on mussels *M. edulis* and *Perna viridis* exposed to PAH, PCB and pesticides (Gowlan et al., 2002; Cheung et al., 2002), whereas Akcha et al. (2000) showed a significant decrease of GST activity in gills of mussels *M. galloprovincialis* exposed to benzo[a]pyrène. The phase II detoxification mechanisms also involved sulfotransferase family members by catalysing sulfoconjugation of small lipophilic endobiotics and xenobiotics. Our work showed an inhibitory effect of hydrocarbon exposure on sulfotransferase mRNA expression. This enzyme has previously exhibited an increased activity after phenolic compounds exposure (Sugahara et al., 2003), but other authors demonstrated a negative regulation by xenobiotics in rat hepatocytes (Runge-Morris, 1998).

Metabolism of xenobiotic pollutants and cyp450 activity generate ROS, entailing the induction of a variety of stress proteins such as HSP and SOD and causing lipid peroxidation or DNA damages (Stegeman and Hahn, 1994). Exposure to hydrocarbons like PCB can cause changes in fatty acid composition due to desaturase activity, such as an increase of *Δ9* desaturase (Matsusue et al., 1997). This study is not in agreement with our observations; indeed we showed in our SSH an inhibition of *Δ9* desaturase mRNA synthesis from 7 to 21 days of exposure. The generation of deleterious ROS requires a catalyst such as iron (Reif, 1992). However, in the presence of transition metals, superoxide can generate the very reactive hydroxyl radical by the iron-catalysed Fenton reaction. Iron is therefore normally tightly controlled by transport and storage proteins. A potential biological source of iron is ferritin, the major iron-regulated protein (Reif, 1992), which exhibited a mRNA synthesis inhibition by hydrocarbon exposure in *C. gigas*.

ROS and especially superoxide can cause toxicity against which SOD is protective. Exposure to hydrocarbons induced SOD in *C. gigas* as shown in this study and previously in oysters and other marine organisms such as mussels, crab and fish (Orbea et al., 2002). No introns were detected in the genomic DNA sequence coding for Cu/Zn SOD in *C. gigas*. This is not consistent with findings in other species SOD genes where introns are present such as in *Ipomoea batatas* (GenBank accession no. L36229), *Caenorhabditis elegans* (GenBank accession no. X77020), *Drosophila willistoni* (GenBank accession no. L132881) or *Homo sapiens* (GenBank accession no. X01784) in which SOD genes contained 7, 3, 1 and 4 introns, respectively. The absence of introns was observed in HSP70 inducible members. The interruption of genes by introns suggests a constitutive expression (Basu et al., 2002; Boutet et al., 2003). It has been suggested that the lack of introns may help to circumvent the block of RNA splicing, allowing the rapid synthesis of proteins (Kay et al., 1987). This characteristic enables preferential expression of these proteins during periods of stress, with the nuclear export signal probably being provided by the mRNA's secondary or tertiary structure.

Stress response due to hydrocarbon exposure was studied by analysis of the HSP70 expression by RT-PCR and ELISA. By themselves, HSP70 induction is marker of multiple stress exposure and cannot indicate exposure to any specific contaminant without direct observation under carefully controlled conditions (Snyder et al., 2001). In our work, we showed a significant increase of HSP70 levels (mRNA and proteins) in exposed oysters (compared to the control). Quantity of HSP70 in control oysters was estimated to about 7% of total proteins. We previously showed a rate of 6% of total proteins in *C. gigas* (Boutet et al., 2003) and Feige and Polla (1994) reported a figure of about 5% under normal conditions (without stress) in other organisms. In their study, Snyder et al. (2001) observed an increase of HSP70 level in the digestive glands of abalone (*Haliotis rufescens*) exposed to the chlorinated pesticide heptachlor and in mussels (*M. galloprovincialis*) exposed to degraded-oil. They also showed that HSP90 levels were elevated in the foot of *H. rufescens*. This agrees with our observation of this stress protein in the up-regulated SSH library after 21 days of exposure. Previous studies have shown elevated HSP90 induction in brain, liver, and lung of rats after oral dosing with polycyclic halogenated hydrocarbons and chlorinated or organophosphate pesticides (Bagchi et al., 1996).

The results given here provide a basis for studying hydrocarbon detoxification processes in the marine bivalve *C. gigas*. To our knowledge, the present report is the first investigation for understanding these mechanisms at the molecular level. The method used allowed specifically hydrocarbon-regulated genes to be identified and our data suggests that oysters present: (A) similar detoxification mechanisms to other organisms (phase I and phase II enzymes), (B) lipid metabolism disruptions (regulation of lipid metabolism enzymes), (C) protection against oxidative stress (induction of specific enzymes) and (D) induction of the molecular chaperones (induction of HSP70 at mRNA and protein level and HSP90).

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