Developmental and Comparative Immunology 35 (2011) 725–735

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, *Crassostrea gigas*, challenged with Ostreid herpesvirus 1

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ARTICLE INFO

Article history:
Received 9 January 2011
Received in revised form 8 February 2011
Accepted 10 February 2011
Available online 1 March 2011

Keywords:
Pacific cupped oyster
Crassostrea gigas
OsHV-1
Immunity
Gene expression
Multicopper oxidase
Macrophage expressed protein
Ig cell adhesion molecule
Interferon-induced protein 44
Virus RNA

ABSTRACT

Virus-induced genes were identified using suppression subtractive hybridisation (SSH) from Pacific cupped oyster, *Crassostrea gigas*, haemocytes challenged by OsHV-1. A total of 304 clones from SSH forward library were sequenced. Among these sequences, some homologues corresponded to (i) immune related genes (macrophage express protein, IK cytokine, interferon-induced protein 44 or multicopper oxidase), (ii) apoptosis related genes (Bcl-2) and (iii) cell signalling and virus receptor genes (glypican). Molecular characterization and phylogenic analysis of 3 immune-related genes (macrophage expressed protein, multicopper oxidase and immunoglobulin domain cell adhesion molecule) were performed. Finally, quantitative PCR revealed significant changes in the expression of immune related genes (multicopper oxidase, macrophage expressed protein, myeloid differentiation factor 88 and interferon-induced protein 44) in oysters experimentally challenged with OsHV-1.

These findings provide a first basis for studying the role of innate immunity in response to viruses in bivalves and identified genes may serve as markers of interest in breeding programs in order to obtain selected oysters presenting OsHV-1 resistance.

1. Introduction

Meanwhile the development of mollusc aquaculture worldwide (FAO, 2008), infectious diseases due to an extended variety of pathogens including viruses remain a major concern. A wide host range is reported for herpes and herpes-like viruses among bivalves including oysters, clams and scallops (Renault and Novoa, 2004). A virus infecting the Pacific cupped oyster, *Crassostrea gigas*, in France has been purified from naturally infected larvae (Le Deuff and Renault, 1999) and its genome entirely sequenced (Davison et al., 2005). This virus was classified as the unique member of the Malacoherpesviridae under the name Ostreid herpesvirus 1 (OsHV-1) (Davison et al., 2005, 2009). OsHV-1 is a large DNA enveloped virus that infects several bivalve species. Although two OsHV-1 genotypes were previously reported in France (OsHV-1 reference and OsHV-1 var) (Arzul et al., 2001b), the presence of a third genotype, termed OsHV-1 μVar, was reported in France since 2008 in association with massive mortality outbreaks among French *C. gigas* (Segarra et al., 2010).

Despite the impact that herpes virus infections may have on shellfish, no information is, however, available on the immune responses of oysters to these viruses. The main objective of the present study was to provide knowledge of anti-viral innate immunity of the Pacific cupped oyster, *C. gigas*. For this purpose, virus-induced genes were first searched in Pacific cupped oyster haemocytes challenged by OsHV-1 using suppression subtractive hybridisation (SSH). Comparing the transcriptome changes was expected to identify cellular pathways and genes that are important to OsHV-1 resistance. Molecular characterization and phylogenic analysis of 3 selected immune-related genes were then performed and the expression of candidate genes was monitored by real time PCR in oysters experimentally challenged with the virus. Finally, results obtained in the present study were discussed in relation to existing knowledge on the role of innate immunity against viruses in vertebrates and invertebrates.

2. Materials and methods

2.1. Challenge of oyster haemocytes with OsHV-1

Pacific oysters, *C. gigas*, two-year old, 8–10 cm in shell length were purchased from a shellfish farm located in Marennes-Oleron

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doi:10.1016/j.dci.2011.02.004

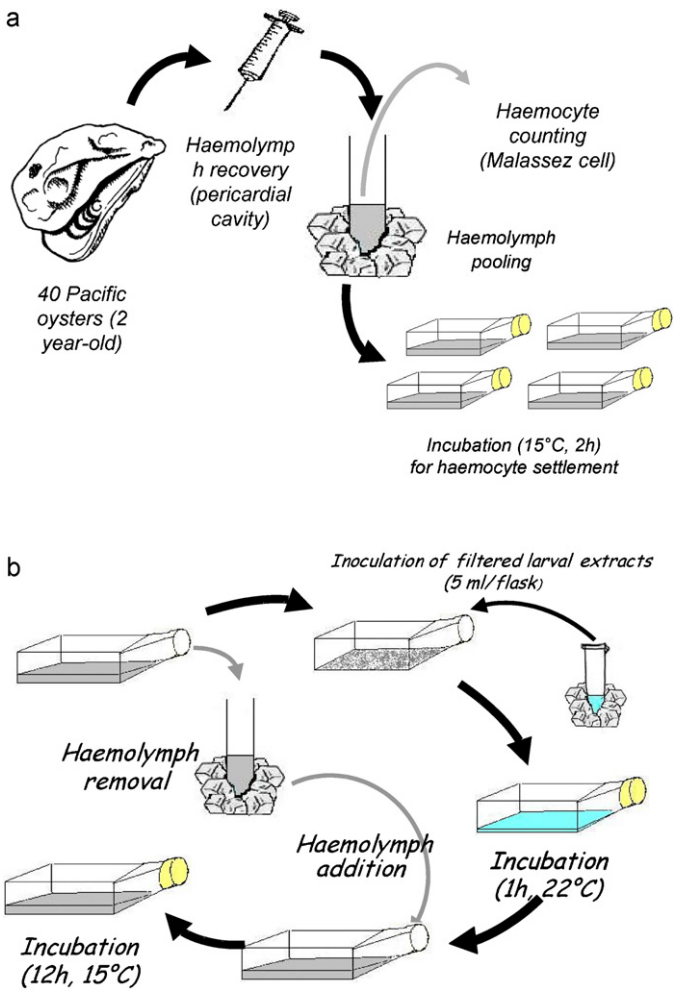

Fig. 1. Challenge of Pacific cupped oyster haemocytes with OsHV-1. (A) Oyster haemocyte collection and settlement in culture flasks. (B) Oyster haemocyte challenge with OsHV-1 (contact with filtered larval homogenate).

Bay (France). The haemolymph was withdrawn from the pericardial cavity with a sterile syringe of 1 mL equipped with a needle (0.9× 25 mm). Haemolymphs collected from 40 individuals were pooled. Oyster haemocytes were divided into 4 tissue culture flasks (25 cm^2, Nunc) adjusted to 7×10^6 viable cells each one. The culture flasks were incubated at 15 °C for 2 h for cell settlement (Fig. 1A). The haemolymph (the acellular counterpart) was then removed from the flasks and kept on chilled ice. Two flasks were inoculated with the filtered tissue homogenate prepared from experimentally infected axenic larvae. Infected axenic larvae were obtained using a previously described protocol (Le Deuff et al., 1994). The two other flasks received non-infected axenic larval homogenate. After a 1 h 30 contact at 22 °C, filtered larval homogenates were removed from flasks and the haemolymph kept on ice was added again on cells after filtration through 0.22 μm filter. The culture flasks were then incubated 12 h at 15 °C and were used for total RNA extraction (Fig. 1B).

2.2. Suppression subtractive hybridisation (SSH)

After washing twice with cold PBS, 780 μL of the Trizol® (Invitrogen) reagent were added on cells in culture flasks and RNA extracted. Briefly, cDNA was obtained from 1 μg of haemocyte RNA (control and challenged haemocytes) using the SMART PCR cDNA Synthesis Kit (Clontech), which allowed the amplification of cDNA from mRNA transcripts. The SSH assay was then performed using the PCR-Select cDNA Subtraction Kit (Clontech) following manufacturer's instructions. The cDNA from the tester and from the driver were digested with Rsa I, and the tester cDNA was then ligated to either two different cDNA adaptors.

PCR mixtures of forward substraction were ligated using TA cloning kit (Invitrogen). PCR products from selected clones were spotted onto duplicate membranes which were screened for virus-induced genes using the PCR Select Differential Screening Kit (Clontech). Clones that hybridised to the forward-subtracted probe and to both subtracted probes when the difference of signal intensity was higher than 2 were interpreted as differentially expressed, their expression being induced in oyster haemocytes in contact with OsHV-1 compared to control haemocytes.

Selected clones were sequenced using a BigDye terminator Cycle Sequencing Ready Reaction Kit and a 3100 Avant Genetic analyzer ABI Prism (Applied Biosystem, Hitachi). Sequences were compared against the GenBank database using BLAST programs on the NCBI website (http://www.ncbi.nlm.nih.gov/blast/).

2.3. 5′/3′ RACE PCR and sequence analysis

The full-length cDNAs were obtained by 5′ and 3′ RACE PCR from the sequences identified by SSH. SMART RACE cDNA Amplification Kit was used according to the manufacturer's instructions. 5′ and 3′ primers were designed using primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and synthesized by Eurogentec (Table 1). After transformation in top 10 competent bacteria (Invitrogen), clones were sequenced from both ends with TA forward and reverse primers as described above. Sequences were blasted using NCBI-BLAST software (http://www.ncbi.nlm.nih.gov/blast/). Open reading frames and protein conserved domains were predicted (ORF finder, Search conserved Domain NCBI, SMART, ScanProsite). Multiple sequence alignments (Clustal W) and phylogenic analysis (neighbour-joining algorithm) were carried out using MEGA4 software.

2.4. Oyster challenges with OsHV-1

Two experiments were carried out. For the first experiment, two-year-old Pacific oysters (adults) were purchased from an oyster farm in La Tremblade (Charente Maritime, France). For the second experiment, 14-month-old Pacific oysters (juveniles) were obtained from the same farm. For each experiment, 90 healthy individuals were randomly distributed in 6 tanks supplied with 12 L of filtered (1 μm) seawater and acclimatizated at 22 °C during a period of one week. Oysters were then placed out of water for 24 h at 22 °C and then anesthetized (4 h at 22 °C) in a solution of seawater (1 v)/distilled water (4 v) containing 7% (w/v) of magnesium chloride ($MgCl_2$, 50 g L^{-1}).

Fifty microlitres of a 0.22 μm filtered larval homogenate were injected into the pericardial cavity of 45 animals. OsHV-1 infected larvae (*C. gigas*) kept frozen (−20 °C) served as the virus source. The 45 remaining oysters received an injection of 50 μL of sterile seawater and served as negative controls. Inoculated oysters were then placed for 48 h at 22 °C in 12 L tanks supplied in filtered (1 μm) seawater without food supply or seawater change.

In order to evaluate gene expression, 3 oysters from each tank were sacrificed at each collecting time. Haemolymphs were collected 0 h, 24 h and 48 h post-injection for the first experiment and 0 h, 12 h, 24 h and 48 h post-injection for the second experiment. Haemolymphs were pooled for each tank (3 oysters per tank) at each collecting time and then haemocytes were pelleted by centrifugation (1500 × *g* for 10 min, 4 °C). Haemocyte RNAs were extracted from collected haemocytes as reported above.