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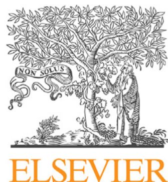

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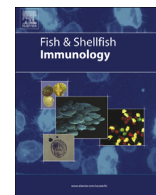

Transcriptome analysis reveals strong and complex antiviral response in a mollusc

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A B S T R A C T

Viruses are highly abundant in the oceans, and how filter-feeding molluscs without adaptive immunity defend themselves against viruses is not well understood. We studied the response of a mollusc *Crassostrea gigas* to Ostreid herpesvirus 1 μVar (OsHV-1μVar) infections using transcriptome sequencing. OsHV-1μVar can replicate extremely rapidly after challenge of *C. gigas* as evidenced by explosive viral transcription and DNA synthesis, which peaked at 24 and 48 h post-inoculation, respectively, accompanied by heavy oyster mortalities. At 120 h post-injection, however, viral gene transcription and DNA load, and oyster mortality, were greatly reduced indicating an end of active infections and effective control of viral replication in surviving oysters. Transcriptome analysis of the host revealed strong and complex responses involving the activation of all major innate immune pathways that are equipped with expanded and often novel receptors and adaptors. Novel Toll-like receptor (TLR) and MyD88-like genes lacking essential domains were highly up-regulated in the oyster, possibly interfering with TLR signal transduction. RIG-1/MDA5 receptors for viral RNA, interferon-regulatory factors, tissue necrosis factors and interleukin-17 were highly activated and likely central to the oyster's antiviral response. Genes related to anti-apoptosis, oxidation, RNA and protein destruction were also highly up-regulated, while genes related to anti-oxidation were down-regulated. The oxidative burst induced by the up-regulation of oxidases and severe down-regulation of anti-oxidant genes may be important for the destruction of viral components, but may also exacerbate oyster mortality. This study provides unprecedented insights into antiviral response in a mollusc. The mobilization and complex regulation of expanded innate immune-gene families highlights the oyster genome's adaptation to a virus-rich marine environment.

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1. Introduction

Marine environments are rich in viruses, which account for 94% of the nucleic-acid-containing particles in the ocean and are a major cause of disease and mortality of marine organisms [1]. Molluscs are a major group of animals in ocean and estuaries, and provide key functions in marine and coastal ecosystems [2]. Some molluscs, such as bivalves, are filter-feeders living in estuaries that are rich in potentially pathogenic microbes, which they concentrate as they feed [3]. They lack adaptive immunity and rely on an innate immune system for host defense [4]. How bivalves without adaptive immunity defend against viral infections is of fundamental interest. In the marine environment, studies on host response to viruses are mostly limited to finfish. Studies on molluscs are scarce and hindered by the lack of resources such as cell lines and host genome information. The recently sequenced genome of the Pacific oyster, *Crassostrea gigas* [5], which is a host of a disease-causing virus, Ostreid herpesvirus 1 or OsHV-1 provides an opportunity for studying genome-wide host-responses to viral infections in molluscs.

The first report of a viral infection in a marine mollusc was in 1972, when Farley et al. [6], using electron microscopy, observed a

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herpes-type viruse in eastern oysters, *C. virginica*, that were experiencing mortality in the elevated temperature of a power plant effluent. Subsequently, herpesviruses were identified in many bivalves around the world including oysters, scallops, clams and abalone, where it can cause serious mortalities [7–14]. In 2008, heavy mortalities, often reaching 90–100% within a few days, of spat (<1 year old) and juvenile (1–2 years old) Pacific oysters were reported in the oyster-growing regions of France, and associated with a newly emergent genotype of OsHV-1, termed OsHV-1μVar [15–17]. In China, massive mortality, typically 85–90%, of cultured scallops since 1995 has been associated with another variant of OsHV-1 [18,19]. The ability of herpesvirus to infect a wide range of molluscs and cause massive mortality raises the question: how does the innate immune system of molluscs, particularly oysters, respond to OsHV-1 and other viruses?

Our understanding of antiviral defense comes primarily from humans and other vertebrate systems, where the innate immune defense collaborates with the adaptive immune system to provide the host protection. Innate immune receptors or pattern recognition receptors (PRRs) recognize viral pathogen-associated molecular patterns (PAMPs) and activate complex signaling pathways leading to the production of pro-inflammatory cytokines and antiviral factors. Adaptive immune response employs virus-specific receptors, or antibodies, that provide immunological memory upon initial exposure and enhanced response in future encounters. While molluscs and other invertebrates do not have adaptive immunity, they do possess innate immune systems that appear to be complex. Many PRRs and other components of the innate immune system are conserved in molluscs, and some PRR gene families are even greatly expanded compared with vertebrates [20–22]. The expansion and diversification of PRRs may be important for molluscs, helping to cope with diverse pathogens in the absence of adaptive immunity. The oyster genome contains a rich set of genes related to apoptosis, cytokine activity and inflammatory response [5].

The oysters' response to OsHV-1μVar has been studied with suppression subtractive hybridization [23] and with qPCR analyses of selected genes [24–29]. Genome-wide responses have also been studied in OsHV-1μVar infected oysters collected from the field with microarray [30] or RNA-seq [31] analyses. However, gene expression in infected oysters from the field may be influenced by multiple factors. Genome-wide responses in time-course samples from controlled laboratory challenges have not been characterized, but are essential to our understanding of the dynamics of oyster's antiviral responses and their association with mortalities during the course of infection.

In this study, we challenged Pacific oysters with OsHV-1μVar and characterized their transcriptome response at different time points post-inoculation by transcriptome sequencing. We also characterized the transcriptome behavior of the virus at the same time. Our analyses show that OsHV-1μVar enlists or orchestrates a strong and complex response from the oyster that involves all major innate immune pathways often equipped with expanded and novel genes. Host response, viral gene expression and replication, and mortality data all indicate that OsHV-1μVar can replicate very rapidly after challenge of oysters, causing heavy mortalities, but that some oysters are resistant and can effectively control OsHV-1μVar infection within a relatively short period post-infection.

2. Materials and methods

2.1. Virus challenge

In 2012 and 2013, 4 viral challenge experiments were conducted at the Oyster Reference Center, University of Caen Basse-Normandie, using hatchery-produced Pacific oysters, either spat (<1 year old, 1.5–2.3 cm shell height) or juveniles (~18 months old, 4–6 cm shell height), injected with OsHV-1μVar and analyzed for transcriptome responses. The oysters were obtained from a rack and bag system near Cricqueville-en-Bessin, on the coast of Lower Normandy (France), a location at least 4 km from any known oyster-growing area and established as a site where experimental oysters could be kept free of OsHV-1μVar infections. Oysters were transported to the laboratory and placed in seawater within 2 hours of collection. They were maintained as described below until injected, 2–7 days later.

Highly infected spat from culture sites on the west coast of the Cotentin Peninsula in Normandy were used as a source of virus. Viral inoculum was prepared by thoroughly mincing infected tissue in 0.20-μm filtered seawater (FSW, salinity = 33) and passing the resulting suspension through 11-μm mesh, then 0.45-μm and 0.20-μm filters. DNA, extracted from subsamples of the filtrates, was subjected to qPCR amplification using primers for OsHV-1 to estimate viral concentration [32] and diluted with FSW to obtain the desired dosages. This protocol (see Section 2.3) amplifies both the reference OsHV-1 and OsHV-1μVar, but we assume that the virus we injected was OsHV-1μVar because recent evidence from the Normandy coast indicates that essentially all detected herpes virus is of the variant genotype [33,34]. Juvenile oysters were injected into the adductor muscle, via a notch in the shell, with 100 μL of inoculum. Spat were injected into the pallial cavity with 50 μL of inoculum. The estimated viral load injected into each individual, as determined by the qPCR assay (see below), ranged from 1.6×10^6 to 1.5×10^9 viral genomic units (VGU) in the four experiments (Fig. 1A, Supplementary Materials). Controls were injected with the same volume of FSW. Salinity was maintained at 33–35 in all trials and temperature was between 21 and 25 °C, depending on experiment. Tanks were inspected at least twice daily at which time dead or dying oysters were removed and recorded for mortality calculations.

2.2. Tissue processing

One juvenile oyster experiment was chosen for transcriptome analysis (Fig. 1A). At each sample time, live oysters were shucked and the soft tissues placed in a Petri dish. Mantle, gill, digestive gland and adductor muscle were dissected, placed separately in individual, labeled Eppendorf tubes and immediately frozen in liquid nitrogen. Shucking knives and scalpels were wiped clean between oysters and tissues, respectively. The frozen tissues were then stored at −80 °C for subsequent RNA extraction and transcriptome sequencing. The remaining tissues were thoroughly minced in FSW and the supernatant was stored at −20 °C for virus-load determination by qPCR.

2.3. Determination of viral loads

Viral inoculum and viral loads post challenge were estimated with qPCR. DNA from the minced remaining-tissue supernatant of each oyster was extracted on an EpMotion5075 automated pipetting system (Eppendorf®) with the Nucleospin 8 Blood kit (Macherey Nagel®) according to manufacturer's protocol.

Quantification of viruses was based on the TaqMan qPCR protocol described by Martenot et al. [32]. Quality and quantity of template DNA was estimated on a Nanodrop 2000 spectrophotometer (Thermoscientific®) and was used to normalize the VGU [35]. Two μL of extracted DNA from each oyster was amplified. DNA quantity varied considerably, but averaged about 70 ng μL^{-1} and was mostly >20 ng μL^{-1}. The 260/280 ratios were >1.8.

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