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Poly I:C induces a protective antiviral immune response in the Pacific oyster (*Crassostrea gigas*) against subsequent challenge with Ostreid herpesvirus (OsHV-1 μvar)

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ABSTRACT

In-vivo studies were carried out to investigate the protective effect of a synthetic viral analogue (poly I:C) against Ostreid herpes virus (OsHV-1 μvar). Pacific oysters (*Crassostrea gigas*) were immune-primed by intramuscular injection of 240 μg of poly I:C or sterile seawater at 1 day prior to infection with OsHV-1 μvar. Poly I:C injection induced an antiviral state in *C. gigas* as the percentage of viral-infected oysters at 48 h post infection was significantly lower in the poly I:C treatment (11%) compared to seawater controls (100%). In an additional experiment, we demonstrated that the protective role of poly I:C is reproducible and elicits a specific antiviral response as immune-priming with heat-killed *Vibrio splendidus* provided no protection against subsequent viral infection. In both experiments, genes homologous to a toll-like receptor (TLR), MyD88, interferon regulatory factor (IRF) and protein kinase R (PKR) were up-regulated in oysters immune-primed with poly I:C compared to seawater controls ($p < 0.05$). The MyD88, IRF and PKR genes were also significantly up-regulated in response to OsHV-1 μvar infection ($p < 0.05$), which is suggestive that they are implicated in the antiviral response of *C. gigas*. Our results demonstrate that *C. gigas* can recognise double-strand RNA to initiate an innate immune response that inhibits viral infection. The observed response has striking similarities to the hallmarks of the type-1 interferon response of vertebrates.

1. Introduction

Innate immunity, the ancient immune system present in both invertebrates and vertebrates, is the first line of defence against microbial invaders, such as viruses. In vertebrates, the interferon signalling pathway is crucial for innate immunity against viral infection [1]. Most viruses produce double-stranded RNA (dsRNA) during viral replication and vertebrate cells recognise dsRNA (viral or synthetic) via toll-like receptor 3, which triggers a complex signal-transduction pathway resulting in the translocation of transcription factors (NF-κB and interferon regulatory factor) to the cell nucleus and the up-regulation of a number of genes, principally interferon. Interferon exerts an antiviral state in neighbouring cells by inducing the expression of antiviral proteins, such as protein kinase R (PKR), 2′,5′-oligoadenylate synthetase (OAS), and Myxovirus resistance protein (Mx) [1]. Until recently, it was a generally accepted paradigm that the interferon-signalling pathway was absent from invertebrates, as genes homologous to interferon or its major effectors were absent in several fully sequenced invertebrate genomes [2]. Instead, it was believed invertebrates blocked viral replication by RNA interference (RNAi) and this mechanism of antiviral immunity is considered the primary and most important antiviral response in insects and crustaceans, but has a lesser role in vertebrate innate immunity [3].

The Pacific oyster (*Crassostrea gigas*) is an economically valuable species farmed worldwide. Historically, Ostreid herpesvirus 1 (OsHV-1) has regularly been detected in *C. gigas* from France, United States and New Zealand [4–6]. OsHV-1 has typically only caused sporadic mortality events in larval and juvenile developmental stages of *C. gigas* [summarised by [5]]. Since 2008, *C. gigas* have experienced increased mortality in France due to a new genotype of the Ostreid herpes virus, termed OsHV-1 μvar [7]. Subsequently, the OsHV-1 μvar genotype has been confirmed to cause recurring mass mortalities in juvenile and adult developmental stages of *C. gigas* in Ireland, England, Spain, New Zealand and

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Australia [8]. The mortality caused by OsHV-1 μvar is influenced by environmental factors and the survival capacity of the host [9]. Oysters have no acquired immune system, but they do have efficient defence mechanisms for eliminating invading agents, based on an innate immune system. Over the last ten years, diverse feature of this defence system have been characterised in *C. gigas*, including signalling pathways, antimicrobial peptides and effectors [reviewed by [10]]. However, knowledge of the antiviral immune response of molluscs is poorly understood and it would not be surprising if it differed from other model invertebrate species. This lack of knowledge has hampered the development of successful management solutions against this disease. Several genes induced by OsHV-1 infection have been reported in *C. gigas* [11], but thus far, no significant mechanistic insight into the molecular basis for antiviral immunity in *C. gigas* has been described.

We propose that an "interferon-like pathway" exists in molluscs (see Fig. 1): Pearl oysters (*Pinctada fucata*) have receptors for interferon-Ω on the surface of their immuno-competent cells (hemocytes) [12] and injection of recombinant interferon-Ω prior to challenge with Akoya virus provides increased protection [13]. Furthermore, treatment of hemocytes with interferon-γ was shown to activate a STAT-like pathway in the bivalve, *Mytilus galloprovinciallis* [14]. Expressed sequence tags with high homology to interferon regulatory factors (IRFs) have been identified from flat oysters (*Ostrea edulis*), Pacific oysters (*C. gigas*) and mussels (*Mytilus edulis*) [15–18], and the Mx gene, a classic interferon induced antiviral effector of vertebrates was reported in a gastropod (*Haliotis discus discus*) [19]. Complete genome sequences of primitive invertebrates indicate ancient origins for many components of the vertebrate innate immune system with significant gene loss likely having occurred in model invertebrate species [20,21]. It appears that the OAS gene was lost from Ecdysozoa, as OAS activity was shown in crude extracts from the sponge, *Geodia cydonium* [22]. Furthermore, the OAS gene was recently isolated from the sponge, *Lubomirskia baicalensis* and its expression was significantly up-regulated following exposure to poly I:C, a known powerful inducer of type-1 interferon response in vertebrates [23].

Studies conducted in fish have demonstrated that poly I:C injection induces an interferon response [24] that can provide protection against subsequent viral infection [25,26]. Previously, it was demonstrated that poly I:C injection was a powerful inducer of putative antiviral gene expression in the Sydney rock oyster, *Saccostrea glomerata* [27]. Synergies in the pathways and genes expressed by *S. glomerata* following poly I:C injection were observed with *C. gigas* experimentally infected with OsHV-1 [11]. It is also known that injection of dsRNA (poly G:C) in marine shrimp (*Litpenaeus vannamei*) induces an innate immune response that provides increased protection against two unrelated viruses, white spot syndrome virus and Taura syndrome virus [2]. Therefore, we investigated whether poly I:C injection could induce *C. gigas* into an antiviral state that provided protection against subsequent OsHV-1 μvar infection. In parallel, we measured the expression of nine putative antiviral genes to gain insight into the immune response of *C. gigas*. These genes included a TLR and IRF identified in a cDNA library generated from short read sequences of *C. gigas* exposed to varying degrees of anthropogenic impacts [15] and homologues of PKR and OAS, which were identified in the recently completed oyster genome [18]. We also selected genes from the NF-κB pathway that have been identified in *C. gigas:* the NF-κB homologue *Cg*-Rel and its inhibitor, *Cg*-IκB [28,29]. The remaining genes chosen for qRT-PCR analysis were shown to be up-regulated in *C. gigas* in response to OsHV-1 infection [11].

2. Materials and methods

2.1. Experimental animals poly I:C protection studies

Specific pathogen free *C. gigas* were produced on the 5th of March, 2012, at the Ifremer oyster hatchery in La Tremblade, Charente Maritime, France. These oysters were confirmed to be free of OsHV-1 and its variants throughout the larval and spat production cycle. Oyster spat were on-grown in a biosecure nursery facility before being transferred to Ifremer's Aquaculture Research Facility in Palavas-les-Flots (Laboratoire Aquaculture en Languedoc—Roussillon, LALR), Southern France. These oyster spat (mean weight = 3.8 g) were used for experimentation in this manuscript, unless otherwise stated.

2.2. Preparation of OsHV-1 μvar and control oyster homogenates

An initial oyster homogenate was prepared according to Schikorski and Colleagues [30] from five moribund oyster spat collected during a mortality event on the 30th of May, 2012 from Thau Lagoon, France. Briefly, gill and mantle tissue was excised, pooled

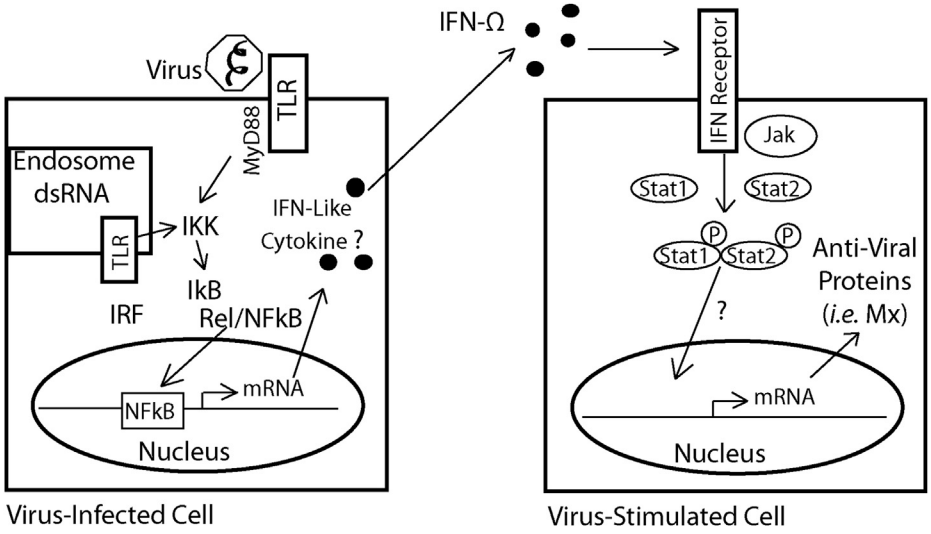

Fig. 1. Conceptual diagram of the "*interferon-like*" pathway in molluscs [adapted from [24]]. Homologues of toll-like receptor, NF-κB and interferon regulatory factors are conserved in molluscs [15,28,29,44]. Mammalian interferon activates a STAT-like pathway in molluscs [14] and induces them into an antiviral state [13]. Classic interferon stimulated effector molecules, such as Mx, are present in molluscs and their corresponding mRNA levels are upregulated in response to virus injection [19].