Cloning a new Cytochrome P450 isoform (CYP356A1) from oyster *Crassostrea gigas*

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Abstract

We have cloned the full-length cDNA of the first member of a new cytochrome P450 (CYP) family from the Pacific oyster *Crassostrea gigas*. This new *CYP* gene was obtained based on an initial 331 bp fragment previously identified among the list of the differentially expressed genes in oysters exposed to untreated domestic sewage. The full-length *CYP* has an open reading frame of 1500 bp and based on its deduced aminoacid sequence was classified as a member of a new subfamily, CYP356A1. A phylogenetic analysis showed that CYP356A1 is closely related to members of the CYP17 and CYP1 subfamilies. Semi-quantitative RT-PCR was performed to analyze the *CYP356A1* expression in different tissues of the oyster (digestive gland, gill, mantle and adductor muscle). Results showed slightly higher *CYP356A1* expression in digestive gland and mantle, than the other tissues,

indicating a possible role of the CYP356A1 in the xenobiotic biotransformation and/or steroid metabolism.

Keywords: Cytochrome P450; Pacific oyster; Crassostrea gigas; biotransformation enzymes, CYP356A1, CYP17.

The cytochromes P450 (CYP) superfamily is one of the largest and functionally most diverse protein families. CYP enzymes are associated with xenobiotic biotransformation and other processes, including homeostasis, hormone biosynthesis and degradation, and oxidative stress (Stegeman and Hahn, 1994). CYP enzymes are the most important oxidative (phase I) biotransformation enzymes in terms of catalytic versatility and breadth of xenobiotic biotransformations carried out (Guengerich, 1987). In this study we cloned the full-length cDNA of a member of a new CYP subfamily from the gill of the Pacific oyster *Crassostrea gigas*.

Methods. An initial fragment of 331 bp was previously identified among the list of the differentially expressed genes in oysters exposed to untreated domestic sewage (Medeiros et al., this volume). Amplification of 5' and 3' cDNA ends were performed by SMART RACE (Clontech), using specific primers (forward 5'-CCAGAAGAATTTGACCCACTTCG-3' and reverse 5'-TTTGTAATCGGACGGAAGCTCTAC-3'). Reactions were set to 25 cycles of: 30 s at 94ºC, 30 s at 51ºC, and 2 min at 72ºC. PCR products were analyzed in 1.2% agarose gel and the 550 bp and 400 bp expected products were purified, cloned and sequenced on ABI3730 (Applied Biosystems). The results were manipulated using BioEdit software.

Amplification of the internal region was carried out using the primers forward 5'-GAAAGGCTCTCAGGCATTATCT-3' and reverse 5'-CCTCTTGACATTTTGCTTGG-3'. Amplification conditions were 2 min at 94ºC initial denaturation, followed by 30 cycles: 30 s at 94ºC, 45 s at 47ºC, and 60 s at 72ºC. PCR product was directly sequenced on MEGABACE 1000 (GE Healthcare). Phylogenetic studies were carried out using Bayesian techniques as implemented in the software MrBayes, which estimates posterior probabilities using Metropolis-Hastings coupled Monte Carlo Markov chains (MC^3). Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out in order to analyze the *CYP* expression in different tissues (digestive gland, gill, mantle and adductor muscle) using the forward and reverse primers (forward: 5'-CCAGAAGAATTTGACCCACTTCG-3', reverse: 5'-TTTGTAATCGGACGGAAGCTCTAC-3'). In order to avoid individual variability, each tissue was pooled from 5 oysters and the total RNA was used for this analysis. The densitometry of products was quantified using Scion Image software.

Results and discussion. The full-length sequence of the new *CYP* gene has 1500 bp (Figure 1, Genbank access no. **ABR45717**). The deduced amino acid sequence shows conserved motifs typical of CYP enzymes, such as the heme group binding region, helix-C, helix-I and helix-K motifs (Figure 1). This sequence was classified by the CYP Nomenclature Committee as a member of a new subfamily, CYP356A1. The phylogenetic analysis demonstrates a close relationship between the CYP356A1 and CYP1 and CYP17 subfamilies (Figure 2a). CYP356A1 may be classified as an invertebrate CYP Clan 2 (*CYP17*-like) gene, sharing 32-36% amino acid identity (masking out regions of alignment uncertainty) with vertebrate CYP17s. In contrast, CYP356A1 shares a lower percentage identity (30-33%) with CYP1 and CYP2 genes. The CYP17 family is associated with

steroid metabolism, while CYP1A is classically used as biomarker of exposure to polycyclic aromatic hydrocarbons (Hahn, 2002). No *CYP17* genes have been found in non-chordate invertebrates, although *CYP17* is present in amphioxus (Mizuta and Kubokawa 2007), and *CYP17*-like genes were identified in the genome of the purple sea urchin, *Strongylocentrotus purpuratus* (Goldstone et al., 2006). Similarly, *CYP1* and *CYP1*-like genes have been detected in tunicates (CYP1E and CYP1F subfamilies; Goldstone et al submitted) and sea urchins (Goldstone et al., 2006 and submitted), but no *CYP1* sequences have been found in non-deuterostome invertebrates.

RT-PCR results showed that higher expression of *CYP356A1* was observed in digestive gland and mantle when compared to gill and adductor muscle (Figure 2b). The digestive gland and the mantle are important tissues for both biotransformation and steroid metabolism. Interestingly, previous studies (Medeiros et al., submitted) showed a 1.9-fold increase in the *CYP356A1* expression in oysters exposed to untreated domestic sewage which might be associated to increased xenobiotic biotransformation and/or to the presence of endocrine disruptors, given the high similarity of this gene to *CYP1* and *CYP17* genes. Endocrine disruptors are commonly found in domestic sewage (Quinn et al., 2004), and during biotransformation can interfere in the steroid metabolism. The characterization of the *CYP356A1* gene in *C. gigas* suggests the possibility of its use as a biomarker of exposure to domestic sewage in oysters. Further studies include expression, purification, and characterization of the CYP356A1 protein, analysis of *CYP356A1* gene expression after exposure to endocrine disruptors in the laboratory, and field studies of oysters in sewage contaminated sites.

Acknowledgements

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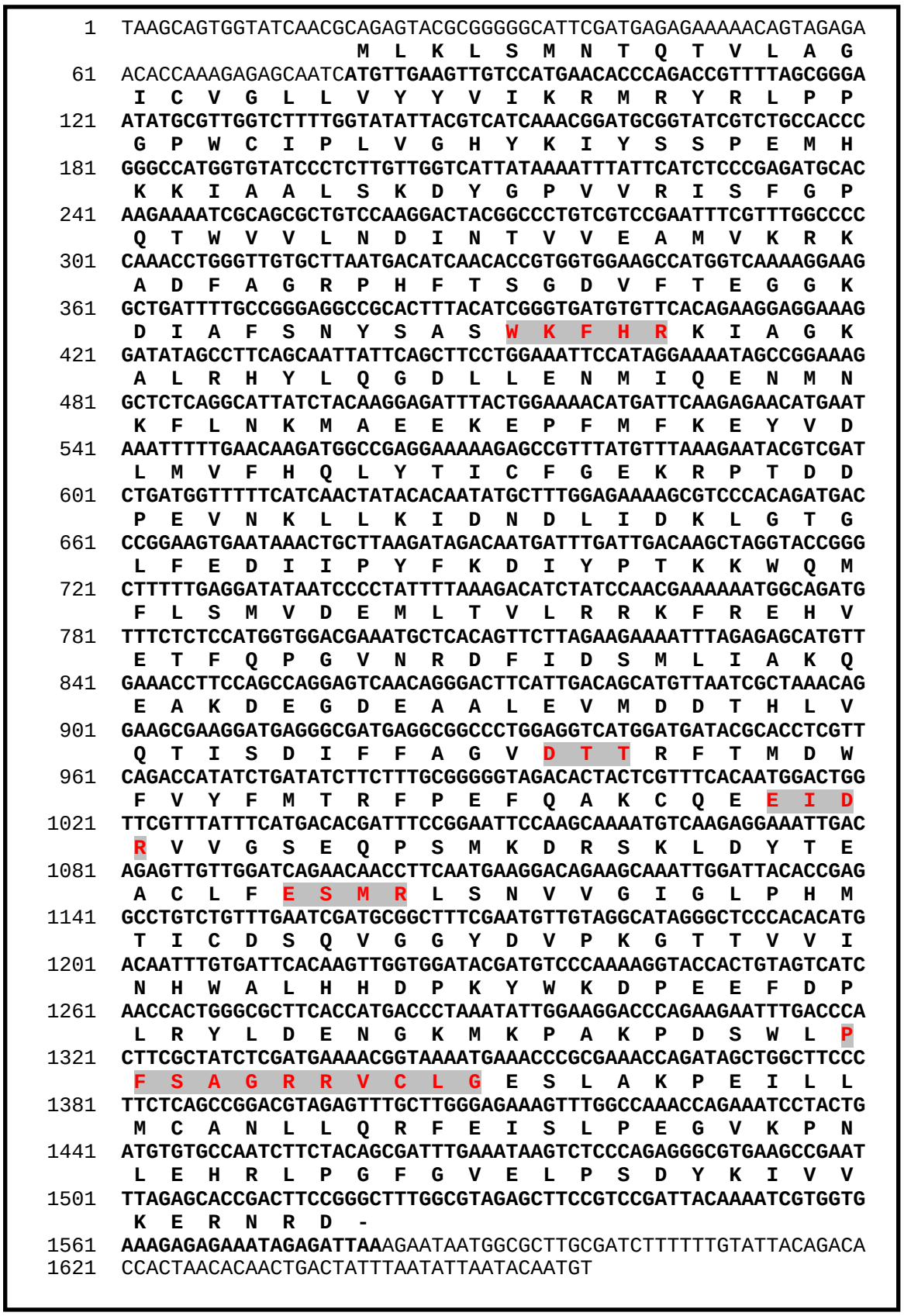
Legend of the Figures

Figure 1. Nucleotide and deduced amino acid sequences of CYP356A1 from *Crassostrea rhizophorae.* Some motifs of CYP signatures are indicated, including the C, K, and I helices, and the heme-binding region.

Figure 2a. Phylogenetic relationships using Bayesian techniques. Phylogenetic relationships were estimated with uninformative prior probabilities using the WAG model of amino acid substitution and prior uniform gamma distributions approximated with four categories (WAG+I+G). Four incrementally heated, randomly seeded Markov chains were run for $3x10^6$ generations, and topologies were sampled every 100th generation. Burnin values were conservatively set at $1x10^6$ generations.

Figure 2b. Semi-quantitative RT-PCR and densitometry of CYP356A1 in different tissues of oyster *Crassostrea gigas.* Pool of 5 individuals were used for the analysis in each tissue Legend: G, gill; DG, digestive gland; MT, mantle; M, muscle.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M L K L S M N T Q T V L A G
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I C V G L L V Y Y V I K R M R Y R L P P
121 ATATGCGTTGGTCTTTTGGTATATTACGTCATCAAACGGATGCGGTATCGTCTGCCACCC
G P W C I P L V G H Y K I Y S S P E M H
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K K I A A L S K D Y G P V V R I S F G P
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D I A F S N Y S A S W K F H R K I A G K
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P E V N K L L K I D N D L I D K L G T G
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L F E D I I P Y F K D I Y P T K K W Q M
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Q T I S D I F F A G V D T T R F T M D W
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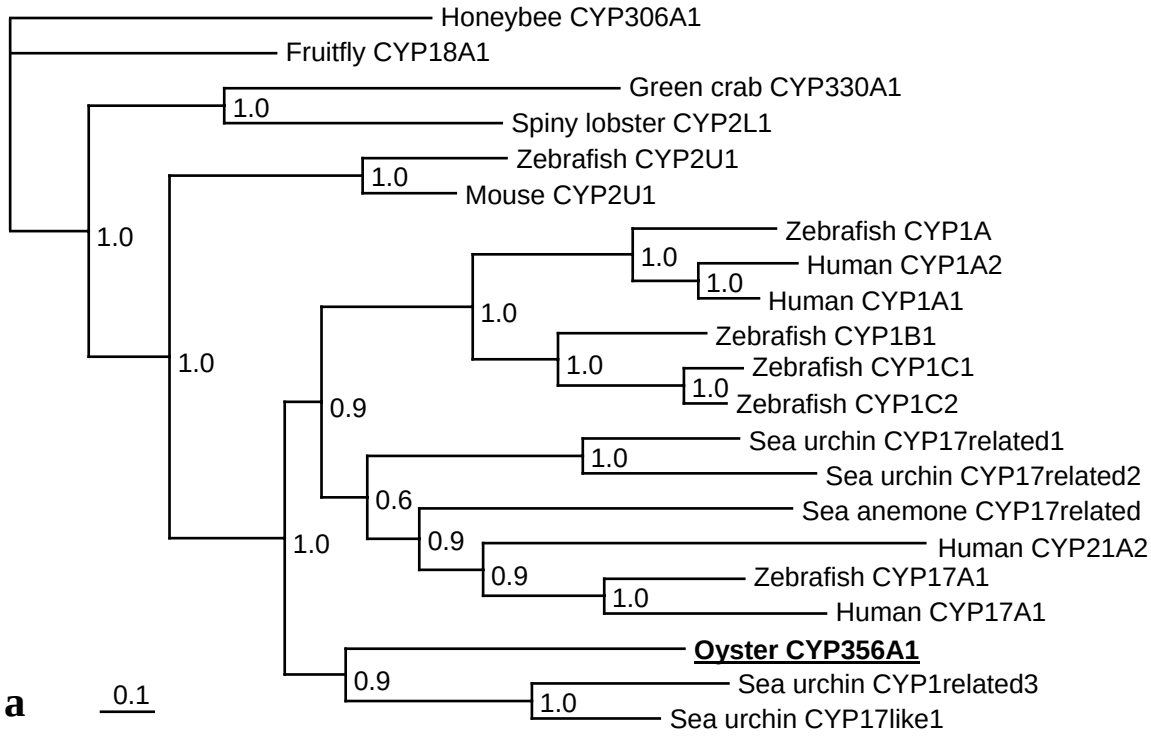
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Green crab CYP330A1
Spiny lobster CYP2L1
Zebrafish CYP2U1
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Human CYP1A1
Zebrafish CYP1B1
Zebrafish CYP1C1
Zebrafish CYP1C2
Sea urchin CYP17related1
Sea urchin CYP17related2
Sea anemone CYP17related
Human CYP21A2
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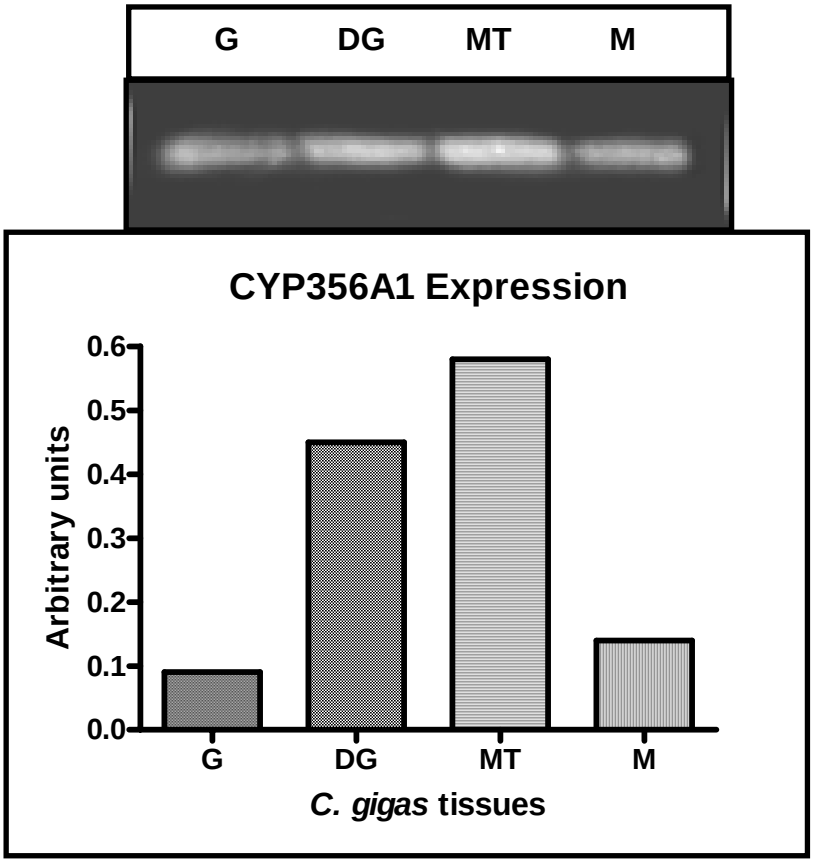
a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M
CYP356A1 Expression
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G
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MT
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C. gigas tissues

b