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P-glycoprotein and its inducible expression in three bivalve species after exposure to *Prorocentrum lima*

Lu Huang[a,b], Su-Li Liu[a], Jian-Wei Zheng[a], Hong-Ye Li[a], Jie-Sheng Liu[a], Wei-Dong Yang[a,*]

[a] College of Life Science and Technology, Key Laboratory of Aquatic Eutrophication and Control of Harmful Algal Blooms of Guangdong Higher Education Institute, Jinan University, Guangzhou 510632, China
[b] Department of Pathology, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China

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ABSTRACT

P-glycoprotein (*P*-gp or ABCB1) belongs to the family of ATP-binding cassette (ABC) transporters responsible for multixenobiotic resistance (MXR) in aquatic organisms. To provide more information of *P*-gp in shellfish, in this study, complete cDNA of *P*-gp in three bivalve species including *Ruditapes philippinarum*, *Scapharca subcrenata* and *Tegillarca granosa* were cloned and its expressions in gill, digestive gland, adductor muscle and mantle of the three bivalves were detected after exposure to *Prorocentrum lima*, a toxogenic dinoflagellate. The complete sequences of *R. philippinarum*, *S. subcrenata* and *T. granosa* *P*-gp showed high homology with MDR/*P*-gp/ABCB proteins from other species, having a typical sequence organization as full transporters from the ABCB family. Phylogenetic analyses revealed that the amino acid sequences of *P*-gp from *S.* subcrenata and *T. granosa* had a closest relationship, forming an independent branch, then grouping into the other branch with *Mytilus californianus*, *Mytilus galloprovincialis* and *Crassostrea gigas.* However, *P*-gp sequences from *R. philippinarum* were more similar to the homologs from the more distantly related *Aplysia californica* than to homologs from *S. subcrenata* and *T. granosa*, suggesting that bivalves *P*-gp might have different paralogs. *P*-glycoprotein expressed in all detected tissues but there were large differences between them. After exposure to *P. lima*, the expression of *P*-gp changed in the four tissues in varying degrees within the same species and between different species, but the changes in mRNA and protein level were not always synchronous.

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1. Introduction

Aquatic organisms are constantly exposed to a multitude of natural and anthropogenic toxicants dissolved in the sea water. These contaminants can be accumulated in tissues of marine animals by ingestion, water exchange, and absorption by body surfaces, causing a variety of health problems (Pinot et al., 2000; Gerssen et al., 2010). However, marine organisms possess some defense systems against the detrimental effect induced by these external substances (Eufemia and Epel, 2000; Minier et al., 2002; Achard et al., 2004; Luckenbach and Epel, 2008). It has been shown that multixenobiotic resistance (MXR) confers an effective protection against deleterious effects caused by various toxic compounds, representing a 'first line of defense' against xenobiotics (Epel, 1998). Several studies have demonstrated that some species of bivalves possess MXR mechanisms similar to the multidrug resistance (MDR) phenomenon in tumor cells (Lüdeking and Köhler, 2002; Luckenbach and Epel, 2008; Huang et al., 2014). It is proposed that level of MXR activity might be, at least partly, responsible for the resistance of a particular molluscs species to organic pollutions and phycotoxins (Smital et al., 2000).

MXR defense in bivalves is mediated by at least three ABC subfamilies including *P*-glycoprotein (*P*-gp) or ABCB family, the multidrug resistance protein (MRP) or ABCC family, and the breast cancer resistance protein (BCRP) transporter (Epel et al., 2008; Luckenbach and Epel, 2008). It has been shown that *P*-gp is an important constituent of cellular protection arsenal, restricting the uptake of xenobiotic substrates in bivalves (Luckenbach and Epel, 2008). Smital et al. (2000) found that there was a different, species-specific, inherent level of the basal MXR activity in several marine and freshwater bivalves. Navarro et al. (2012) reported that ABCB1/ABCC1 transporters were expressed and active in larvae and adult stages of zebra mussel but regulation differed in larvae and tissue of adult stages after exposure to dacthal and mercury. These data indicated that the regulation of *P*-gp expression might be species- and tissue-specific in bivalves, and that *P*-gp might

* Corresponding author. Fax: +86 020 85225183.
E-mail addresses: tywd@jnu.edu.cn, jnuywd@163.com (W.-D. Yang).

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play a major role in the variations in xenobiotics responses like humans (Sharom, 2008), however, very few papers have concerned the interspecies difference in pollutant response of *P*-gp in bivalves due to the genetic insufficiency of *P*-gp.

Ruditapes philippinarum, the Manila clam, is an edible species of saltwater clams in the family Veneridae, which is becoming one of the most exploited bivalves in the world (Lassudrie et al., 2014). The ark shell, *Scapharca subcrenata*, is a suspension feeding bivalve that inhabits the muddy sediments of the shallow coasts of Korea, Japan and China (Chen et al., 2009; Nakamura, 2005). The Malaysian cockle *Tegillarca granosa* (also known as *Anadara granosa*) is an intertidal infauna bivalve belonging to the family Arcidae, which is known as the blood cockle due to the red haemoglobin liquid inside (Lee et al., 2012). The three species of bivalves are all important economically bivalves, widely distributed in the Indian and Pacific Oceans. Some papers have reported that the three bivalves can accumulate phycotoxins in their tissues (Wang and Wu, 2006).

Prorocentrum lima is a toxic benthic, epiphytic, photosynthetic dinoflagellate usually found attached to or associated with macrophytes, floating detritus, debris, or other substrates and, less commonly, found in plankton (Steindinger, 1983). As a known producer of diarrheic shellfish poisoning (DSP) toxins, it has been associated to DSP episodes in different parts of the world, and has been extensively used in aquatic toxicological studies (Carvalho Pinto-Silva et al., 2005; Flórez-Barrós et al., 2011; de Jesús Romero-Geraldo et al., 2014). The DSP toxins are lipophilic polyether compounds, including okadaic acid (OA), dinophysistoxin-1 (DTX1), DTX2 and other derivative forms (Gerssen et al., 2010). These toxins can be ingested and accumulated by bivalve mollusks, causing DSP in humans through food chain (Yasumoto and Murata, 1993). However, bivalves can survive and grow well during the periods of DSP producing dinoflagellate blooms, which demonstrating that they may have some mechanisms to protect them from harmful effects of OA both in field and laboratory areas. Interestingly, it has been suggested ABC transporters including *P*-gp might be associated with OA defense in bivalves (Svensson et al., 2003; Huang et al., 2014; Xu et al., 2014). *P. lima* exposure could induce expression of *P*-gp mRNA in gills of bivalves (Huang et al., 2014; Xu et al., 2014). However, the addition of *P*-gp-specific inhibitors had no effect on the OA accumulation in gills of *Perna viridis*, but CsA, a broad-spectrum inhibitor of ABC transporters increased OA accumulation in gills of *P. viridis* (Huang et al., 2014). These suggested that there might be a potential compensatory mechanism in *P*-gp and ABC transporters like MRP mediated resistance to DSP toxins, or *P*-gp might play a limited role in efflux of OA. In addition, it is of note that various bivalve species exhibit different responses to the algal toxins as many papers reported (McCarthy et al., 2014), so it is imperative to learn the expression responses of *P*-gp in different species of bivalves against DSP toxins.

Here, to provide more information on the character of *P*-gp and its response to DSP toxins in different bivalves, complete cDNA sequences of *P*-gp gene in these three bivalves were cloned by RACE. Additionally, the expressions of *P*-gp in gill, digestive gland, mantle and adductor muscle were detected after exposure to *P. lima*.

2. Materials and methods

2.1. Experimental organisms

Three species of bivalves including *R. philippinarum*, *S. subcrenata* and *T. granosa* were purchased from Huangsha Seafood Market in Guangzhou, China. They were all farmed along the coast of Zhanjiang, Guangdong Province in China. After collection, the bivalves were scrubbed clean to remove sediment and epibiont, and immediately transferred to tanks (300 mm × 450 mm × 300 mm) filled with aerated filtered natural seawater at 20 ± 1 °C, which was renewed at a regular time every day. The bivalves were fed with *Platymonas subcordiformis* (1×10^7 cell L^{-1}) for a week period of acclimation (Murray et al., 2009; de Jesús Romero-Geraldo et al., 2014). After that, each species of bivalves were randomly divided into two groups, half of them were fed with cultures of *P. lima* (1×10^6 cell L^{-1}) and *P. subcordiformis* (1×10^7 cell L^{-1}), and the others were fed with *P. subcordiformis* (1×10^7 cell L^{-1}) as controls. After exposure to *P. lima* for 24 h, gills, digestive glands, mantles and adductor muscles were dissected from 18 individuals of each bivalve species in each treatment. Subsequently, tissues from 6 individuals within the same treatment were pooled together as one sample. Accordingly, three samples in each treatment of each bivalve species were obtained for quantitative real-time PCR (qRT-PCR) and protein analyses.

2.2. Cloning of P-glycoprotein cDNA

Total RNA was extracted from approximate 100 mg of gill tissue using a TRIzol reagent (TaKaRa, China), and retro-transcribed to cDNA using an AMV First Strand cDNA Synthesis Kit (TaKaRa, China).

To obtain full lengths of *P*-gp cDNA from *R. philippinarum*, *S. subcrenata* and *T. granosa*, rapid amplification of cDNA ends (RACE) were used. Prior to RACE, initial cDNA fragments of *P*-gp cDNA were obtained from *R. philippinarum*, *S. subcrenata* and *T. granosa* using degenerate or specific primers listed in Table 1, which were designed based on the conserved amino acid sequence of *P*-gp from different bivalve species available in NCBI database including *R. philippinarum* (FJ612109), *Mytilus edulis* (AF159717.1), *Mytilus galloprovincialis* (EF057747.1) and *Crassostrea gigas* (AJ422120). Using these primers, PCR products with lengths of 375- (CTF406), 350- (CTF408) and 178-bp (CTF410) were generated in *R. philippinarum*, *S. subcrenata* and *T. granosa*, respectively. PCR reactions were conducted as follows: 94 °C for 3 min, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 72 °C for 10 min. The PCR products were sequenced, and homology of these sequences with *P*-gp/MDR/ABCB cDNAs from other organisms was confirmed with NCBI blastn.

Based on the partial sequences obtained above, 3′-Full RACE and 5′-Full RACE were performed to generate full-length cDNA sequence of *P*-gp using a 3′-Full RACE Core Set Ver.2.0 and a 5′-Full RACE Kit (TaKaRa, China) according to the manufacturer's specifications. Related primers for 3′-RACE and 5′-RACE are listed in Table 1. All PCR amplifications were performed using a TaKaRa LA Taq® with GC Buffer Kit according to manufacturer's protocol.

In *R. philippinarum*, based on the CTF406 PCR sequence, primers 5′-ATG ATC CCG AGG AAG GTG TTG T-3′ and 5′-ATT GGA ATY GTC TCR CAR GAA C-3′ were designed for 3′-RACE, and a PCR product (CTF406-P1, ~0.9 kb) was obtained. The cycling conditions were as follows: 94 °C for 3 min; 30 cycles of 94 °C (30 s), 55 °C (30 s) and 72 °C (2 min); 72 °C for 10 min. As for 5′-RACE, total RNA was treated with CIAP and TAP, then ligated with 5′-RACE adaptor using T4 RNA ligase. Based on the primers 5′-TAC TTT TCT CGT ATT GTC ACC GT-3′ /5′-ACG GGT TCY TGY GAG ACR ATT CC-3′, PCR reactions were conducted as follows: 94 °C for 1 min; 30 cycles of 98 °C (10 s), 60 °C (15 s) and 68 °C (2 min), and a new PCR product (CTF407 P1, ~3.6 kp) was generated. Same procedures were performed for *S. subcrenata* and *T. granosa* with primers listed in Table 1. In some cases, the second 3′-RACE was carried out. Except for some products that could be directly sequenced, most fragments were purified using a TaKaRa Agarose Fragment Purification Kit Ser. 2.0 (TaKaRa, China), then cloned into the vectors pGEM20-T (TaKaRa, China) according to the manufacturer's instructions, and sequenced.

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