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Contrasted survival under field or controlled conditions displays associations between mRNA levels of candidate genes and response to OsHV-1 infection in the Pacific oyster *Crassostrea gigas*

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ABSTRACT

Pacific oyster *Crassostrea gigas* suffers from chronic or sporadic mortality outbreaks worldwide, resulting from infectious diseases and/or physiological disorders triggered by environmental factors. Since 2008, ostreid herpesvirus OsHV-1 μVar has been identified as the main agent responsible for mass mortality of juvenile oysters in Europe. Previous studies of genome-wide expression profiling have provided candidate genes that potentially contribute to genetically-based resistance to summer mortality. To assess their value in determining resistance to the juvenile mass mortality that has occurred in France since 2008, we analyzed the expression of 17 candidate genes in an experimental infection by OsHV-1 μVar, and in an *in vivo* field experiment. Individual quantification of mRNA levels of 10 out of the 17 targeted genes revealed significant variation, of which 7 genes were showed differences between conditions that created significant differences in mortality, and 6 depended on the number of OsHV-1 genome copies individually quantified in mantle tissue. Complex SOD metalloenzymes known to be part of the antioxidant defense strategies may at least partly determine susceptibility or resistance to OsHV-1-associated mortality. Furthermore, inhibitor 2 of NF-κB, termed *CgIκB2*, exhibited highly significant variation of mRNA levels depending on OsHV-1 load in both experiments, suggesting its implication in the antiviral immune response of *C. gigas*. Our results suggest that *CgIκB2* expression would make a good starting point for further functional research and that it could be used in marker-assisted selection.

1. Introduction

Mass mortalities of oysters have been reported for decades in many countries throughout the world but, in many cases, no precise pathological causal factors were identified (reviewed in Samain and McCombie, 2008). Sporadic outbreaks usually occur during the summer months, when oysters are undergoing sexual maturation, and result from a combination of the effects of pathogens, environmental factors and host physiological and genetic characteristics (Samain and McCombie, 2008). Indeed, taking advantage of the high heritability estimated for resistance to summer mortality (Boudry et al., 2008; Dégremont et al., 2007, 2010), lines of oysters selected in 2001 for resistance ("R") or susceptibility ("S") to summer mortality were examined by genome-wide expression profiling studies (Huvet et al., 2004; Fleury et al., 2010; Fleury and Huvet, 2012). Genes identified as differentially expressed between these two groups mainly fall into the categories: energy metabolism (carbohydrates, lipids), reproduction, response to stress and immune response. Besides the energetic requirements of reproduction, which can lead to a phase of energetic weakness for oysters, these studies highlighted antioxidant defense and signaling in innate immunity as constitutive pathways involved in resistance to summer mortality. Indeed, the kinetics of mRNA levels, especially for molecules regulating the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway, assayed under field conditions before a mortality peak, suggested that resistant oysters had the capacity to modulate signaling in innate immunity, whereas susceptible oysters did not (Fleury and Huvet, 2012). Additionally, a few of these differentially expressed candidate genes were also found to be in statistically

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significant co-location with the first Quantitative Trait Loci (QTL) identified for resistance to OsHV-1-associated summer mortality (Sauvage et al., 2010).

Since spring 2008, a new pattern of mass mortality has occurred of *Crassostrea gigas* of less than one year old along all coasts of France. These mortality episodes are different from the previous sporadic phenomenon by their higher intensity and more widespread geographic distribution (Jolivel and Fleury, 2012). Oysters analyzed during mortality outbreaks in 2008 showed an elevated prevalence of the ostreid herpesvirus OsHV-1 compared with previous years (Segarra et al., 2010). These authors characterized a genotype of OsHV-1 not previously reported, which was named OsHV-1 μVar. Official sample collection from the National Reference Laboratory in France revealed that the OsHV-1 μVar genotype has replaced the previously observed OsHV-1 as almost the only strain detected during oyster mortality events in France since 2008 (Renault et al., 2012). Experimental infection trials indeed confirmed the high pathogenicity of OsHV-1 μVar (Schikorski et al., 2011a, 2011b). Finally, a recent paper showed that the selection previously made for resistance to summer mortality, leading to the R and S lines presented above, still conferred a strong advantage to descendant batches in this new mortality context when survival of juvenile *C. gigas* was tested in 2009 (Dégremont, 2011). The highest survival reported for R batches (95% against 6% for the S) was associated with herpesvirus resistance in R oysters displayed by a low OsHV-1 prevalence in oyster tissues (Dégremont, 2011).

The main objective of the present study was to examine the expression profiles of the 17 previously-identified candidate genes in the novel context of mass mortalities of young oysters. The expression profiles of these candidate genes were evaluated in oysters (1) injected with OsHV-1 μVar in the laboratory, and (2) naturally exposed to the disease in the Thau lagoon, a farming area where mortalities are among the highest in France (Jolivel and Fleury, 2012). Variation of mRNA levels was explored by comparing expression between conditions that created a significant difference in mortality, and by looking at the relationship between the gene expression and the OsHV-1 load. The present study will help us to prioritize these candidates for further functional and genetic studies of resistance to summer mortality.

2. Material and methods

2.1. Experiments

2.1.1. Exp. 1

This trial aimed to infect healthy oysters with OsHV-1 through intramuscular injection of virulent inoculum obtained by filtering tissue homogenates from contaminated oysters (following Schikorski et al., 2011b). The control group consisted of oysters from the same experimental population but injected with non-virulent inoculum. Virulent and control non-virulent inocula were obtained by crushing the whole soft tissues of three oysters from two commercial stocks, one showing mortality in progress and the other not. Soft tissues were crushed on ice; homogenates were then diluted with sterile seawater (1 g of fresh tissues for 40 mL) and filtered through 0.1-μm syringe filters to prevent bacterial transmission.

The oysters to be challenged were triploids purchased from a commercial hatchery. These had been produced by a cross between diploid females and tetraploid males in summer 2008 and then cultured in the Aber Benoit (Finistère, France). They were transferred at 8 months old to the Ifremer experimental facilities in Plouzané (Finistère, France) in March 2009 for acclimation. The oysters were then anesthetized as described by Suquet et al. (2009) and injected (see Saulnier et al. (2010) for details of the injection protocol) with 100 μl inocula that were considered either virulent or non-virulent/very weakly virulent, corresponding to 2.10^9 and 4.10^6 copies of viral genome injected, respectively (quantified in the inocula by real-time PCR). After injection, oysters from challenged and control groups were divided into 2×3 batches of 45 individuals. Each batch was placed in a 20-L tank filled with 1-μm filtered seawater and maintained at 20 °C with aeration and no seawater renewal.

Two and six days after injection, 10 live oysters were collected in each tank (to constitute 10 biological replicates per condition; 40 samples in total), opened and the entire flesh of each animal immediately frozen in liquid nitrogen, crushed to a fine powder at −196 °C with a Dangoumau mill and stored in liquid nitrogen until DNA and RNA extractions were performed. The remaining oysters in each group continued to be reared under the same experimental conditions. Mortality was estimated by counting live and dead individuals daily. Dead oysters were removed from the tanks after counting.

2.1.2. Exp. 2

This experiment corresponds to "experiment 3" previously reported in Pernet et al. (2012) which initially was designed to test for rearing structures and site effects on the mortality of juvenile oysters. As for Exp. 1, this trial used triploid oysters, produced by diploid × tetraploid crosses in summer 2008, which were purchased from a commercial hatchery. Juveniles were reared in the open sea close to the Thau lagoon. At this time, rearing conditions allowed these oysters to be maintained without any mortality, so that they were healthy at the start of the experiment. On 27 May 2010, these individuals were transferred to two different locations inside the lagoon that showed extremely closed environmental conditions (reported for seawater temperature in Pernet et al., 2012) as only separated by a maximum of 200 and 50 m, and deployed in three different combinations of rearing structure and placement, which gave three different experimental conditions (for details of the procedure see Pernet et al., 2012). One part of the experimental stock was placed within the farming area of Marseillan, in baskets (condition 1) or cemented on ropes (condition 2), both of which are common rearing methods in the Thau lagoon. The remaining individuals were kept in baskets outside the farming area of Marseillan, but within the Thau lagoon (condition 3).

At 8 (4 June 2010), 11 (7 June) and 15 (11 June) days after deployment, mortalities were estimated by counting live and dead oysters, and nine live individuals per condition were randomly sampled that constitute 9 biological replicates per condition (81 samples in total). The valves were opened and discarded, and the soft tissues of each animal was immediately frozen in liquid nitrogen, crushed to a fine powder at −196 °C with a Dangoumau mill and stored in liquid nitrogen until DNA and RNA extractions.

2.2. Methods

2.2.1. Detection and quantification of OsHV-1

Total DNA was extracted from oyster tissues as described in Schikorski et al. (2011a, b) for real-time PCR assay analyses. The detection and quantification of OsHV-1 DNA were carried out in triplicate using real-time PCR according to Pepin et al. (2008), with specific primers that amplified both reference and μVar types. Briefly, absolute quantification of OsHV-1 DNA copies was carried out by comparing the Ct values obtained from tested samples with a standard curve based on a ten-fold dilution of a stock solution of OsHV-1 genomic DNA (5×10^6 copies/μL) extracted from purified virus particles. In Exp. 1, the results were expressed as virus DNA copy numbers per ng total DNA. Standardization of OsHV-1 genomic DNA quantity was reached using DNA concentrations measured using an ND-1000 spectrophotometer (Nanodrop Technologies) at 260 nm with the conversion factor of 1 OD = 50 μg/mL DNA. In Exp. 2, the quantity of OsHV-1 genomic DNA was expressed per mg of wet tissues. Standardization was different between the two experiments, and better adapted in Exp. 1 (standardization being more accurate and stable per ng of extracted DNA), which was published between analyses of Exp. 2 in 2010 and those of Exp. 1 in 2011 (Schikorski et al., 2011b).

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