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Cellular and molecular responses of haemocytes from *Ostrea edulis* during in vitro infection by the parasite *Bonamia ostreae* ☆

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ABSTRACT

Bonamia ostreae is a protozoan, affiliated to the order Haplosporidia and to the phylum Cercozoa. This parasite is intracellular and infects haemocytes, cells notably involved in oyster defence mechanisms. Bonamiosis due to the parasite *B. ostreae* is a disease affecting the flat oyster, *Ostrea edulis*. The strategies used by protozoan parasites to circumvent host defence mechanisms remain largely unknown in marine bivalve molluscs. In the present work, in vitro experiments were carried out in order to study the interactions between haemocytes from *O. edulis* and purified parasite, *B. ostreae*. We monitored cellular and molecular responses of oyster haemocytes by light microscopy, flow cytometry and real-time PCR 1, 2, 4 and 8 h p.i. Light microscopy was used to measure parasite phagocytosis by oyster haemocytes. Parasites were observed inside haemocytes 1 h p.i. and the parasite number increased during the time course of the experiment. Moreover, some bi-nucleated and tri-nucleated parasites were found within haemocytes 2 and 4 h p.i., respectively, suggesting that the parasite can divide inside haemocytes. Host responses to *B. ostreae* were investigated at the cellular and molecular levels using flow cytometry and real-time PCR. Phagocytosis capacity of haemocytes, esterase activity and production of radical oxygen species appeared modulated during the infection with *B. ostreae*. Expression levels of expressed sequence tags selected in this study showed variations during the experiment as soon as 1 h p.i. An up-regulation of galectin (*OeGal*), cytochrome p450 (*CYP450*), lysozyme, omega GST (OGST), super oxide dismutase Cu/Zn (*Oe-SOD Cu/Zn*) and a down-regulation of the extracellular super oxide dismutase SOD (*Oe-EcSOD*) were observed in the presence of the parasite. Finally, the open reading frames of both SODs (*Oe-SOD Cu/Zn* and Oe-EcSOD) were completely sequenced. These findings provide new insights into the cellular and molecular bases of the host–parasite interactions between the flat oyster, *O. edulis*, and the parasite, *B. ostreae*.

1. Introduction

Diseases due to infectious agents (virus, bacteria or protozoa) might curb oyster production and contribute to financial losses. A better understanding of interactions between the immune system of oysters and pathogens is necessary to control the development of these diseases. However, these interactions are poorly documented in oysters in contrast with other invertebrate species such as insects and nematodes (D'Argenio et al., 2001; Gravato-Nobre and Hodgkin, 2005). The immune defence of invertebrates, including oysters, is based on innate immune responses.

In Europe, since 1979 flat oyster production has experienced high mortality outbreaks associated with protozoans including *Bonamia ostreae* (Pichot et al., 1979). This protozoan is an intracellular parasite and belongs to the order Haplosporidae and the phylum Cercozoa (Carnegie et al., 2000; Cochennec et al., 2000; Cavalier-Smith and Chao, 2003). *Bonamia ostreae* infects haemocytes, cells notably involved in oyster defence mechanisms (Cheng, 1981). Three different types of haemocytes have been described in the flat oyster, *Ostrea edulis*: granulocytes, large hyalinocytes and small hyalinocytes (Bachère et al., 1991; Chagot et al., 1992; Mourton et al., 1992; Xue et al., 2001). These cells are responsible for wound repair, phagocytosis and encapsulation (Cheng, 1981; Fisher, 1986). The haemocytes are carried by the haemolymph in a semi-open system. Some soluble immune components such as lectins, lysosomal enzymes and antimicrobial peptides have already been identified in the haemolymph of different bivalves species (Olafsen et al., 1992; Bachère et al., 2004; Xue et al., 2004).

The flat oyster *O. edulis* and its parasite *B. ostreae* represent a suitable model to study host–pathogen interactions in molluscs due to the possibility of reproducing the associated disease under experimental conditions (Mourton et al., 1992).

Intracellular parasites have developed sophisticated strategies to escape host defence mechanisms, thereby finding unique niches where they can survive, and from which they can establish

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successful infection. As previously described, the internalisation of *B. ostreae* by flat oyster haemocytes induces a diminution of esterase activity and reactive oxygen species (ROS) production after 2 h of in vitro infection (Morga et al., 2009). Esterases are enzymes belonging to the group of hydrolases catalysing the hydrolysis of ester bonds. The respiratory burst is a series of biochemical reactions that produce highly microbicidal ROS including superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^+). Although radicals produced during the respiratory burst are known to be involved in the destruction of parasites in different host species including molluscs (Toreilles et al., 1996; Hahn and Bayne, 2001; Humphries and Yoshino, 2008), some intracellular parasites such as *Leishmania* spp., *Toxoplasma gondii* and *Perkinsus marinus* develop strategies to evade this process which allow them to invade and multiply within host cells (Dermine and Desjardins, 1999; Schott et al., 2003; Shrestha et al., 2006). Similar strategies seem to be developed by the parasite *B. ostreae*. Recently, new data obtained on the flat oyster genome have allowed identification of some expressed sequence tags (ESTs) potentially involved in the host–parasite interactions, including the genes selected in the present study (Morga et al., 2011).

Previously reported in vitro studies examined the specificity of the host responses to infection with the parasite at the cellular or at the molecular level after 2 h, but never by considering both simultaneously and over time (Morga et al., 2009, 2011). The specificity of the haemocyte response has been tested using dead and live parasites.

In the present study, we studied the kinetics of the response of haemocytes of *O. edulis* to an in vitro infection with the parasite *B. ostreae*. For that purpose, we combined flow cytometry to measure some haemocyte activity and real-time PCR analysis to measure expression levels of some genes of interest including galectin (*Oe-Gal*), super oxide dismutase (*Oe-SOD Cu/Zn*), extracellular super oxide dismutase (*Oe-EcSOD*), cytochrome p450 (*CYP450*), heat shock protein 90 (*HSP90*), omega GST (*OGST*) and lysozyme. In addition, the complete open reading frames (ORFs) of two *O. edulis* genes related to detoxification (*Oe-SOD Cu/Zn* and *Ec Oe-SOD*) were determined and characterised.

This integrated study of the kinetics of the immune response of the *O. edulis* to *B. ostreae* may contribute to better understanding of the strategies developed by the parasite to escape host responses and by the oyster to eliminate the parasite and finally survive.

2. Materials and methods

2.1. Biological material

2.1.1. Haemolymph collection

Two year old flat oysters, *O. edulis*, were collected from the Bay of Quiberon (Southern Brittany, France), an endemic zone for bonamiosis.

Haemolymph was withdrawn from the adductor muscle using a 1 mL syringe equipped with a needle (0.40 × 90 mm) and then filtered on 75 μm mesh to eliminate debris. Ten to 15 samples of haemolymph were pooled together and maintained on ice to prevent aggregation (Auffret and Oubella, 1997). Haemocyte counts were performed using a Malassez cell chamber.

2.1.2. Parasite

Bonamia ostreae were purified according to the protocol of Mialhe et al. (1988). Briefly, heavily infected oysters were selected by examination of heart tissue imprints using light microscopy. After homogenisation of all the organs except the adductor muscle, the parasites were concentrated by differential centrifugation on sucrose gradients and then purified by isopycnic centrifugation on a Percoll gradient. Finally, the purified parasites were suspended in filtered sea water (FSW). *Bonamia ostreae* cells were then counted using a Malassez cell chamber. Suspensions of purified parasites were stored at 4 °C and used within 24 h following the purification for in vitro infection experiments.

2.2. In vitro infection protocol

Haemocytes (5×10^5 cells mL^{-1}) were incubated with live parasites at the ratio of 5:1 (parasites per haemocyte) at 15 °C and analysed after 1, 2, 4 and 8 h of in vitro infection. The control consisted of haemocytes alone suspended in FSW (5×10^5 cells mL^{-1}). The whole experiment was carried out three times in duplicate ($n = 6$).

2.3. Flow cytometry analysis

Protocols and methods used for the flow cytometry analyses have previously been described in Morga et al. (2009). For each sample, 5,000 events were counted using an EPICS XL 4 (Beckman Coulter). Based on size discrimination, parasites or other small particles were not counted; only haemocytes were taken into account for cell activity measurements. Results were depicted as cell cytograms and reported as log scale fluorescence levels for each marker used. Fluorescence depended on the monitored parameters: non-specific esterase activity, ROS production and phagocytosis were measured using green fluorescence while cell mortality was measured using red fluorescence.

2.4. Light microscopy

One hundred μl of cell suspensions were cytocentrifugated (100*g*, 4 °C, 1 min), stained with Hemacolor® (Merck) and examined using light microscopy. Before the infection experiment, pools of haemolymph were examined in order to check for absence of the parasite. Then, for time-point, 150 haemocytes were observed and the number of infected haemocytes, the number of parasites per infected haemocyte and the total number of bi- and tri-nucleated parasites were reported.

2.5. Gene selection

Seven ESTs were selected from *O. edulis* cDNA databases due to their potential involvement in responses to infection with *B. ostreae*. Among these genes, one encodes a cell recognition protein, OeGal and four are involved in post-phagocytosis degradation and cellular protection mechanisms: *Oe-SOD Cu/Zn*, *Oe-EcSOD*, *CYP450*, *OGST*. The expression of two other genes implicated in defence mechanisms (lysozyme) and stress, *HSP90*, was also studied.

2.6. RNA extraction and reverse transcription

Total RNA was extracted from haemocytes using Trizol (Trizol® reagent, Invitrogen™) and treated with RQ1 RNase-free DNase (Promega) to remove remaining genomic DNA. RNA concentrations were measured before and after DNase treatment. Reverse transcription (RT) was carried out as previously described by Morga et al. (2010) using the oligo(dT) anchor primer (5′-GACCACGCGTATCGATGTCGACT(16)V-3′). Reverse transcription was performed using SuperScript III (Invitrogen) according to the manufacturer's recommendations.

2.7. Expression analysis of the selected ESTs by real-time quantitative PCR

For each of the selected ESTs, forward and reverse primers were designed using primer3 software (http://biotools.umassmed.edu/