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.

Graphical abstract :

Highlights

► Multiplication of parasite Bonamia ostreae occurs inside haemocytes at 2 h p.i. ► Diminution of esterases and reactive oxygen species in the host at 1 h p.i. ► Over-expression of the galectin gene (OeGal) at 1 h p.i. ► Up-regulation of cytoplasmic super oxide dismutase (OeSOD Cu/Zn) at 2 and 4 h p.i.

Keywords : Bonamia ostreae ; Protozoan ; Ostrea edulis ; Haemocytes ; Real-time PCR ; Flow cytometry ; Super oxide dismutase
1. Introduction

Diseases due to infectious agents (virus, bacteria or protozoan) 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 (Gravato-Nobre and Hodgkin, 2005; D’Argenio et al., 2001). The immune defence of invertebrates, including oysters, is based on innate immune responses.

In Europe, since 1979 the 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 of Haplosporidae and the phylum of cercozoan (Cochennec et al., 2000; Carnegie et al., 2000; Cavalier-Smith and Chao 2003). *B. ostreae* infects the 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 (Auffret, 1985; 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 because of the possibility to reproduce the associated disease in 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 successful infection. As previously described, the internalisation of *Bonamia ostreae* by flat oyster haemocytes induces a diminution of esterase activities and reactive oxygen species (ROS) production after 2 hours 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$_{2}$O$_{2}$), and hydroxyl radical (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 like *Leishmania sp*, *Toxoplasma gondii* and *Perkinsus marinus* develop strategies to evade this process which allows 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 flat oyster genome have allowed identifying some ESTs potentially involved in the host-parasite interactions, including the genes selected in the present study (Morga et al., 2010a).

Previous *in vitro* studies have examined the specificity of the host responses to infection with the parasite at the cellular or at the molecular level after 2 hours, but never by considering both simultaneously and throughout time (Morga et al., 2009, Morga et al., 2010a). The specificity of the haemocyte response has been tested using dead and live parasite.

In the present study, we have 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 activities and real-time PCR analysis to measure expression levels of some genes of interest including galectin (OeGal), super oxide dismutase (Oe-SOD Cu/Zn), extracellular super oxide dismutase (Oe-EcSOD), cytochrome p450 (CYP450), heat shock protein
90 (HSP90), omega glutathione S transferase (OGST) and lysozyme. In addition, the complete open reading frames of two *Ostrea edulis* genes related to detoxification (Oe-SOD and Ec Oe-SOD) were determined and characterized.

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

### 2. Material and methods

#### 2.1 Biological material

**2.1.1 Haemolymph collection**

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

Haemolymph was withdrawn from the adductor muscle using a 1mL syringe equipped with a needle (0.40x90 mm) and then filtered on a 75 µm mesh to eliminate debris. 10-15 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.

**2.1.2 Parasite**

*B. ostreae* was 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
homogenization 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. Suspensions of purified parasites
were stored at 4°C and used within the 24 hours following the purification for *in vitro* infection
experiment.

2.2 *In vitro infection protocol*

Haemocytes (5.10^5 cells mL^{-1}) were incubated with live parasites at the ratio 5:1 (parasites per
haemocyte) at 15 °C and analysed after 1h, 2h, 4h and 8h 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 and included each time 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, 5000 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 measures. Results were depicted as cell
cytograms and reported in log scale fluorescence levels for each marker used. Fluorescence
depended on the monitored parameters: non specific esterase activities, 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 the absence of the parasite. Then, for each time of contact, 150 haemocytes were observed, and number of infected haemocytes, number of parasites per infected haemocyte and the total number of di and trinucleated parasites were reported.

2.5 Gene selection

Seven ESTs were selected from O. edulis cDNA databases because of their potential involvement in response to infection with Bonamia ostreae. Among these genes, one encodes a cell recognition protein, the galectin (OeGal) and four are involved in post-phagocytosis degradation and cellular protection mechanisms: super oxide dismutase (Oe-SOD Cu/Zn), extracellular super oxide dismutase (Oe-EcSOD), cytochrome p450 (CYP450), omega glutathione s transferase (OGST). The expression of two other genes implicated in defence mechanisms (lysozyme) and stress (heat shock protein 90, 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 Morga et al., (2010) using the oligo(dT) anchor primer (5’-GAC
CACGCGTATCGATGTCGACT(16)V-3'). Reverse transcriptase was performed using SuperScript III (Invitrogen) according to the manufacturer’s recommendations.

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

For each of the selected ESTs, forward and reverse primers were designed using primer3 software (http://biotools.umassmed.edu/bioapps/primer3 www.cgi) (Table 1). Real-time quantitative PCR reactions were duplicated and performed in a total volume of 25 μL using a Mx3000 Thermocycler sequence detector (Stratagene) in 96-microwell plates. Each well (25 μL) contained 5 μL of cDNA dilution (1/30), 12.5 μL of Brilliant® SYBR® Green II PCR Master Mix (Stratagene), 2.5 μL of each diluted primer (3µM) and 2.5 μL of distilled water. Thermal cycling conditions were: 1 cycle of activation of Hot start Taq polymerase at 95 °C for 10 min; 40 cycles of amplification at 95°C for 10 s, 60°C for 30 s; and melting temperature curve analysis at 95°C to 60°C by 0.5°C decrease of the temperature every 10 s. In all cases, negative controls (without cDNA) were included to rule out DNA contamination.

For each candidate gene, melting curve and gel picture were analysed in order to verify the specificity of the amplified products and that a single PCR product was amplified. PCR efficacy (E) was calculated for each primer pair by determining the slopes of standard curves. These curves were generated using a serial dilutions analysis of plasmid containing the insert of interest. Amplification efficacies were calculated according to the following equation $E=10^{(1-slope)}$ (Pfaffl, 2001).

The expression of the candidate genes was normalized using the elongation factor 1 alpha (GenBank, accession n° EU651798) as housekeeping gene. The elongation factor alpha has previously been identified as the most stable housekeeping gene for similar study (Morga et al. 2010b). Haemocytes alone were used as calibrator. Primers are shown in Table 1. Fold units were calculated using the double delta Cts method described by Pfaffl (2001).
2.8 Identification and characterization of immune-related genes Oe-SOD Cu/Zn and Oe-EcSOD

In order to obtain the complete open reading frame (ORF) of Oe-SOD Cu/Zn and Oe-EcSOD, RACE reactions were carried out using SMART RACE cDNA Amplification Kit from Clontech according to the manufacturer’s instructions. 5’ and 3’ primers were designed using primer 3 software (http://biotools.umassmed.edu/bioapps/primer3 www.cgi) (3’Oe-SOD Cu/Zn TGATTAACCTGGCTGGTCCACAGTC, 5’Oe-SOD Cu/Zn GACTGTGGACCAGCCAGGTTAATCA and 3’Oe-ECSOD GTGCACATGGAGGTCATGTTGACTG, 5’Oe-ECSOD TGACAGGATGGTCCTCTCTCCTCCTC) and synthesized by Eurogentec. Race PCR products were cloned using TOPO Vector System (Invitrogen). Several clones were selected and amplified with TOPO R and F primers in order to control the size of the insert in the plasmid.

2.8.1 Sequencing and sequence analysis

Selected clones were first amplified using TempliPhi™ DNA Sequencing Template Amplification Kit (Amersham’s). PCR products isolated from individual clones were sequenced in both ends with TOPO R and F primers (Table 1) with the sequencing kit ABI BigDye® terminator version 3.1 using an ABI PRISM® 3130 XL-Avant Genetic Analyzer, a 36 cm capillary array and POP 7 polymer. Chromatograms were analyzed with Chromas 231 software. Sequences were then analyzed with BlastX algorithm available from the National Center for Biotechnology Information (NCBI) and the EST sequences were then submitted to dbEST and GenBank databases.
Complete sequences (Oe-SOD and Oe-EcSOD) were deposited in GenBank and assigned under the accession numbers GU320695 and GU320696.

2.8.2 Phylogenetic analysis of Oe-SOD Cu/Zn and Oe-EcSOD

The sequence of the complete ORF of Oe-SOD Cu/Zn was aligned with homologous genes from GenBank database (Homo sapiens sapiens NP000445.1, Rattus norvegicus CAA79925.1, Bos taurus NM174615, Salmo salar NP00111780.1, Pagrus major AA015363.1, Mytilus edulis CAE46443.1, Crassostrea ariakensis ABF14366.1 and Crassostrea gigas CAD427221).

The sequence of the complete ORF of Oe-EcSOD was aligned with homologous genes from GenBank database (Homo sapiens sapiens NP0003093.2, Rattus norvegicus CAA64149.1, Bos taurus NP001076079.1, Salmo salar NP001134324.1, Perna canaliculus AF273766.1, Crassostrea virginica BAF30874.1 Crassostrea gigas AAY60161.1 Saccostrea glomerata ACQ73551.1 and Barbula unguiculata BAC53790.1 (outgroup)).

Sequences were aligned using ClustalW (Thompson et al., 1997) from MEGA 4 (Tamura et al., 2007). Phylogenetic trees based on deduced amino acid sequences were constructed using the Neighbour-Joining (NJ) (Saitou et al., 1987) and the Maximum parsimony (MP) method (Eck and Dayhoff 1966) with the MEGA 4 software program. Statistical confidence on the inferred phylogenetic relationships was assessed by bootstrap of 1000 replicates.
2.9 Statistical analysis

A Mann-Whitney test was performed to test the difference between the mean numbers of parasites in infected haemocyte during the kinetic experiment. Flow cytometry results were expressed as percentages of positive cells. In order to detect an effect of tested conditions, a two factors ANOVA was performed using XLSTAT-Pro® version 7.5.3 software. Values were converted into angular arcsin square root (% of positive cells) before analysis to ensure the respect of a priori assumptions of normality and homogeneity. In the case of rejection of $H_0$, an a posteriori Tukey test was used to compare differences between means. Quantitative PCR results were also analysed by a two factor ANOVAs using XLSTAT-Pro® version 7.5.3 software. In the case of rejection of $H_0$, an a posteriori Tukey test was used to compare differences between means.

3. Results

3.1. Detection of parasites in haemocytes

*Bonamia ostreae* appeared engulfed by haemocytes whatever the tested contact period was. Percentages of infected haemocytes are 18% to 27% and mean number of parasites per infected haemocyte from 1.3 to 2.4 between 1h and 8h of contact (Fig. 1). The number of parasites observed inside the haemocyte was significantly more important at 4h and 8h compared to 1h ($p<0.036$ and $p<0.016$, respectively). Number of binucleated parasites inside the haemocytes increased after one
hour of contact (Fig. 1). Moreover, two intrahaemocytic trinucleated parasites could be observed after 4h.

3.2 Flow cytometry analysis

3.2.1 Haemocyte mortality

Prior to haemocyte activity measurement, cell survival was checked by deducing cell mortality based on propidium iodide labelling. Whatever the tested condition was, percentage of live cells was never below 89.1%. Cell mortality was considered equivalent between all the tested conditions.

3.2.2 Esterases, ROS and phagocytosis activity

In presence of parasites, the percentages of positive haemocytes showing esterase activities were significantly lower compared to the control after 1h (p < 0.033), 2h and 4h (p < 0.004) of \textit{in vitro} infection (Fig. 2a). Percentages of ROS positive haemocytes were significantly lower in presence of parasites than in controls at 1h, 2h and 8h (p < 0.013) (Fig. 2b). Lastly, a significant decrease of the percentage of haemocytes which have engulfed three beads or more was observed in haemocytes in contact with \textit{Bonamia ostreae} after 2h of contact (p < 0.02) (Fig. 2c).

3.3 Identification and characterization of immune-related genes

3.3.1 Ostrea edulis Super Oxide Dismutase (Oe-SOD Cu/Zn)

RACE-PCR reactions allowed obtaining the full cDNA sequence of \textit{Ostrea edulis} Super Oxide Dismutase Cu/Zn (\textit{Oe-SOD Cu/Zn}). This sequence of 898 nucleotides contains a short 5′-
untranslated region (1-48 nt) followed by an ORF of 471 nucleotides (49-519 nt) and finished by a
3’-untranslated region (520-898 nt) showing a polyadenylation signal (AATAAA) starting 79 bp
upstream from the polyA tail (Fig 3). The deduced amino acid sequence presented a molecular mass
of 15 863.70 Da and a predicted isoelectric point of 5.93 (http://www.expasy.ch/tools/pi_tool.html).
SignalP analysis further confirmed that Oe-SOD Cu/Zn was a cytoplasmic protein. The deduced
amino acid sequence was devoid of signal peptide.

Multiple alignments of intracellular SOD identified in different invertebrate and vertebrate species
revealed the presence of several conserved motifs and SOD signature sequences in Oe-SOD Cu/Zn.
Two cysteine residues (C\textsuperscript{60} and C\textsuperscript{149}), involved in the formation of internal disulfide bond were fully
conserved in Oe-SOD Cu/Zn. Four histidine residues (H\textsuperscript{50}, H\textsuperscript{52}, H\textsuperscript{66}, and H\textsuperscript{123}) predicted to be
critical for copper (Cu) binding, and highly consistent with other invertebrate or vertebrate SODs,
are present in Oe-SOD Cu/Zn (Fig. 3). Moreover, four residues involved in Zinc (Zn) binding,
notably three histidine (H\textsuperscript{66}, H\textsuperscript{74}, H\textsuperscript{83}) and one aspartate (D\textsuperscript{86}), were also identified in Oe-SOD
Cu/Zn. Finally, Oe-SOD Cu/Zn amino acid sequence shows two Cu/Zn-SOD family signatures:
GFHVHQFGDNT and GNAGGRLACGVI.

3.3.2 Ostrea edulis Extacellular Super Oxide Dismutase Oe-EcSOD

RACE-PCR reactions allowed obtaining the full cDNA of Ostrea edulis Extracellular Super Oxide
Dismutase (Oe-EcSOD). Oe-EcSOD showed a 985 nucleotide sequence containing a short 5’-
untranslated region (1-42 nt) followed by an ORF of 794 nt (43-837 nt) and finished by a 3’-
untranslated region (838-985 nt) (Fig 4). The deducted amino acid sequence of the ORF region
showed a predicted molecular mass of 29 478.38 Da and an isoelectric point of 4.93
(http://www.expasy.ch/tools/pi_tool.html). Analysis of the deducted amino acid sequence using the
SignalP3.0 software (www.cbs.dtu.dk/services/SignalP/) revealed the presence of a putative signal
peptide of 20 amino acids (MQSLILVLAALFYVA), suggesting that Oe-EcSOD can be secreted. The mature peptide contained a putative N-glycosylation site \( \text{NLS}^{41} \) (www.cbs.dtu.dk/services/NetNGlyc/), suggesting that Oe-EcSOD is a glycoprotein (Fig. 4).

3.3.3 Phylogenetic analysis of both Ostrea edulis SOD

The phylogenic trees based on amino acid sequences of Oe-SOD and Oe-EcSOD from different species have been generated using Neighbour-Joining (NJ) and the Maximum Parsimony (MP) methods.

Both phylogenic trees (NJ) (Fig. 5) and (MP) (data not shown) show two distinct groups including the cytoplasmic SOD sequences and the extracellular SOD sequences. Extracellular SOD group and cytoplasmic SOD present two sub group, the mammal group and the invertebrate group in both trees (Fig. 5). In the extracellular SOD group, “mollusc species” formed a unique branch including Perna canaliculus, Crassostrea virginica, Ostrea edulis, Crassostrea gigas and Saccostrea glomerata (Fig. 5). In the cytoplasmic SOD group, “mollusc species” formed a unique branch including Mytilus edulis, Crassostrea ariakensis, Ostrea edulis and Crassostrea gigas.

3.4 Expression patterns of selected genes

At 1h of in vitro infection, Oe-Gal was significantly \( (p<0.01) \) over expressed (3.9) and its expression slowly decreased afterwards. A significant over expression of OGST \( (p<0.02) \) was observed at 1h (2.1) while it appears stable after that time. The lysosyme gene was significantly \( (p<0.01) \) over expressed at 1h, 2h and 4h with a maximum expression at 2h (4.1). The CYP450 expression was significantly \( (p<0.01) \) up regulated at 1h (2.3) and 8h (2.5). Oe-SOD was significantly \( (p<0.006 \text{ and } 0.001) \) over expressed at 2h (1.9) and 4h (1.4). Oe-EcSOD was down...
regulated at 1h and not significantly regulated at 2h, 4h and 8h. Finally, HSP 90 was not significantly regulated during the course of the experiment (Fig. 6).

4. Discussion

The present work aimed at better understanding the interactions between flat oyster O. edulis haemocytes and the parasite Bonamia ostreae. Cellular and molecular responses were measured by light microscopy, flow cytometry and real time PCR assays at different times post incubation between haemocytes and parasites: 1h, 2h, 4h and 8h. In vitro infection was performed at the ratio of five parasites per haemocyte at 15°C. The internalization of the parasite by the haemocytes was monitored by examination of cytocentrifugated cells.

Parasites were observed inside haemocytes as soon as 1h of infection. Previous studies demonstrated that B. ostreae was internalized after 30 min of contact with haemocytes and was not degraded after phagocytosis (Chagot et al., 1992 ; Mourton et al., 1992). Percentages of infected haemocytes increased during the course of the experiment. Due to the difficulty to identify with certainty haemocyte types for some cytocentrifugated cells, we did not compare percentages of infected haemocytes considering cell types. However, all types of haemocytes (granulocytes, small and large hyalinocytes) were found infected as it was previously observed in several studies (Chagot, 1989 ; Cochennec et al., 2003). The mean number of parasites observed inside the infected haemocytes was significantly higher after 4h and 8h compared to 1h. This result suggests that the parasite multiplies inside the haemocytes, or that the parasites accumulate within a particular cell during the time course of the experiment. Binucleated and trinucleated parasites were observed inside haemocytes suggesting that the multiplication of the parasite rapidly occurs after the internalization.
Haemocyte activities were monitored by flow cytometry according to protocols previously described (Morga et al., 2009). Haemocyte mortality never exceeded 89.1% during the experiment. The internalization of the parasite did not induce haemocyte death even after 8h of contact. Previous in vitro studies have examined the specificity of the host responses to infection with the parasite at the cellular level after 2 hours of contact, the specificity of the haemocyte response has been tested using live and dead parasite (Morga et al., 2009).

Non specific esterase activities were recognized as reflecting the global activity of haemocytes. Esterases may also be involved in the degradation of internalised particles after phagocytosis. In the present study, parasites induced a decrease of the percentage of esterase positive cells after one hour and more significantly after two and four hours of contact. These results, in addition to the lack of haemocyte mortality, suggest a direct impact of parasites on host cell esterase activities. Catalytic enzymes present in B. ostreae (Hervio et al., 1991) might be able to inhibit hydrolases (ie esterases) produced by haemocytes.

Similarly, the percentage of ROS positive haemocytes was lower after 1h, 2 h and 8h of incubation with parasites compared to the control. As no cell mortality was detected, parasites may have a direct impact on haemocyte ROS production. The inhibition of oxygen radical production has already been described and should facilitate intracellular survival of protozoan parasites including Trypanosoma sp (Penketh et al., 1987), Toxoplasma sp (Murray et al., 1980, Shrestha et al., 2006), Leishmania sp (Murray, 1981), B. ostreae (Morga et al., 2009) and P. marinus (Volety and Chu, 1997).

P. marinus and rickettsia-like organisms possess acid phosphatase, partly responsible for the suppression or inhibition of ROS production (Le Gall et al., 1991 ; Volety and Chu 1997). Hervio et al., (1991) demonstrated the presence of acid phosphatase activity in haplosporosomes of Bonamia ostreae. Acid phosphatase can inactivate the production of NAD(P)H oxidase and thus prevents
ROS production. The level of acid phosphatase activity in *B. ostreae* is equivalent (Hervio et al., 1991) to levels found in several *Leishmania* species (Lovelace and Gottlieb, 1987) in which the inhibitor role of the NAD(P)H oxidase has been shown.

Bead phagocytosis capacity of haemocytes was significantly lower after 2 hours in presence of *Bonamia ostreae* compared to haemocytes alone. Cytocentrifugation observation revealed an increase of the parasite number inside infected haemocytes between 1 and 2 hours post infection. The decrease of bead phagocytosis capacity by infected haemocytes observed after 2 hours of contact with parasites could be due to the presence of parasites inside haemocytes. Goedken et al., (2005) have shown that the internalisation of the parasite *P. marinus* by haemocytes from the oyster *Crassostrea virginica* did not induce a difference of bead internalisation by haemocytes. The decrease of bead phagocytosis capacity and the increase of infected haemocytes during the course of the experiment support the hypothesis that the parasite *B. ostreae* mediates its own phagocytosis.

In addition to this cellular analysis of haemocytes response to an infection with the parasite *B. ostreae*, some genes have been selected for their potential interest to better understand host parasite interactions. Previous *in vitro* studies have examined the specificity of the host responses to infection with the parasite at the molecular level after 2 hours of contact, the specificity of the haemocyte response has been tested using live and dead parasite (Morga et al., 2010a).

Galectins are able to bind glycans present at the surface of micro organisms and favour their entrance of the pathogens inside host cells (Tasumi and Vasta, 2007). In the present study the expression level of Oe-Gal was highly up regulated at 1h and slightly up regulated at 2h. This result suggests the participation of oyster galectin to the parasite internalization process facilitating its binding to the host cell surface.
The expression level of the lysozyme gene appeared up regulated in haemocytes after 1, 2 and 4h of contact with the parasite. Similar results were observed in *Mercenaria mercenaria* infected with the parasite QPX (Quahog parasite X) (Perrigault et al., 2009). Lysozyme is a lysosomal enzyme with an important defence role as it can hydrolyse bacterial components. *O. edulis* lysozyme was previously identified by Matsumoto et al., (2006). Lysozyme activity has been detected in the body fluids and tissues of many bivalve molluscs and is believed to play a role in host defence and digestion (Takahashi et al., 1986, Cronin et al., 2001). Lysozyme concentrations were unchanged in clams *Ruditapes decussatus* infected by *Perkinsus atlanticus* (Ordás et al., 2000) and in oysters *C. virginica* infected by *P. marinus* (Chu et al., 1993). However, a subsequent investigation in oysters showed a slight decrease in lysozyme concentration in *P. marinus* infected oysters (La Peyre et al., 1995).

Four genes involved in degradation and cellular protection (OGST, CYP450, Oe-SOD Cu/Zn and Oe-EcSOD), were also studied by real time PCR. CYP450 and OGST were up regulated after 1h of infection. This result can be related to parasite internalization and the production of toxic components produced by the haemocyte and the parasite. Indeed, glutathione S-transferases play an important role in the detoxification of toxic compounds of endogenous and exogenous origin (Brophy and Pritchard, 1992 ; Brophy and Pritchard, 1994) and cytochrome p450 is involved in various functions notably oxidative metabolism. Cytochrome p450 has been studied in other bivalves subjected to parasite challenges (Tanguy et al., 2004, Perrigault et al., 2009). Perrigault et al., (2009) reported an up regulation of this gene after 14 days post QPX infection with in *M. mercenaria*.

Two genes (Oe-SOD Cu/Zn, Oe-EcSOD) involved in the oxidative stress were completely characterized and their expression levels were studied. Oe-SOD Cu/Zn homologous genes have been recently characterized in other oyster species including *C. gigas* (Huvet et al., 2004) and
Crassostrea virginica (Tanguy et al., 2004). Oe-EcSOD appeared similar to the permin or cavortin previously identified in other bivalve species such as Perna canaliculus, C. gigas and C. virginica (Scotti et al., 2001; Scotti et al., 2007). Surprisingly, these two genes presented a different expression pattern during the experiment. The cytoplasmic SOD appeared slightly up regulated after 2h and 4h while the extracellular SOD was down regulated after 1h and not significantly modulated at 2h, 4h and 8h. The down regulation observed after 1h of contact could be induced by the parasite in order to facilitate its own internalization and to escape degradation before internalization. The EC-SOD in Saccostrea glomerata was found up regulated in Marteilia sydneyi resistant oyster (Green et al., 2009). Cytoplasmic SOD up regulation might reflect the cell response to parasite internalization. ROS production showed a decrease after 1h, 2h and 4h. Decrease of ROS production could be related to the up regulation of the cytoplasmic SOD at 2h and 4h of haemocyte incubation with the parasite.

The present work is the first study combining cellular and molecular approaches to investigate the interactions between haemocytes from O. edulis and the parasite B. ostreae. Results showed internalisation of the parasite after 1h of contact and an increase of the number of the parasites observed inside infected haemocytes during the time course of the experiment suggesting a multiplication of the parasite especially after 2h of contact. Flow cytometry revealed a general decrease of esterase activities, ROS production and phagocytosis capacity in infected cells. Real time PCR measures showed an up regulation of some genes potentially involved in the entrance of the parasite within host cells (e.g. OeGal) or involved in the detoxification and degradation like lysozyme or cytochrome P450. Such results contribute to better understand how the flat oyster reacts against the infection with B. ostreae and how the parasite may escape these defence mechanisms.
Acknowledgement

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References


**Figure captions**

Figure 1 Mean number of parasites observed inside infected haemocytes and total number of binucleated cells observed inside haemocytes. Bars represent standard deviation of the mean number of parasites observed inside infected haemocytes.

Figure 2 Flow cytometry analysis for esterase’s activities, ROS and phagocytosis activity.

2a Percentages of positive haemocytes for non specific esterase activities after 1h, 2 h, 4h and 8h of incubation with parasites at 5:1 ratio. Values are means of three replicates and bars represent standard deviation (n=6). * indicates a significant difference of positive cells compared to the control.

2b Percentages of positive haemocytes for ROS production after 1h, 2 h, 4h and 8h of incubation with parasites at 5:1 ratio. Values are means of three replicates and bars represent standard deviation (n=6). * indicates a significant difference of positive cells compared to the control

2c Percentages of positive haemocytes for phagocytosis after 1h, 2 h, 4h and 8h of incubation with parasites at 5:1 ratio. Values are means of three replicates and bars represent standard deviation (n=6). * indicates a significant difference of positive cells compared to the control

Figure 3 Complete ORF nucleotide and deduced amino acid sequences of the flat oyster *Oe-SOD Cu/Zn*. ORF contains some conserved (boxed) amino acids (C$^{60}$, C$^{149}$, H$^{50}$, H$^{52}$, H$^{66}$, H$^{66}$, H$^{74}$, H$^{83}$ and H$^{123}$ and D$^{86}$) and signature (surrounded) amino acids (GFHVHQFGDNT and GNAGGRLACGVI).
Figure 4 Complete ORF nucleotide and deduced amino acid sequences of the flat oyster *Oe-EcSOD*. ORF contains a signal peptide (MQSLILVLAALFYV\textsuperscript{20}) and a glycosylation site (NLS\textsuperscript{41}) boxed and surrounded respectively on the sequence. The polyadenylation signal (AATAAA) is underlined in grey.

Figure 5 Neighbour-joining (NJ) tree showing phylogenetic analysis of both SOD (cytoplasmic and extracellular) amino acid sequences from various species. The tree is based on 1000 bootstrap replications. The scale for branch length (0.1 substitutions/site) is shown below the tree.

Figure 6 Relative expression by quantitative PCR of selected genes (HSP 90, CYP450, OGST, Oe-SOD Cu/Zn, Oe-EcSOD, OeGal and lysozyme) after 1h, 2h, 4h and 8h of incubation with *Bonamia ostreae*. Expression levels were normalized to EF1-\(\alpha\) and presented as relative expression to controls (mean \(\pm\) SD, \(n = 4\)). Controls are arbitrarily assigned a value of 1. * indicates a significant difference of gene expression compared to controls.
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<th>Concentration of Reverse primer (µM)</th>
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Table 1 Combinations of primers used in quantitative PCR assays
Fig1
Fig 3
Fig 4
Fig 5

Cytoplasmic SOD

Extracellular SOD

Fig 5
Fig 6