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Identification and expression of multiple *CYP1-like* and *CYP3-like* genes in the bivalve mollusk *Mytilus edulis*

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ABSTRACT

Various sequencing projects over the last several years have aided the discovery of previously uncharacterized invertebrate sequences, including new cytochrome P450 genes (CYPs). Here we present data on the identification and characterization of two *CYP1-like* and three *CYP3-like* genes from the bivalve mollusk *Mytilus edulis*, and assess their potential as biomarkers based on their responses to several known vertebrate aryl hydrocarbon receptor (AHR) agonists. Quantitative real-time PCR was used to measure *CYP* transcript levels in digestive gland, labial palps, adductor muscle, gill, foot, and different regions of the mantle. Levels of both *CYP1-like* genes were highest in digestive gland, whereas labial palps had the highest expression levels of the three *CYP3-like* genes followed by digestive gland and outer margin of the mantle. Mussels were exposed by injection to the AHR agonists, β-naphthoflavone (BNF; 25 $\mu g\,g^{-1}$), 3,3′,4,4′,5-polychlorinated biphenyl (PCB126; 2 $\mu g\,g^{-1}$), or 6-formylindolo[3,2-b]carbazole (FICZ; 0.1 $\mu g\,g^{-1}$), or to Aroclor 1254 (a mixture of PCBs; 50 $\mu g\,g^{-1}$) for 24 h, followed by *CYP* expression analysis. There was no statistically significant change in expression of either of the *CYP1-like* genes after exposure to the various AHR agonists. The *CYP3-like-1* gene was significantly up-regulated by BNF in gill tissues and the *CYP3-like-2* gene was up-regulated in digestive gland by PCB126 and in gill tissue by BNF. These results suggest that distinct mechanisms of *CYP* gene activation could be present in *M. edulis*, although the importance of the *CYP1-like* and *CYP3-like* genes for xenobiotic and endogenous lipids biotransformation requires additional investigation.

1. Introduction

The cytochrome P450s (CYPs) comprise one of the largest and most versatile protein families in living organisms, and they catalyze an extraordinary range of important biochemical reactions. Vertebrate CYPs are distributed in 18 families and a growing number of subfamilies, with for example, 57 genes arranged into 42 subfamilies in humans (Nelson et al., 2004). A large diversity of *CYP* genes also occurs in invertebrates, including members of the prebilateria such as the sea anemone (Goldstone, 2008), early-diverging deuterostomes (e.g., sea urchin (Goldstone et al., 2006)), and protostomes, including the ecdysozoa (e.g., *Daphnia pulex* (Baldwin et al., 2009) and *Caenorhabditis elegans* (Gotoh, 1998)), and the lophotrochozoa (e.g., the gastropod *Lottia gigantea* (Gotoh, 2012; Nelson, 2011; Nelson et al., 2013)). In all animals CYPs are thought to have roles in xenobiotic metabolism and bioactivation, and in development and maintenance of homeostasis, via regulation of signaling molecules (e.g., steroid hormones, eicosanoids and retinoic acid) (Nebert and Dalton, 2006; Nebert and Russell, 2002; Rewitz et al., 2006). However, even in vertebrates not all CYPs have known functions, and for most species many CYPs can be considered as "orphans", enzymes for which the biological role is unknown.

In vertebrates, members of the CYP1 and CYP2 families (all in CYP Clan 2) and the CYP3 family (in CYP Clan 3) are known for the oxidative transformation of xenobiotics, e.g., drugs, pesticides and polycyclic aromatic hydrocarbons (PAHs) (Nebert and Russell, 2002). The induction of vertebrate *CYP1* genes occurs via the aryl-hydrocarbon receptor (AHR), activated by xenobiotic chemicals such as planar halogenated hydrocarbons and PAHs, and by dietary compounds such as flavonoids (Denison et al., 2002; Hahn, 2002). The expression of CYP1 family members, especially of the CYP1A

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subfamily in fish, birds and mammals, has been used for decades as a marker of environmental or experimental exposure to AHR agonists (Bucheli and Fent, 1995; Payne, 1976; Stegeman et al., 1986).

Bivalves have long been known to accumulate foreign organic chemicals (Stegeman and Teal, 1973) and metals (Huggett et al., 1973) and have been employed extensively as sentinels in monitoring programs, e.g., the Mussel Watch Program in the USA and the MEDPOL and BIOMAR programs in Europe (Dondero et al., 2006; Venier et al., 2006). Many studies have examined total microsomal cytochrome P450 content, levels of CYP enzyme activity, or CYP protein by immunoassay in bivalve mollusks exposed to chemicals (Canova et al., 1998; Livingstone, 1998; Livingstone et al., 1989; Monari et al., 2007, 2009; Shaw et al., 2002; Snyder et al., 2001; Sole and Livingstone, 2005; Wootton et al., 1995). The interpretation of those measurements has been complicated by low catalytic activity of microsomal enzymes with diagnostic substrates for CYP1A-like activity, such as ethoxyresorufin (ethoxyresorufin-O-deethylase activity, EROD) and benzo[a]pyrene (benzo[a]pyrene hydroxylase; BPH), and lack of specific cross-reactivity of antibodies for CYP1As (Chaty et al., 2004; Jonsson et al., 2006a,b). Thus, the use of CYP protein or activity as a marker of exposure to or effects of environmental contaminants in bivalves has met with limited success and weak inference at best, reflecting the limited characterization of *CYP* genes and proteins in mollusks.

The sequencing of the gastropod *L. gigantea* genome revealed numerous *CYP* genes, and bivalve EST libraries have provided a new basis of comparison into molluscan CYP diversity (Craft et al., 2010; Fiedler et al., 2010; Gotoh, 2012; Lockwood and Somero, 2011; Milan et al., 2011; Nelson, 2011; Nelson et al., 2013). We recently identified more than 50 unique *CYP* sequences in *Mytilus californianus* (Zanette et al., 2010) from previously sequenced EST libraries (Connor and Gracey, 2011). Some of these sequences clustered with *CYP1* sequences of vertebrates and with *CYP1-like* genes in *L. gigantea* (Zanette et al., 2010). In the present study we focused on *Mytilus edulis* CYP sequences, seeking possible homologs of vertebrate CYPs that respond to chemical exposure. We cloned full-length sequences for multiple *CYP* genes, and found them related to genes in vertebrate CYP1 and CYP3 families. We examined organ distribution of expression and the responses to selected AHR agonists, including 3,3′,4,4′,5-polychlorinated biphenyl congener 126 (PCB126), a mixture of PCBs (Aroclor 1254), and the synthetic compound β-naphthoflavone (BNF). Notably, we also tested the endogenous tryptophan metabolite 6-formylindolo[3,2-b]carbazole (FICZ), a highly potent and rapidly metabolized AHR agonist suggested to be a physiological ligand for AHR (Wincent et al., 2009). The results are foundational for studies of *CYP1*- and *CYP3-like* genes in bivalves.

2. Methods

2.1. Animal sampling and laboratory care

Mussel *M. edulis* of both sexes, 6–8 cm in shell length, were sampled from a clean reference site (Scorton Creek, MA, USA) in August and September 2008, transferred to the laboratory and acclimated at 20 °C in flowing seawater for one week. Food was given twice a day as a mixture of microalgae *Thalasiosira weissflogi*, *Tetraselmis chuil* and *Isochrysis* sp., cultured for this purpose.

2.2. Cloning of CYP1-like and CYP3-like transcripts in M. edulis

Digestive gland was dissected from one randomly selected untreated mussel. Total RNA was isolated using the Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories Inc., Hercules, CA), which eliminates genomic DNA by DNAse treatment. RNA quantity and quality were determined spectrophotometrically (Nanodrop ND 1000; NanoDrop Technologies, Wilmington, DE). cDNA was synthesized from 2 μg of total RNA, using the Omniscript Reverse Transcriptase kit (Qiagen Inc., Valencia, CA), anchored oligo(dT) primer (MWG Biotech, Inc., High Point, NC) and RNasin RNase inhibitor (Promega Corp., Madison, WI).

Table 1
Mytilus californianus primers used for the cloning of the new *CYP1-like* and *CYP3-like* genes in *Mytilus edulis*.

Primer name	Primer sequence 5′–3′
CYP1-1 forward	TGACGTGTTTAAAATTCGCATGG
CYP1-1 reverse	CTGCGTCCAATACCAAATGTAAG
CYP1-2 forward	TTGGGAAATACGAAAGCTACCTCCA
CYP1-2 reverse	TCCAGCAACAATGAAATCCCGTA
CYP3-3 forward	TTCCTGGTCCGGAACCAACTCC
CYP3-3 reverse	GCAGACCTTAGCGCCGTGTCG

Primers used in the identification of the *M. edulis CYP1-like-1*, *CYP1-like-2* and *CYP3-like-3* sequences were designed with Primer3 (Rozen and Skaletsky, 2000). The primers were designed based on the *CYP1-like* and *CYP3-like* transcripts from the mussel *M. californianus*, previously identified by phylogenetic analysis (Zanette et al., 2010), and were to regions of the transcripts expected to be highly conserved. *CYP1-like-1* primers were designed based on one *M. californianus* contig obtained from EST sequences in the GenBank with accession numbers ES401002, ES398625, ES407183, ES400274, ES401422, ES400520, ES403691, ES405860, ES401376, ES402773, ES404163, ES398150 and ES407922. Primers for *CYP1-like-2* and *CYP3-like-3* were designed based on *M. californianus* EST sequences with the accession numbers ES399754 and ES400411, respectively. Primer sequences are shown in Table 1. The full-length sequences of the two other *M. edulis CYP3-like* genes analyzed in the present study were found in GenBank, accession numbers AB479540 and AB479539. We did not clone those sequences but used them to design qPCR primers (below). A paper describing these latter two *M. edulis CYP3-like* genes was published recently (Cubero-Leon et al., 2012), after our study was completed. The sequences we refer to as *CYP3-like-1*and *CYP3-like-2* are referred to as *CYP3A-like isoform 2* and *CYP3A-like isoform 1*, respectively, in that paper.

The PCR amplified products for the new *CYP* sequences were resolved on 1.5% agarose gel, isolated and ligated in pGEM-T Easy Vector (Promega), and transformed into *E. coli* (TOP 10 Kit, Invitrogen). Plasmids were purified from cultures of positive clones (QiaPrep™, Qiagen) and sequenced (MWG Biotech). The 5′ and 3′ ends of those transcripts were obtained by rapid amplification of cDNA ends (RACE) with the BD Smart™ RACE cDNA Amplification Kit (Clontech) and gene-specific primers described in Table 2.

Table 2
RACE specific-primers used for full-length cloning of *CYP1-like-1*, *CYP1-like-2* and *CYP3-like-3* sequences in *Mytilus edulis*.

Primer name	Primer sequence 5′–3′
CYP1-1 forward 1	GCAGAGAGGCAATACGTGAAGCGTTG
CYP1-1 forward 2	AACCCACTTCAGCCCCCATGATGAC
CYP1-1 reverse 1	GTTCCGGCGAATTTCCGTATTGTTCC
CYP1-1 reverse 2	GGCATAACAAATCGCAACCATGGCA
CYP1-2 forward 1	TGGGTAAGTGGCCGACCATAGTGC
CYP1-2 forward 2	AAATCACCAGCGTGGAATGGTTGATG
CYP1-2 reverse 1	TTCCCCAAACACAAGGCCTTTCA
CYP1-2 reverse 2	TCATGGTGAACTTCTTTCTCGGCGTA
CYP3-3 forward 1	CCCAGGGAACACATTGGAAGTTTCT
CYP3-3 forward 2	GGAAAGAAAGCAGCCATTGGACAAC
CYP3-3 reverse 1	GTCTCCCCGCTGTGAGTGGATGTT
CYP3-3 reverse 2	CTTAGCGCCGTGTCGAAGAATTCAA