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Key metabolic pathways involved in xenobiotic biotransformation and stress responses revealed by transcriptomics of the mangrove oyster *Crassostrea brasiliana*

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ABSTRACT

The Brazilian oyster *Crassostrea brasiliana* was challenged to three common environmental contaminants: phenanthrene, diesel fuel water-accommodated fraction (WAF) and domestic sewage. Total RNA was extracted from the gill and digestive gland, and cDNA libraries were sequenced using the 454 FLX platform. The assembled transcriptome resulted in ~20,000 contigs, which were annotated to produce the first *de novo* transcriptome for *C. brasiliana*. Sequences were screened to identify genes potentially involved in the biotransformation of xenobiotics and associated antioxidant defence mechanisms. These gene families included those of the cytochrome P450 (CYP450), 70kDa heat shock, antioxidants, such as glutathione *S*-transferase, superoxide dismutase, catalase and also multi-drug resistance proteins. Analysis showed that the massive expansion of the CYP450 and HSP70 family due to gene duplication identified in the *Crassostrea gigas* genome also occurred in *C. brasiliana*, suggesting these processes form the base of the *Crassostrea* lineage. Preliminary expression analyses revealed several candidates biomarker genes that were up-regulated during each of the three treatments, suggesting the potential for environmental monitoring.

1. Introduction

Mangrove oysters, *Crassostrea brasiliana* (sin. *Crassostrea gasar*, Lazoski et al., 2011), are common species along the Brazilian coast, where they are both economically and ecologically important. These sessile, filter feeders are known to accumulate water contaminants in their tissues and are therefore ideal bioindicator species for pollution monitoring in coastal waters (Lüchmann et al., 2011; Lüchmann et al., 2011). However, along with other Ostreidae species, little is known regarding the specific genomic and transcriptomic adaptations to these contaminants. The monitoring endpoints are based on a small number of biomarkers with origins in human toxicology, and are therefore not bivalve-specific (Forbes et al., 2006). In excess of the ecological and economical importance of oysters to the coastal areas and the aquaculture industry, understanding their biology, susceptibility to pollutants and differential stress resistance has become an important issue for modern

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ecotoxicology. In particular, genomic resources such as genome or transcriptome sequences would greatly facilitate studies into the cellular mechanisms under-pinning biological responses in this species and enable the development of molecular markers for bioaccumulative pollution monitoring.

Due to their economic importance, oysters have been the subject of several large-scale expressed sequence tags (EST) projects (Fleury et al., 2009; Joubert et al., 2010; Tanguy et al., 2008; Wang and Guo, 2007). Indeed whole genome or transcriptome sequencing has proved a very efficient and cost effective method for expanding the sequence database for bivalves and other non-model species (*i.e.* Clark et al., 2010; Craft et al., 2010; Hou et al., 2011; Joubert et al., 2010; Meyer et al., 2009), and so was applied here to the transcriptome of the mangrove oyster *C. brasiliana.* In the last few years, several oyster genomes have become available, including the Pacific oyster *Crassostrea gigas* (Zhang et al., 2012a) and Pearl oyster *Pinctada fucata* (Takeuchi et al., 2012), significantly enriching the genomic resources for this animal model.

Here we aimed to sequence the transcriptome of the mangrove oyster *C. brasiliana* to both improve the genomic resources for this species, and to explore gene transcription for biotransformation of xenobiotics, antioxidant and stress response during exposure to three different environmental contaminants: phenanthrene (PHE), diesel fuel water-accommodated fraction (diesel WAF) and domestic sewage. All three contaminants are key chemical models for ecotoxicological studies. Phenanthrene, a 3-ring compound included in the US-EPA priority pollutant list, is one of the most abundant aquatic PAH(polycyclic aromatic hydrocarbon), as a result of human activities (US EPA, 2009). It is lipophilic and has a low molecular weight, making it easily taken-up by aquatic organisms (Oliveira et al., 2007), with a greater bioaccumulation rate in bivalve molluscs (Hannam et al., 2010; Lüchmann et al., 2014). In contrast, diesel WAF comprises a model for complex mixtures derived from petroleum industry activities. Diesel fuel is one of the most common aquatic contaminants, and has recently been shown to exert biochemical effects and bioaccumulation trends in *C. brasiliana* (Lüchmann et al., 2011). Domestic sewage was chosen based on the high inputs of untreated sewage discharges in coastal ecosystems around the world and its potential effects on transcriptional levels of oysters (Medeiros et al., 2008).

C. brasiliana was challenged to each contaminant separately, total RNA was extracted from the gill and digestive gland with the resulting cDNA libraries sequenced using the 454 FLX platform. The sequence data was assembled into a reference transcriptome, which was then screened to identify genes potentially involved in the biotransformation of xenobiotics and associated antioxidant defence and stress mechanisms. The results demonstrated differences between the responses to the different toxicants, with promising relevance for ecotoxicology studies and aquatic monitoring programs.

2. Material and methods

2.1. Oyster collection and chemical exposures

Mangrove oysters (*C. brasiliana*) of similar shell length (5–8 cm) were collected from an oyster farm at Sambaqui beach (Marine Mollusks Laboratory, UFSC) in Florianópolis, southern Brazil. This criterion was strictly adhered to and therefore limited availability of oysters throughout the course of the study meant that certain experiments were unfortunately run without replication. After collection, the animals were covered with wet towels and immediately transported in coolers by road approximately 20 km to the laboratory. In the lab, the oysters were transferred into 50 L aquaria containing 0.45 μm-filtered, aerated seawater, at 21 °C, and salinity 25. Oysters were fed twice a day on microalgae (*Chaetoceros muelleri* and *Isochrysis* sp.) at a density of 3.3×10^6 cels mL^{-1} and 2.2×10^6 cels mL^{-1}, respectively, and water was changed daily for one week prior to experiments. Oyster were then randomly divided into the glass exposure tanks (1 animal per 1 L of seawater) and held (without feeding) for 24 h prior to the exposures. During the exposure periods, control and exposed organisms were not fed to prevent potential bioaccumulation of chemicals by food.

There were 4 exposure experiments: diesel WAF for 24 h, diesel WAF for 72 h, PHE for 24 h and sewage for 24 h, which were carried out in different occasions but the oysters were supplied from the same brood stock of the mollusc farm, and were submitted to the same acclimatization process as described above. For each set of experiment, there was a control group where a separate set of oysters was kept under control conditions in normal seawater, with the exception of the PHE exposure control group, where the seawater also included 0.01% DMSO, as this was the solvent used to dissolve the PHE (please see details of the exposure condition below). The diesel WAF exposure was carried out in duplicate, and PHE and sewage exposures were performed without replication.

Diesel fuel was purchased at a PETROBRAS petrol station and WAF was obtained according to Singer et al. (2000) with minor modifications. Briefly, one part (1 L) of fresh diesel fuel was diluted with nine parts (9 L) of 0.45 μm-filtered seawater (salinity 25) in a sealed 14 L glass flask which was protected from light, in order to minimize evaporation and degradation of the fuel components. The diesel-water mixture was stirred for 23 h with the homogenizer Glas-Col (LLC) using a steel modified pestle at 1600 rpm at a constant temperature of 21 °C. The mixture was then allowed to settle for 1 h before the lower layer of water (diesel WAF) was transferred into the glass tanks. The 10% diesel WAF was prepared through dilution of the WAF with the control seawater. The diesel WAF concentration was chosen based on previous results of biochemical biomarkers measured in *C. brasiliana* (Lüchmann et al., 2011). No mortality was observed in the control and treated groups. The levels of individual and total PAHs bioaccumulated after 24 h are summarized in the Supplementary Table S1.

Phenanthrene (PHE) (Sigma–Aldrich, P1, 140-9) was first dissolved in dimethyl sulfoxide (DMSO), and then added to 0.45 μm-filtered seawater (salinity 25) to achieve final nominal PHE concentration of 1000 $\mu g.L^{-1}$ (equivalent to 5.6 μM), and a final maximum DMSO concentration of 0.01% (v/v). The concentration of PHE added to the test media was chosen based on previous reports carried out with bivalves (*i.e.* Hannam et al., 2010; Lüchmann et al., 2011; 2014). Oysters were then randomly divided into the glass exposure tanks, which were individually aerated and covered with glass and sealed to avoid evaporation of PHE, and held (without feeding) for 24 h prior to the exposure. The control oysters were subjected to the same conditions as the exposed groups, except for the addition of 0.01% (v/v) DMSO only without PHE. No mortality was observed in the control and treated groups.

Sewage exposure was performed according to Medeiros et al. (2008) with minor modifications. Briefly, domestic sewage was collected at the influent duct of the downtown wastewater treatment plant (Florianópolis, southern Brazil) after solid material grid removal, and diluted to 33% (v/v) using 0.45 μm-filtered seawater (salinity 25). Oysters were placed in the exposure glass tanks which were individually aerated using glass Pasteur pipettes and were covered with glass. No mortality was observed in the control and treated groups.

After chemical exposures, twelve oysters from each of the diesel fuel WAF and PHE experiments, and seven from the domestic sewage experiment were sacrificed and the gill and digestive gland were immediately excised, flash frozen in liquid nitrogen and individually stored at −80 °C until further analysis. Three oysters from the control groups of each treatment were pooled, totalizing 12 ani-