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Molecular identification and expression of two non-P450 enzymes, monoamine oxidase A and flavin-containing monooxygenase 2, involved in phase I of xenobiotic biotransformation in the Pacific oyster, Crassostrea gigas

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Abstract: Marine bivalve metabolism can be perturbed by hydrocarbon and pesticide pollution in coastal ecosystems. In this study, in the Pacific oyster, Crassostrea gigas, full-length cDNAs encoding two non-P450 phase I enzymes, flavin-containing monooxygenase 2 (FMO-2) and monamine oxidase A (MAO A), were characterized. Both sequences contained the co-factor fixation motifs characteristic of their respective enzyme families. Using reverse transcription polymerase chain reaction (RT-PCR), the messenger RNA (mRNA) transcription levels of these two enzymes in tissues of oysters exposed, under experimental conditions, to hydrocarbons and two pesticide treatments were investigated. The pesticide treatments were exposure to either glyphosate or to a mixture composed of atrazine, diuron and isoproturon. The results showed a strong differential expression of FMO-2 and MAO A that was both tissue-specific as well as time- and treatment-dependent. It was also clearly demonstrated that the transcription levels of MAO A (generally considered a constitutive enzyme without external regulation) were induced by hydrocarbons and pesticides in digestive gland and inhibited by pesticides in gill tissue. Furthermore, the transcription levels of FMO-2 and MAO A mRNA in digestive gland might be useful as a marker of hydrocarbon or pesticide exposure in monitoring programs.

Keywords: Monoamine oxidase A; Flavin-containing monooxygenase 2; Crassostrea gigas

1. Introduction

Marine organisms are continuously exposed to a wide variety of anthropogenic contaminants from industry, agriculture or urban effluents. The marine organisms, and especially the Pacific oyster *Crassostrea gigas*, living in coastal ecosystems have developed defence mechanisms against xenobiotic contamination, such as stress proteins (molecular chaperoning) (Boutet et al., 2003), metallothioneins (metal detoxification) (Tanguy et al., 2001; Tanguy and Moraga, 2001; Tanguy et al., 2002; Boutet et al., 2002), biotransformation enzyme system (organic compounds detoxification) (Boutet et al., in press), etc. The biotransformation system essentially involved enzymes capable of transform organic compounds in more soluble and easily excreted molecules than parent compound. These mechanism is divided into three phases. The first one is characterised by the oxygenation of the organic compound via microsomal monooxygenase enzymes, among them the cytochrome P450 pathway constitute the major detoxification system, followed by flavin-containing monooxygenase (FMO; Schlenk, 1998), monoamine oxidase (MAO; Strolin Benedetti, 2001), oxidase molybdenum hydroxylase (aldehyde and xanthine oxidase; Beedham, 1997) and alcohol and aldehyde dehydrogenase (Beedham, 1997). The resulting products are either directly excreted or more reactive than the parent product, entailing cell damages (DNA, lipids, protein) (Michel et al., 1992). The second phase is characterised by the enzymatic conjugation of phase I resulting products to endogenous compounds via glutathione S-transferases (GSTs), UDP-glucuronyl transferase and sulfotransferases (Van der Oost et al., 2003). The third phase included membrane proteins, known as multi-drug resistance or multi-xenobiotic resistance (McFadzen et al., 2000), and acting as a pump involved in the export of xenobiotics out of the cell (Lüdeking and Köhler, 2002).

Flavin-containing monooxygenases catalyse the oxygenation of a wide variety of xenobiotics. They are involved in the four-electron reduction of dioxygen with two electrons derived from reduced nicotinamide cofactor and two electrons derived from substrate (Poulsen and Ziegler,

1979). FMOs are membrane-bound enzymes found in the smooth endoplasmic reticulum in eucaryotic cells. Several studies reported FMO enzyme activity in molluscs, such as mussels, *Mytilus edulis* and *Mytilus galloprvincialis* (Kurelec, 1985; Kurelec and Krca, 1987) or oysters, *C. gigas* (Schlenk and Buhler, 1989, 1990). To our knowledge, no information are available in database concerning molecular characterisation of FMO in mollusc species.

Monoamine oxidases exist into two forms in mammalian tissues, MAO A and B, differing in their substrate specificity and inhibitor sensitivity (Strolin benedetti, 2001). These enzyme are essentially mitochondrial, although some MAO activity has been reported in micosomes (Wouters, 1998). MAO A enzyme activity has been reported in several tissues and central nervous system of squid in relation with hydrostatic pressure (Youdim et al., 1986; Antipov et al., 1996), but to our knowledge, no investigations were conducted to characterise MAO genes or to detect MAO mRNA expression or enzyme activity in other mollusc species.

In this paper, we characterised, for the first time, the complete cDNA sequences of two non-P450 phase I enzymes, flavin-containing monooxygenase 2 and monoamine oxidase A, in the marine bivalve *C. gigas*. The mRNA expression of these two enzymes, and their potential use as biomarkers of contaminant exposure were investigated. We also discussed on the regulatory effect of contaminant on FMO-2 and MAO A mRNA expression. We used RT-PCR to analyse FMO-2 and MAO A mRNA expression in oysters exposed to hydrocarbons and two pesticide treatments. One pesticide exposure designated ADI was to a mixture of 2-chlor-4-ethylamino-6-isopropylamino-1,3,5,-triazin (atrazine), 3-(3,4-dichlorphenyl)-1-1-dimethyl-harnstoff (diuron) and 3-(4-isopropylphenyl)-1,1-dimethylharnstoff (isoproturon) and the second was to *N*-(phosphonomethyl)glycine (glyphosate).

2. Materials and methods

2.1. Experimental design

Adult oysters (10-11 cm), *Crassostrea gigas*, were collected from La Pointe du Château (Brittany, France) in Winter 2001 for the hydrocarbon experiment and in Fall 2002 for the pesticide experiments. After an acclimatisation period of 7 days in aerated 0.22µm-filtered seawater at constant temperature and salinity (15°C and 34‰, respectively), oysters were challenged as follows. Groups of 20 oysters were exposed for 3 weeks to a 0.1% mixture of hydrocarbon consisting of the water-soluble fraction of domestic fuel homogenised for three days in filtered seawater (Snyder et al., 2001). Other groups of 20 oysters were exposed for four weeks to either a mixture of three herbicides (atrazine 2µg/l; diuron 0.5µg/l and isoproturon 1µg/l, mixture called ADI) or to 2µg/l of glyphosate. Another group of 20 oysters was maintained in seawater, without contaminant, as a control. No mortality was observed in the control or treated oysters.

2.2. Extraction of total RNA and cDNA synthesis (reverse transcription)

Total RNA were extracted from digestive gland of control and hydrocarbon exposed oysters after 0, 7, 15 and 21 days of exposure and from digestive gland and gills of control and pesticide exposed oysters after 0, 7, 15, 21 and 30 days of exposure according to the method based on extraction in guanidium isothiocyanate (Strohman et al., 1977). Ten µg RNA were submitted to reverse transcription using oligo dT anchor primer (GAC CAC GCG TAT CGA TGT CGA $CT_{(16)}V$) and M-MLV reverse transcriptase (Promega).

2.3. Cloning and sequencing of 5' and 3' flanking regions of monoamine oxidase A and flavin-containing monooxygenase 2 cDNA

The procedures for the generation of MAO A and FMO-2 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) using specific primers designed from the sequences obtained in suppressive subtraction hybridisation libraries (Boutet et al., in press) and reported in table I. The 3' UTR of MAO A and FMO-2 were amplified as follows: 200 ng of reverse transcription product plus 2 mM $MgCl_2$ and 10 pmol each of PCR anchor primer (GAC CAC GCG TAT CGA TGT CGA C) and specific sense primer was submitted to amplification using one cycle at 94°C for 2 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. 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 antisense primer. The resulting cDNA fragments corresponding to the 5' and 3' UTRs were cloned into pGEM-T vector (Promega) and sequenced using a Li-COR IR² (Sciencetech) and Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Bioscience).

2.4. Monoamine oxidase A and flavin-containing monooxygenase 2 expression study by RT-PCR

MAO A and FMO-2 mRNA expression was analysed in digestive gland of hydrocarbon exposed oysters and in gills and digestive gland of pesticide exposed oysters. PCR was performed using one cycle consisting of denaturation at 94°C for 2 min, hybridisation at 58°C for 2 min and elongation at 72°C for 1 min, then 35 cycles for FMO-2 and 30 cycles for MAO A at 94°C for 30 s,

58°C for 30 s, 72°C for 30 min, and a final step at 72°C for 7 min with 200 ng of reverse transcription product for FMO-2 amplification and 100 ng of reverse transcription product for MAO A amplification plus 2 mM $MgCl_2$ and 10 pmoles each primers (Table I). A PCR amplification control (28S ribosomal DNA) was used for all experiments and was amplified as described above with primers sense AAG GGC AGG AAA AGA AAC TAA C and antisense TTT CCC TCT AAG TGG TTT CAC. The resulting PCR products were electrophoresed in a 0.5X TBE/1.5% agarose gel, and visualised with U/V light after ethidium bromide coloration. Quantification of band intensities was measured by using Gene Profiler 4.03 Software (Scanalytics Inc.).

3. Results

3.1. Identification of the cDNA encoding MAO A in C. gigas

The cDNA encoding MAO A from *C. gigas* is contained an open reading frame of 1566 bp (521 amino acids) with a 88 bp-length 5'UTR and a 258 bp-length 3'UTR (Figure 1). The corresponding amino acid sequence contained the characteristic fixation motif of the flavin-adenine dinucleotide (FAD) cofactor (SGGCY, via the cystein 402) and the cystein 370 involved in MAO A enzyme activity. We also observed multiple ATTTA(G) motifs in the untranslated regions of the MAO A correlated with transcript stability. The cDNA encoding MAO A had one ATTTG motif in the 3' UTR. Moreover, the cDNA contained 3 polyadenylation signals in its 3' UTR. A search for amino acid sequence homologies showed that our sequence displayed an homology of 56% with mammal sequences and dropped to 18% with *Escherichia coli* sequence.

Figure 1: The nucleotide sequence and predicted amino acid sequence of monoamine oxidase A from C. gigas (GenBank accession number AJ556989). Stop codon is marked by an asterisk and untranslated regions are in lower cases. Polyadenylation signals are underlined and messenger stability determining motif ATTTG is in bold characters. Amino acids involved in the fixation of the cofactor FAD are double underlined and the cysteine essential for MAO A activity is boxed.

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cttgaggagagcttgcgacaacggtttgggtgaaggcttaggtgttctagataacgtgtctcgactgtctat     72
                  M   T   M   E   E   D   Q   L   V   D   V   I   V   V      14
acgaccacatatacac ATG ACT ATG GAG GAA GAT CAG CTG GTG GAT GTT ATT GTT GTA    130
 G   A   G   L   S   G   L   A   A   A   K   L   L   Q   E   T   G   L      32
GGG GCA GGT CTG AGT GGT TTG GCG GCT GCT AAG CTT TTA CAA GAG ACA GGA CTG    184
 D   V   L   V   L   E   A   R   D   R   V   G   G   R   T   L   T   E      50
GAT GTG TTG GTT CTT GAA GCA CGT GAC AGA GTG GGT GGA CGG ACA CTT ACA GAG    238
 H   N   S   H   V   G   Y   V   D   L   G   G   A   Y   V   G   P   T      68
CAC AAC TCT CAT GTT GGC TAT GTT GAT TTG GGC GGA GCC TAT GTT GGT CCA ACA    292
 Q   N   R   L   L   R   L   A   D   E   F   G   I   K   T   Y   F   T      86
CAG AAC AGA CTC CTG CGT CTG GCG GAT GAA TTC GGG ATT AAA ACT TAT TTT ACC    346
 N   E   V   E   D   L   V   F   Y   T   K   G   K   S   K   R   Y   H     104
AAT GAA GTG GAA GAC CTG GTG TTT TAC ACT AAG GGG AAA TCC AAG AGG TAC CAC    400
 G   A   F   S   P   A   S   G   F   F   E   Y   L   D   M   N   N   F     122
GGC GCC TTT TCC CCA GCA AGT GGG TTT TTC GAG TAT TTG GAC ATG AAT AAT TTT    454
 F   R   L   L   D   K   M   G   E   E   I   P   P   D   A   P   W   R     140
TTC AGA CTT TTG GAT AAA ATG GGT GAA GAG ATT CCT CCC GAT GCC CCT TGG AGG    508
 A   P   H   A   K   E   W   D   Q   M   T   M   Q   Q   F   L   D   K     158
GCC CCA CAT GCC AAA GAA TGG GAC CAA ATG ACC ATG CAG CAA TTT CTT GAC AAA    562
 H   V   W   T   K   Q   I   Y   R   F   C   K   T   F   V   S   V   N     176
CAT GTT TGG ACC AAA CAA ATC TAC CGC TTC TGT AAA ACC TTC GTC AGT GTT AAC    616
 V   T   S   E   P   Y   E   A   S   V   L   W   F   L   W   Y   I   K     194
GTC ACT TCG GAG CCC TAT GAA GCC TCA GTA CTT TGG TTT CTG TGG TAT ATC AAA    670
 C   C   G   G   Q   K   R   I   F   S   T   T   N   G   G   Q   E   R     212
TGT TGT GGC GGA CAA AAG CGA ATT TTC TCA ACA ACA AAT GGC GGA CAG GAG AGA    724
 K   F   V   G   G   S   Q   Q   I   S   K   R   I   A   E   K   L   G     230
AAG TTT GTT GGC GGC TCA CAA CAA ATC AGC AAA AGA ATT GCC GAA AAG CTG GGC    778
 N   D   R   V   L   L   S   H   P   V   C   H   I   S   Q   T   T   D     248
AAC GAC CGA GTC CTC CTG AGT CAC CCT GTG TGC CAT ATC AGC CAG ACA ACT GAT    832
 G   V   T   V   S   V   T   G   G   Q   Q   F   R   A   K   R   V   I     266
GGA GTG ACG GTG TCC GTT ACT GGT GGA CAA CAA TTC AGG GCT AAA CGT GTC ATT    886
 I   A   S   P   L   P   L   Q   N   K   I   T   Y   D   P   P   L   P     284
ATT GCC TCT CCG CTA CCT TTA CAA AAT AAG ATC ACA TAT GAC CCT CCC TTG CCA    940
 S   L   R   N   Q   L   I   Q   R   I   P   M   G   S   V   I   K   T     302
AGT CTT AGG AAT CAA TTA ATA CAA AGA ATC CCG ATG GGT TCC GTC ATC AAA ACA    994
 F   C   Y   Y   K   T   P   F   W   K   E   K   G   Y   C   G   S   T     320
TTT TGC TAC TAC AAG ACC CCT TTT TGG AAG GAG AAA GGT TAT TGT GGG TCC ACT   1048
 A   I   D   D   D   A   A   I   V   E   F   T   L   D   D   T   K   H     338
GCA ATA GAC GAT GAT GCA GCC ATA GTT GAA TTC ACA TTG GAT GAT ACG AAG CAT   1102
 D   G   S   H   P   A   L   M   G   F   V   L   A   D   K   A   K   R     356
GAC GGA AGT CAC CCA GCG TTA ATG GGA TTT GTT CTT GCA GAT AAG GCA AAA CGT   1156
 F   V   S   M   T   P   E   E   K   K   E   S   I   C   R   L   Y   A     374
TTT GTT TCT ATG ACA CCG GAA GAG AAA AAA GAG AGT ATA TGT CGT CTG TAT GCT   1210
 K   V   F   K   S   D   E   A   L   Y   P   I   H   Y   E   E   K   N     392
AAA GTC TTC AAA TCA GAC GAA GCG TTA TAT CCA ATA CAC TAT GAA GAG AAG AAC   1264
 W   L   G   E   Q   W   S   G   G   C   Y   T   A   M   M   P   P   G     410
TGG CTC GGG GAA CAG TGG TCT GGG GGT TGT TAC ACA GCC ATG ATG CCC CCG GGA   1318
 F   L   T   N   F   G   E   E   I   R   R   P   V   G   N   L   Y   F     428
TTC CTG ACC AAC TTC GGC GAA GAA ATA AGA AGA CCC GTT GGT AAT TTA TAT TTC   1372
 A   G   T   E   T   A   T   Q   W   S   G   Y   M   E   G   A   V   Q     446
GCG GGG ACA GAG ACA GCC ACC CAG TGG TCG GGG TAC ATG GAA GGG GCG GTC CAG   1426
 A   G   E   R   A   A   R   E   I   L   F   D   M   K   K   I   P   K     464
GCC GGG GAG AGG GCG GCC AGA GAG ATT TTA TTT GAC ATG AAA AAA ATT CCC AAA   1480
 Y   E   I   W   Q   D   E   E   E   N   T   L   V   R   A   R   P   F     482
TAT GAA ATT TGG CAA GAC GAA GAA GAG AAT ACT CTT GTG AGA GCC CGT CCG TTT   1534
 E   S   T   F   W   E   R   N   L   P   S   V   G   G   F   L   K   C     500
GAG AGC ACG TTT TGG GAG AGA AAC CTA CCG TCA GTT GGG GGG TTT CTG AAG TGT   1588
 V   S   V   T   T   A   L   A   V   G   S   A   G   L   C   L   Y   W     518
GTA TCC GTT ACA ACA GCT TTA GCA GTC GGA TCG GCG GGA CTG TGT CTT TAT TGG   1642
 W   K   R   *                                                           522
TGG AAA CGT TAA  catgctaattcaattaaaaaatgaccagattcaaaataacctagggttttatctt  1709
aaaagatcaaattaaataaggctcagtaaatcaaaaaataaatcaaacattcaagtat atttg tttgcatct  1781
cccaaccaaatatgtgcaaacaaaaataaactgtcttctttatagtatttcggttaatattacttaatgttg  1853
ctggattcttttgacacttgaaataaaagatattgaataaaaaaaaaaaaaaaaaaaaa               1912
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3.2. Identification of the cDNA encoding FMO-2 in C. gigas

The cDNA encoding FMO-2 from *C. gigas* is contained an open reading frame of 1356 bp (451 amino acids) with a 24 bp-length 5'UTR and a 36 bp-length 3'UTR (Figure 2). The corresponding amino acid sequence contained the characteristic fixation motif of the FAD cofactor (GAGPAG). The FMO-2 cDNA sequence showed a short 3'UTR and the polyadenylation signal overlapped the third base of the last codon and the stop codon (AGA TAA A). The amino acid sequence homology with mammal sequences is 24%.

Figure 2: The nucleotide sequence and predicted amino acid sequence of flavin-containing monooxygenase 2 from *C. gigas* (GenBank accession number AJ585074). Stop codon is marked by an asterisk and untranslated regions are in lower cases. Polyadenylation signal is underlined. Amino acids involved in the fixation of the cofactor FAD are boxed.

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                                M   S   G   T   T   G   R   Q   R   V   A   V     12
ATACACGTACAGTGAGCAGTAGAG ATG TCG GGG ACA ACT GGC AGA CAG AGG GTR GCG GTT    60
 I   G   A   G   P   A   G   L   C   C   V   K   H   L   A   A   K   P     30
ATC GGG GCC GGA CCG GCG GGG CTG TGC TGT GTC AAG CAT TTG GCT GCG AAA CCC    114
 E   L   F   E   P   V   A   F   E   R   N   F   W   P   G   G   I   W     48
GAG CTC TTT GAA CCT GTT GCC TTT GAG CGT AAC TTT TGG CCG GGC GGG ATT TGG    168
 N   Y   T   D   Q   T   R   K   D   A   F   G   L   P   V   H   S   A     66
AAC TAC ACT GAC CAG ACC CGG AAA GAC GCA TTC GGA CTT CCG GTA CAC TCT GCA    222
 L   Y   N   K   L   K   I   N   V   P   K   E   L   Q   E   F   P   S     84
CTG TAT AAT AAG CTG AAG ATA AAT GTT CCA AAA GAA CTA CAG GAA TTT CCA AGT    276
 F   P   Y   P   K   E   W   K   T   S   Y   I   T   R   Q   Q   C   W    102
TTC CCC TAC CCA AAG GAA TGG AAG ACG TCC TAC ATC ACG CGG CAG CAG TGC TGG    330
 E   Y   L   N   M   F   T   D   H   F   D   I   R   K   Y   I   R   F    120
GAA TAC CTC AAC ATG TTC ACA GAC CAT TTC GAC ATC AGG AAG TAT ATC CGG TTT    384
 H   S   F   V   R   N   V   K   P   L   K   E   M   N   E   N   G   K    138
CAT TCA TTT GTT CGA AAC GTG AAG CCA TTG AAG GAG ATG AAC GAA AAC GGC AAA    438
 P   K   W   L   V   T   F   S   P   V   T   R   M   S   E   V   N   T    156
CCA AAA TGG CTG GTG ACC TTT TCA CCC GTG ACC CGA ATG TCA GAG GTC AAC ACA    492
 E   V   F   D   A   V   L   V   S   N   G   H   D   F   N   D   Y   T    174
GAA GTG TTC GAC GCC GTA CTT GTG AGC AAC GGC CAC GAC TTC AAC GAC TAC ACG    546
 P   N   I   P   G   L   E   L   F   E   G   R   A   I   H   S   K   E    192
CCC AAC ATT CCC GGC CTG GAG CTG TTT GAG GGA CGA GCT ATC CAT AGC AAG GAG    600
 F   R   Y   E   E   H   F   D   G   L   R   V   A   I   L   G   C   H    210
TTC CGG TAC GAG GAA CAC TTT GAC GGA CTT CGT GTG GCG ATC CTG GGC TGC CAC    654
 Y   S   G   E   D   I   S   T   H   V   A   K   F   A   K   K   V   Y    228
TAT TCC GGA GAA GAC ATC TCT ACG CAT GTC GCT AAG TTT GCT AAG AAG GTA TAC    708
 A   C   H   R   R   N   P   K   E   F   P   P   S   F   P   K   E   I    246
GCC TGT CAT CGA AGA AAT CCA AAG GAA TTT CCA CCG TCT TTC CCG AAG GAA ATC    762
 E   Q   R   P   P   F   A   R   M   T   R   D   S   V   V   F   P   D    264
GAA CAA CGA CCA CCG TTC GCC CGC ATG ACC AGA GAT TCA GTG GTT TTC CCG GAC    816
 G   G   S   E   K   V   D   A   V   I   F   C   T   G   Y   R   F   S    282
GGA GGT TCC GAG AAG GTG GAC GCC GTT ATA TTC TGC ACC GGA TAT CGC TTC TCT    870
 Y   P   F   L   K   D   D   V   I   T   I   K   D   E   R   I   E   P    300
TAT CCA TTT TTA AAG GAT GAC GTC ATC ACA ATC AAG GAT GAG AGG ATA GAG CCC    924
 I   Y   K   H   M   V   H   I   E   Y   N   N   L   I   F   V   G   I    318
ATT TAC AAA CAC ATG GTG CAC ATT GAG TAC AAC AAC TTG ATA TTT GTG GGA ATT    978
 P   R   Q   W   S   Y   F   P   H   Y   H   E   M   A   K   L   A   A    336
CCC CGA CAA TGG TCG TAC TTT CCC CAC TAT CAC GAA ATG GCA AAA CTT GCT GCG   1032
 L   I   L   A   E   D   V   K   L   P   S   K   E   I   M   L   A   D    354
TTA ATT TTG GCG GAA GAC GTC AAA CTG CCG TCC AAA GAG ATC ATG CTG GCG GAC   1086
 S   E   A   D   F   Q   S   R   L   K   E   G   K   P   P   S   F   A    372
AGC GAG GCC GAT TTC CAG TCG CGT TTG AAA GAA GGA AAG CCG CCT TCG TTT GCC   1140
 H   Y   M   G   D   I   D   R   Q   F   R   Y   N   E   D   L   A   K    390
CAT TAC ATG GGG GAC ATC GAC CGT CAG TTC CGG TAT AAC GAG GAC CTG GCA AAG   1194
 M   G   G   F   D   P   L   P   P   V   L   E   M   M   W   D   D   V    408
ATG GGC GGG TTT GAC CCA CTT CCG CCT GTC CTG GAG ATG ATG TGG GAT GAC GTC   1248
 M   D   E   R   Y   M   N   L   P   N   C   N   T   F   D   Y   E   I    426
ATG GAT GAG AGG TAC ATG AAC CTC CCG AAC TGT AAC ACT TTT GAC TAT GAG ATC   1302
 T   G   P   Q   S   Y   R   C   L   N   P   E   G   I   K   T   R   L    444
ACA GGA CCA CAG TCC TAC CGC TGT CTG AAC CCC GAG GGA ATC AAA ACC AGG TTG   1356
 C   K   A   E   N   V   K   R   *                                       453
TGT AAG GCT GAA AAT GTG AAG AGA TAA  AAAGTAATGATCCATGTGGAAAAAAAAAAAAAA   1416
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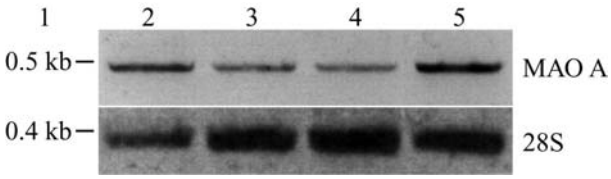
3.3. MAO A expression analysis using RT-PCR

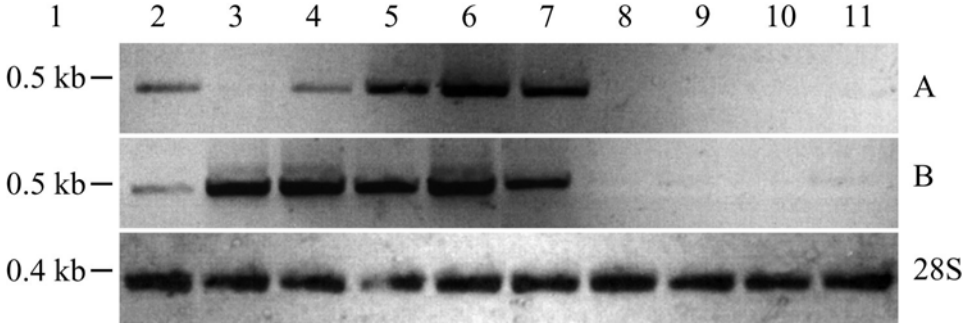
The results of RT-PCR showed a strong differential MAO A expression between tissues and treatments. Analysis of MAO A expression in the digestive gland of oysters exposed to hydrocarbons displayed an induction of MAO A mRNA synthesis after 21 days of exposure (1.4-fold compared with control) (Figure 3).

Figure 3: Expression of MAO A in digestive gland of oysters exposed to hydrocarbons by RT-PCR (*n*=3 for each day). Lane 1: 100 bp marker; Lane 2: control oysters; Lane 3: oysters exposed for 7 days; Lane 4: oysters exposed for 15 days; Lane 5: oysters exposed for 21 days.



Regarding pesticide exposure results, we observed that both treatments inhibit MAO A mRNA synthesis in gills of oysters (Figures 4A and B). Then, we showed that an exposure to ADI entailed an inhibition of MAO A at 7 days, followed by an induction after 15 days, reaching a maximum value at 30 days in the digestive gland (1.8-fold compared with control) (Figure 4A). The expression of MAO A in digestive gland of glyphosate exposed oysters showed a strong induction at 7 days which is maintained until the end of the experiment (2.5-fold compared with control) (Figure 4B).

Figure 4: Expression of MAO A in digestive gland (lanes 2 to 6) and gills (lanes 7 to 11) of oysters exposed to ADI (A) or glyphosate (B) by RT-PCR (*n*=3 for each day). Lane 1: 100 bp marker; Lanes 2 and 7: control oysters; Lanes 3 and 8: oysters exposed for 7 days; Lanes 4 and 9: oysters exposed for 15 days; Lanes 5 and 10: oysters exposed for 21 days; Lanes 6 and 11: oysters exposed for 30 days.



3.4. FMO-2 expression analysis using RT-PCR

The analysis of FMO-2 expression showed differences between tissues and treatments. An exposure to hydrocarbons entailed an induction at 7 days in digestive gland of oysters (2.2-fold compared with control), before dropping to the level observed in control oysters (Figure 5).

Regarding pesticide exposure results, we observed that FMO-2 is not expressed in gills of pesticide-exposed oysters (Figures 6A and B). We observed that both ADI and glyphosate exposure entailed an induction after 30 days in digestive gland (3.5- and 3-fold compared with control, respectively) (Figure 6A and B).

Figure 5: Expression of FMO-2 in digestive gland of oysters exposed to hydrocarbons by RT-PCR (*n*=3 for each day). Lane 1: 100 bp marker; Lane 2: control oysters; Lane 3: oysters exposed for 7 days; Lane 4: oysters exposed for 15 days; Lane 5: oysters exposed for 21 days

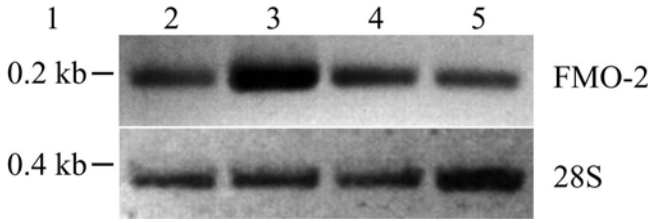
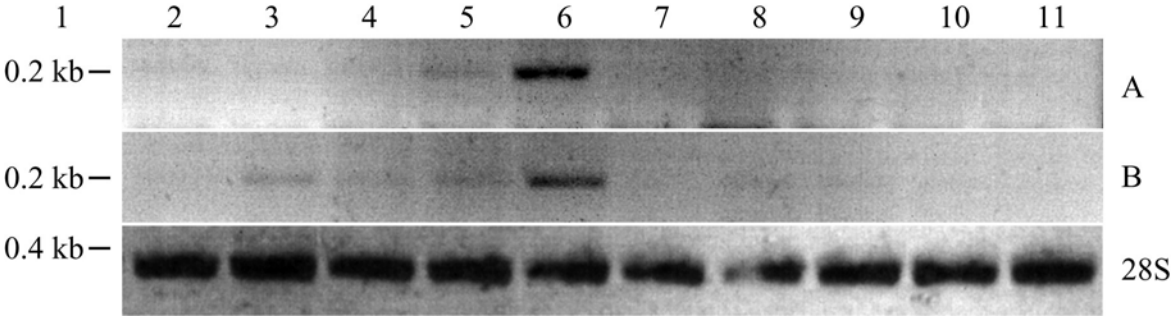


Figure 6: Expression of FMO-2 in digestive gland (lanes 2 to 6) and gills (lanes 7 to 11) of oysters exposed to ADI (A) or glyphosate (B) by RT-PCR (*n*=3 for each day). Lane 1: 100 bp marker; Lanes 2 and 7: control oysters; Lanes 3 and 8: oysters exposed for 7 days; Lanes 4 and 9: oysters exposed for 15 days; Lanes 5 and 10: oysters exposed for 21 days; Lanes 6 and 11: oysters exposed for 30 days.



4. Discussion

In the present paper, we reported, for the first time, the complete sequences of two non-P450 enzymes involved in xenobiotic biotransformation, monoamine oxidase A and flavin-containing

monooxygenase 2, from the Pacific oyster *C. gigas*. We previously obtained two partial sequences in a digestive gland SSH library from oysters exposed to hydrocarbons (Boutet et al., in press). Specific primers were then designed from these sequences to amplified the complete MAO A and FMO-2 cDNAs. The two sequences reported here presented the characteristic FAD-fixation motifs of the two enzyme families studied: SGGCY (via the cyteine 402) for the MAO A (Strolin Benedetti, 2001) and GAGPAG for the FMO-2. We also observed the cystein 370 involved in MAO A enzyme activity (Wouters, 1998). The FAD-fixation motif of the FMO-2 is present in other species sequences as GAGVSG in monkey (Yueh et al., 1997), rabbit (Lawton et al., 1990) and guinea pig (Nikbakht et al., 1992). A fixation motif for β-nicotinamide adenine dinucleotide phosphate (NADP) was reported in monkey and human FMO (GMGNSG) (Phillips et al., 1995; Yueh et al., 1997) and rabbit and guinea pig FMO (GIGNSA) (Lawton et al., 1990; Nikbakht et al., 1992), but this motif was not present in our sequence. The low degree of homology between oyster and mammal sequence certainly explain the lack of similar NADP fixation motif. This motif is probably present in oyster sequence, involving other amino acids that those described above. The cDNA sequence encoding FMO-2 in *C. gigas* presented another interesting feature consisting in a short 3'UTR and a polyadenylation signal which overlapped the third base of the last codon and the stop codon (AGA TAA A). This particularity was previously observed in sequence encoding AMP desaminase in rat (Sabina et al., 1987). The MAO A cDNA sequence presented one ATTTG motif in its 3'UTR. These motifs are known to be correlated with transcript stability (Shaw and Kamen, 1986) and have been observed in other cDNAs, such as the sequence encoding glutamine synthetase in the sea urchin *Paracentrotus lividus* (Fucci et al., 1995), in rat aspartate aminotransferase (AAT) (Pavé-Preux et al., 1988) and in *C. gigas* pi class GST (GenBank accession number AJ557140). Another interesting 3'UTR feature seen was three polyadenylation signals in the MAO A cDNA sequence. Multiple polyadenylation sites have already been observed for other genes (Leff et al., 1986). Caizzi et al. (1990) and Smartt et al. (1998 and 2001) suggest that the multiple polyadenylation sites indicate the presence of multiple transcripts encoding one protein. Pavé-Preux

et al. (1988) found that a single AAT sequence containing two polyadenylation signals encoded two different mRNAs in rat. They postulated that the two mRNAs resulted from the differential use of these signals during the maturation of pre-mRNA. More analysis will be necessary to determine how many different mRNAs are encoding by the *C. gigas* MAO A cDNA sequence.

Analysis of FMO-2 expression by RT-PCR in xenobiotic-exposed oysters, showed an increase of mRNA synthesis in the digestive gland of all treated oysters, whereas an absence of FMO-2 expression was observed in gills. Conversely, Schlenk and Buhler (1989) detected FMO enzyme activity in gills of *C. gigas*. Other studies showed that FMO enzyme activity was normally greater in the digestive gland/hepatopancreas/liver than in other organ (Ziegler, 1988, 1993). It is also assumed that gills contained a limited yield of microsomes (Schlenk and Buhler, 1989) and it could explain the lack of FMO mRNA expression in gills of pesticide-exposed oysters in our analysis. More, FMO activity was detected in digestive gland and visceral mass of mussels and oysters (Kurelec, 1985; Kurelec and Krca, 1987; Livingstone et al., 1990; Schlenk and Buhler, 1990). Detection of FMO activity and mRNA expression in the digestive gland of bivalves suggests that this system plays a key role in xenobiotic detoxification.

The second Phase I enzyme studied here is the monoamine oxidase A. In the present report, we investigated mRNA expression by RT-PCR in hydrocarbon and pesticide exposed oysters. We observed a strong induction of MAO A in digestive gland of all treated oysters, while pesticide exposure entailed an inhibition of MAO A expression in gills. We also shown a similar inhibitory effect of pesticides on mRNA synthesis of two glutathione S-transferases (GSTs) in *C. gigas* (data not shown). Nevertheless, previous investigations on MAOs showed that these enzymes are constitutive and presented no known external regulation of its expression (Ramsay, 1998). In our study, we confirmed the constitutive status of MAO A in oysters, because mRNA synthesis was detected in both tissues of control oysters. More, we previously obtained MAO A partial sequence in an up-regulated SSH library (Boutet et al., in press), that it could have not been possible if MAO A was only constitutively expressed. Our study clearly demonstrate that MAO A mRNA expression

is regulated by xenobiotic exposure in oysters, either characterised by an induction in digestive gland or by an inhibition in gills.

In this study, we characterised for the first time, two complete cDNAs encoding non-P450 enzymes involved in xenobiotic detoxification, monoamine oxidase A and flavin containing monooxygenase 2, in the Pacific oyster *C. gigas*. The two amino acid sequences contained the characteristic motifs of the enzyme families and some interesting features described in other sequences. Using these two sequences, we investigated mRNA expression in tissues of pesticide- and hydrocarbon-exposed oysters under experimental conditions. These two enzymes are strongly time- and tissue-regulated by all xenobiotics tested here. More, according to the present study and work on GSTs in *C. gigas*, we could hypothesise that pesticides have an inhibitory effect on mRNA synthesis of several biotransformation enzymes in gills. The analysis of MAO A and FMO-2 mRNA level could be use as a biomarker of pesticide or hydrocarbon exposure in oysters from contaminated estuaries.

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