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Expression of candidate genes related to metabolism, immunity and cellular stress during massive mortality in the American oyster *Crassostrea virginica* larvae in relation to biochemical and physiological parameters

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ABSTRACT

Quantification of mRNA of genes related to metabolism, immunity and cellular stress was examined in relation to a massive mortality event during the culture of American oyster larvae, *Crassostrea virginica* which was probably, in regard to previous microbiological analysis, induced by *Vibrio* infection. To document molecular changes associated with the mortality event, mRNA levels were compared to biochemical and physiological data, previously described in a companion paper. Among the 18 genes studied, comparatively to the antibiotic control, 10 showed a lower relative gene expression when the massive mortality occurred. Six of them are presumed to be related to metabolism, corroborating the metabolic depression associated with the mortality event suggested by biochemical and physiological analyses. Relationships between the regulation of antioxidant enzyme activities, lipid peroxidation, and the mRNA abundance of genes linked to oxidative stress, cytoprotection, and immune response are also discussed. Finally, we observed an increase in the transcript abundance of two genes involved in apoptosis and cell regulation simultaneously with mortality, suggesting that these processes might be linked.

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1. Introduction

Massive mortality of bivalve larvae has often been associated with a low quality of water or of microalgae provided as food for aquaculture production, promoting the proliferation of opportunistic pathogenic microorganisms (Olafsen, 2001; Paillard et al., 2004). Many studies have been dedicated to the identification and characterization of pathogenic microorganisms to such mortalities but, to our knowledge, few physiological or molecular studies have yet been carried out to study larvae during such mortality events. This is indeed related to the technical difficulties to study such rapid phenomenon on very small organisms. In addition to biochemical and physiological approach (Tikunov et al., 2010), recently developed genomic tools can now be used to better document larval responses. In the last decade, cDNA collections and expressed sequence tags (EST) have been developed for several bivalve species, and they account for the large number of RNA sequences available in public databases (Collin et al., 2010; Fleury et al., 2009; Jenny et al., 2007; Quilang et al., 2007; Sussarellu et al., 2010; Tanguy et al., 2008; Zapata et al., 2009). Such an increasing number of EST databases provide the opportunity to characterize bivalve mortality events and infections (Fleury et al., 2009; Huvet et al., 2004; Samain et al., 2007; Travers et al., 2010) In the American oyster, EST collections are being used to identify genes with putative functions in the cellular and biochemical processes implicated in the resistance to *Perkinsus marinus* (Tanguy et al., 2004; Wang et al., 2010).

To date, the majority of genomic approaches have been carried out on adult bivalves and little is known about gene regulation at larval stage. Jenny et al. (2002) produced two EST collections from embryos and hemocytes of the American oyster to identify genes that are potentially related to immune and stress responses. More recently, Tirape et al. (2007) studied the expression of immune-related genes during ontogeny and bacterial challenge in *Crassostrea gigas*.

Abbreviations: mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid; EST, expressed sequence tags; MDH, malate dehydrogenase; NADH-1, nicotinamide adenine dinucleotide dehydrogenase subunit 1; CC-1, cytochrome C1; AS-6, adenosine triphosphate synthase f0 subunit6; EDL, endothelial lipase; D9-des, delta 9 desaturase; GS, glutamine syntethase; elF-2B, translation initiator factor; CTB, cathepsin B; ANX, annexin; NK-rec, natural killer receptor; KC-rec, killer cell lectin-like receptor; MYC-h, MYC-homolog; SUP, RAS suppressor; PRDX6, peroxiredoxin 6; HSP70, heat shock protein 70; PRN, pernin; CP450, cytochrome P450; RT-PCR, reverse transcriptase polymerase chain reaction; SOD, superoxide dismutase; GPX, glutathione peroxidase; ROS, reactive oxygen species.

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In this context, we selected 18 candidate genes, presumed to be involved in metabolism (energy production, protein synthesis, and lipid remodeling), immunity (pathogen recognition, proteinase, apoptosis, and inflammatory response) and cellular stress (antioxidant defenses, xenobiotic detoxification and cytoprotection). Relative expression of these 18 genes was studied by quantification of mRNA using real time PCR, during a mortality event in larvae of the American oyster *Crassostrea virginica* and compared with an antibiotic-treated control that showed no significant mortality. Results are discussed in the light of biochemical and physiological data reported in a companion paper (Genard et al., 2011).

2. Materials and methods

2.1. Experimental design and physiological characterization of a bacterial mortality event

Details about the rearing procedure and experimental design are presented in Genard et al. (2011). Briefly, this work was done at the hatchery of the Coastal Zone Research Institute (CRZI, Shippagan, New Brunswick, Canada) during winter of 2006. After fertilization, larvae were reared in a 415 L Xactic tank in filtered seawater. Larvae were fed with a mixture of three microalga species *Isochrysis sp.* (T-ISO), *Pavlova lutheri* (MONO), and *Chaetoceros muelleri* (CHGRA). Two days after fertilization, D-larvae were collected, enumerated and split equally into two experimental treatments. Larvae were cultivated in triplicate with or without antibiotic (chloramphenicol at 4 mg L^{-1}; Fluka, Mississauga, ON). Larval samples (100,000 larvae per sample) were collected at 6, 13, and 20 dpf (days post fertilization).

Mortality event occurred 20 days after fertilization in untreated tanks (see Genard et al., 2011 for further details). Mortality was associated with (1) strong changes in the bacterial community structure, (2) a progressive decrease in feeding activity, (3) higher levels of some lipid classes (free fatty acids, diglycerides, and acetone mobile phospholipids), (4) lower levels of phospholipids and protein, (5) higher contents of non-methylene interrupted dienoic fatty acids (22:2 NMI), (6) a decrease in energy metabolism activity (citrate synthase and cytochrome oxidase activities), (7) a higher oxidative stress (lipid peroxidation level), and (8) an activation of antioxidant defenses before mortality (glutathione peroxidase and superoxide dismutase).

2.2. Real-time PCR

Total RNA was extracted for each replicate (between 2 and 3 depending on larval availability) using TRIzol™ Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was resuspended in RNase-free water and concentrations were determined by OD260 measurements. Ten micrograms of total RNA extracted from each developmental stage was reverse transcribed using the oligo(dT) anchor primer and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). Real-time PCR was performed with 5 μL cDNA (1/20 dilution) in a total volume of 25 μL, using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The concentrations of the reaction components were 1× Absolute QPCR SYBR Green ROX Mix (ABgene, Epsom, UK) and 70 nM of each primer. Reactions began with the activation of Thermo-Start DNA polymerase at 95 °C for 15 min followed by amplification of the target cDNA (50 cycles of denaturation at 95 °C for 30 s, annealing and extension at 60 °C for 1 min), and melting curve analysis was performed with continuous fluorescence acquisition (95 °C to 70 °C at a temperature transition rate of 0.5 °C every 10 s) to determine the amplification specificity. Each run included a negative control (non reverse-transcribed total RNA) and blank controls (water) for each primer pair.

The relative level of target gene expression was based on a comparative method (Livak and Schmittgen, 2001; Pfaffl, 2001). The threshold value (Ct) was determined for each target as the number of cycles at which the fluorescence curve entered exponential phase. The relative quantification value of a sample is expressed as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ (target sample) $- \Delta Ct$ (reference sample) and $\Delta Ct = Ct$ (target gene) $- Ct$ (housekeeping gene). Three genes coding for a ribosomal protein subunit were selected as putative housekeeping genes. All of these reference genes were amplified at all stages and in the two treatments. Ribosomal 18s was found to have the lowest variation during the experiment and was then chosen as the housekeeping gene for the rest of the analyses. Larvae treated with antibiotics from each sampling day were used as reference samples to determine treatment differences.

2.3. Studied genes

Oligonucleotide primer sequences used to amplify specific gene products are shown in Table 1. Selected genes could be classified into four groups: metabolism, immunity, cell regulation, and cellular stress. To monitor the putative metabolic changes induced by mortality emergence, we characterized expression of four genes involved in ATP synthesis (malate dehydrogenase (*MDH*), NADH dehydrogenase subunit 1 (*NDH-1*), cytochrome C1 (*CC-1*) and ATP synthase f0 subunit6 (*AS-6*)), two genes of lipids metabolism (endothelial lipase (*EDL*) and delta9 desaturase (*D9-des*)) and two of protein synthesis (glutamine synthetase (*GS*) and translation initiator factor elF-2B delta subunit (*elF-2b*)).

To investigate immune response and cell regulation, six genes (cathepsin B (*CTB*), annexin (*ANX*), natural killer receptor (*NK-rec*), killer cell lectin-like receptor (*KC-rec*), MYC-homolog (*MYC-h*) and RAS suppressor (*SUP*)) were selected from previous EST collections. Finally, cellular stress was investigated through expression of genes involved in antioxidant defense (peroxiredoxin 6 (*PRDX6*)), cytoprotection (heat shock protein 70 (*HSP70*) and pernin (*PRN*)) and xenobiotic detoxification (cytochrome P450 (*CP450*)).

2.4. Statistical analysis

Analyses were carried out using Software SAS® system (8.2). The significance value for all analyses was set at $p<0.05$. PROC GLM (one-way ANOVA) was used to compare relative gene expression between treatments at each sampling days and to show ontogenetic effects on each treatment. Where differences were detected, LSMEANS (*t*-test) multiple comparisons tests were used to determine which means were significantly different. Residuals were graphically assessed for normality using the PROC PLOT function coupled with univariate procedure (PROC UNIVARIATE). Homogeneity was tested using the O'brien test. When normality precept was not realized, LOG transformation was used. Linear regressions were carried out using Sigma plot 8.0 (SYSTAT Software Inc, USA). Data were presented with mean ± standard deviation.

3. Results

RT-PCR was performed with RNA isolated from larvae of 6 to 20 dpf; transcripts were detected in all larval stages. Transcript levels were compared between treatments at each sampling day (using the antibiotic treatment as a reference sample), which led us to classify the 18 genes into six groups of response (Fig. 1). In the first group, which included *MDH*, *CP450*, *D9-des*, and *NK-rec*, no significant differences were found between treatments. *NDH-1*, *CC1*, *AS-6*, and *EDL* constituted the second group and were characterized by a significantly lower gene expression in untreated larvae at 20 dpf, when massive mortality occurred. The third group included the two genes involved in protein synthesis (*GS* and *elF-2B*); it showed over-expression at 6

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