New insights in flat oyster *Ostrea edulis* resistance against the parasite *Bonamia ostreae*

B. Morga, T. Renault, N. Faury, I. Arzul[*]

Institut Français de Recherche pour l'Exploitation de la Mer (Ifremer), Laboratoire de Génétique et Pathologie (LGP), Avenue de Mus de Loup, 17390 La Tremblade, France

[*]: Corresponding author : Isabelle Arzul, Tel.: +33 5 46 76 26 10; fax: +33 5 46 76 26 11.
email address : iarzul@ifremer.fr ; isabelle.arzul@ifremer.fr

Abstract :

Bonamiosis due to the parasite *Bonamia ostreae* has been associated with massive mortality in flat oyster stocks in Europe. Control of the disease currently relies on disease management practices and transfer restriction. Previously, massal selections based on survival to challenge to infection with *B. ostreae* have been applied to produce flat oyster families with resistant progeny. In an attempt to understand the molecular mechanisms involved in disease resistance, differentially expressed sequence tags between resistant and wild *Ostrea edulis* haemocytes, both infected with the parasite, were identified using suppression subtractive hybridisation. Expression of seven ESTs has been studied using quantitative reverse-transcriptase PCR. The base-line expression of an extracellular superoxide dismutase, inhibitor of apoptosis (OeIAP), Fas ligand (OeFas-ligand) and Cathepsin B was significantly increased, whilst cyclophilin B appeared significantly decreased in resistant oysters. Considering their great interest for further studies, the open reading frames of the OeFas-ligand and OeIAP were completely sequenced.

Graphical abstract
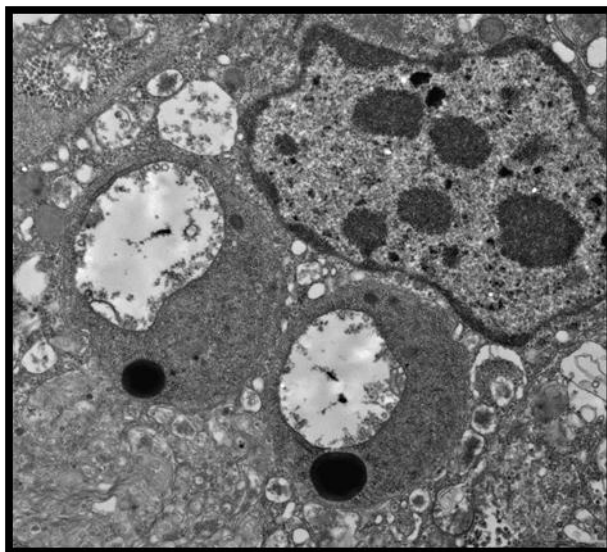
Highlights

► *Bonamia ostreae* is an intra-haemocytes parasite. ► Flat oyster *Ostrea edulis*, is the natural host of *B. ostreae.* ► In this study we have studied genes expressed in response to the parasite. ► Extracellular superoxide dismutase (OeEcSOD) is up regulated in resistant haemocyte. ► Genes involved in apoptosis is up regulated in resistant haemocytes from flat oyster

Keywords : Protozoan ; *Ostrea edulis* ; Flat oyster ; Transcriptomic ; Gene expression

1. Introduction

Oyster farming is currently exposed to heavy mortality associated with the detection of pathogens (virus, bacteria and parasites). Treatment cannot be used because oysters are mainly cultured in open areas and vaccine cannot be developed because molluscs lack lymphocytes and antibodies. Consequently bivalve disease control generally relies on stock management and transfer restriction. Development of resistant animals when possible can also help to mitigate disease impacts.

Bonamia ostreae has contributed to decrease flat oyster *Ostrea edulis* production in France since the end of the seventies [1] and [2]. This protozoan is affiliated to the order of haplosporidia and to the phylum of cercozoan [3]. *B. ostreae* is most often observed inside the haemocytes [1] and [2], but it can also be observed extracellularly in the digestive gland and in the gills [4]. Haemocytes, the circulating cells present in haemolymph, play a key role in the immune response of molluscs. Bivalves lack specific immune system and immune memory. Their immune response relies on innate cellular and humoral mechanisms both operating in coordination to recognize and eliminate pathogens. Phagocytosis is the main cellular immune response against pathogens in molluscs [5]. Thus, the better comprehension of interactions between the immune system of oysters and pathogens are key factors to understand the development of related diseases.

Selective breeding programs were initiated in Ireland and France, with the main objective of producing flat oysters resistant to bonamiosis [6] and [7]. In France, this program was first initiated in 1985 by Ifremer (Intsitut Français de Recherche pour l'Exploitation de la Mer), producing two oyster populations by mass spawning [8]. Individual selection was applied through inoculation tests and field testing, the surviving oysters being used to produce the next generation. Three generations of selection were produced in Ifremer hatchery. At that stage, microsatellite analyses showed that these strains exhibited low genetic diversity due to population bottlenecks, leading to small effective population sizes and subsequent inbreeding [9]. As a result, the second stage of this selective breeding program consisted of the production of bi-parental families combined with within-family selection. Families produced in 1995 consisted of within-strain crosses. Then, in order to maximize genetic variability, families produced in 1998 were issued from among-strain crosses between two families previously produced. These families showed enhanced survival and lower prevalence of the parasite compared with control wild-type oysters in *B. ostreae* contaminated areas [7].

Such material is also helpful to better understand resistance basis. As an example, in the Sydney rock oyster, *Saccostrea glomerrata* the use of selected oyster against the parasite *Marteilia sydneyi* allowed identifying genes involved in the resistance to the disease [10]. In the present study, a comparison of the molecular responses between a resistant population and some wild oysters was undertaken using suppression subtractive hybridization (SSH), a PCR-based technique that allows the identification of genes that are differentially expressed between two conditions. In addition, RT-PCR assays were developed in order to estimate expression level of ESTs of interest during an *in vitro* infection of haemocytes from resistant and wild oysters. Finally, the open reading frames of two selected genes (OeFas-ligand and OeIAP) were completely sequenced and characterized.

2. Material and methods

2.1 Biological material

2.1.1 Oysters

Eighteen-month-old flat oysters *Ostrea edulis* (n=90) were collected from Quiberon Bay (Southern Brittany, France), a bonamiosis endemic zone. They were acclimatized in the quarantine facilities of Ifremer laboratory in La Tremblade (Charente Maritime, France) over 30 days. These oysters are considered as the wild population in this study.

The resistant oysters were produced in the Ifremer hatchery from Argenton (Northern Brittany, France) from spawners previously selected for their resistance to bonamiosis. When they were 18 months old, they were transferred and acclimatized in the quarantine facilities of Ifremer laboratory in La Tremblade (Charente Maritime, France) over 30 days.

Flat oysters were maintained in 120 l raceways supplied with a constant flow of seawater enriched in phytoplankton (*Skeletonema costatum*, *Isochrisis galbana*, *Chaetoceros gracialis* and *Tetraselmis succica*).

2.1.2 Haemolymph collection

Haemolymph was withdrawn from the adductor muscle using a 1mL syringe equipped with a needle (0.40x90mm). Haemolymph samples were filtered on a 75 µm mesh to eliminate debris and maintained on ice to prevent cell aggregation. Haemolymph samples were pooled. Haemocyte counts were performed using a Malassez-cell and cell concentration was adjusted at 2.10^6 cells mL^{-1} with filtered seawater at 0.22 µm (FSW).

2.1.3 Parasites

B. ostreae was purified according to a previously published protocol [11]. 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 FSW. *B. ostreae* cells were then counted using a Malassez-cell and parasite concentration was adjusted at 10.10^7 cells mL^{-1} with FSW.

2.2 *In vitro* infection protocol

In vitro infection experiment including infected haemocytes from resistant oysters and infected haemocytes from wild oysters was performed in order to construct forward and reverse SSH libraries.

The expression level of some genes identified through SSH was then evaluated through a second *in vitro* experiment including two conditions i.e. (1) infected haemocytes from resistant oysters and (2) infected haemocytes from wild oysters.

Whatever was the experiment, haemocytes were maintained in contact with purified parasites during 2 h. For that purpose, 5 ml of haemocyte suspension were introduced in plastic cell culture flasks and incubated at 15°C for two hours until the formation of a cell layer. Supernatant was then withdrawn, preserved after 0.22 µm filtration and replaced by 500 µl of parasite suspension. After 2 hours, supernatant previously filtered at 0.22 µm to eliminate bacteria, was introduced again in flasks. After 12-hour incubation, cells were rinsed twice with phosphate buffer saline (PBS 1X) and were processed for RNA extraction using TRIZOL reagent (Invitrogen) following the manufacturer's instructions.

2.3 Suppression subtractive hybridisation (SSH)

One µg of mRNA from the haemocytes exposed to the parasite and 1 µg of mRNA from the haemocytes alone were used as templates for the SSH following the PCR-select cDNA subtraction kit procedure (Clontech, Palo Alto, CA) [12]. Forward subtraction was carried out using the haemocytes from resistant oysters exposed to the parasite as the tester and the haemocytes from wild oysters exposed to the parasite as the driver. The opposite was performed for reverse subtraction. PCR products were cloned using TOPO TA Cloning Kit (Invitrogen). White colonies were screened by macro-arrays. Inserts were PCR amplified using TOPO F and R primers (Table 3) and 1 µL of PCR products was spotted in duplicate onto nylon membrane (Roche Diagnostic). cDNA was digoxygenin labelled and used as probe in hybridisation experiments using the DIG-labelling and detection kit according to the manufacturer's instructions (Roche Diagnostic).

2.4 Sequencing and sequence analysis

Clones showing a differential digoxigenin labelling intensity between tested conditions were selected and amplified using TempliPhi DNA Sequencing Template Amplification Kit (Amersham's). PCR products isolated from individual clones were sequenced in one way with TOPO F 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 analysed with Chromas 231 software. Sequences were then analysed with BlastX algorithm available from the National Center for Biotechnology Information (NCBI). EST sequences were then submitted to dbEST and GenBank databases (http://www.ncbi.nlm.nih.gov/blast/). Only E values less than 10^{-4} were considered significant.

2.5 Identification and characterization of immune-related genes

In order to obtain the complete open reading frames (ORF) of Fas-ligand (OeFas-ligand) and inhibitor of apoptosis, IAP (OeIAP), RACE PCR 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) (5'racefas TGAGTTAGCCCTGTTTCTCCACCAG, 3'racefas CTGGTGGAGAAACAGGGCTAACTCA; 5'raceIAP CCTCCATGTCAAAAATCACATTAGC, 3'raceIAP ATGTGATTTTTGACATGGAGGAGAG). After ligation and cloning in TOPO Vector System (Invitrogen) and transformation in Top 10 competent bacteria (Invitrogen), several clones were sequenced using the same protocol as described before. Open reading frames were identified using the ORF finder in NCBI. Complete sequences were deposited in GenBank and assigned under the accession numbers GU320694 (OeFas-ligand) and GU814273 (OeIAP). The isoelectric point and molecular mass were calculated in http://www.expasy.ch/tools/pi_tool.html.

2.6 Phylogenic analysis of OeFas-ligand

The sequence of the complete ORF of OeFas-ligand was aligned with Fas ligand, TNF alpha and lymphotoxin alpha genes from GenBank database.
Alignments were performed using the Clustal W [13] including in MEGA 4 [14]. A phylogenic tree based amino acid sequences was designed using the Neighbour-Joining (NJ) [15] algorithm with the MEGA 4 s oftware program. Statistical confidence on the inferred phylogenic relationships was assessed by bootstrap of 1000 replicates.

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

Total RNA was extracted using TRIZOL reagent (Invitrogen) following the manufacturer's instructions. First strand cDNA was synthesized using the oligo(dT) anchor primer (5'-GAC CACGCGTATCGATGTCGACT(16)V-3') and Moloney murine leukaemia virus (M-MLV) reverse transcriptase SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen).
For each of the selected genes, forward and reverse primers were designed using primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Real-time quantitative PCR reactions were duplicated and performed according the previous study presenting in Morga et al., 2010 [17].
For each candidate gene, melting curve and gel picture were analysed in order to verify the specificity of the amplified products and that at 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 $E=10^{(1-slope)}$ [16].
The expression of the candidate genes was normalized using the elongation factor 1 alpha (EU651798) as the housekeeping gene. Primers are shown in Table 1 [17]. Resistant infected haemocytes were used as a control in order to identify the relative expression of selected genes in infected wild haemocyte. Wild infected haemocytes were used as a control in order to identify the relative expression of selected genes in infected resistant haemocyte Fold units were calculated using the method described by [16].

3. Results

3.1 Identification and selection of candidate ESTs

The SSH technique was used to identify transcripts differentially expressed between infected haemocytes from resistant oysters and infected haemocytes from wild oysters. Two thousands and two hundred eight clones were sequenced and 1920 sequences (95% of the sequenced clones) were obtained and analysed (see Table 2). These sequences ranging from approximately 152 to 670 bp in length were analysed for the redundancy by multiple alignments using the Lasergene software. Finally, 909 unique ESTs were identified including 333 contigs and 606 singletons. Among these, 382 ESTs showed similarities with genes available in databases with an E value< 10-4 clustering in 8 main categories according to their putative functions predicted by NCBI using GO (Gene Ontology) (Fig. 1); (1) detoxification and stress, (2) cell communication, membrane receptor and immune system, (3) ribosomal protein, (4) cystoskeleton structure, (5) respiratory chain, (6) cell cycle, DNA repair, protein regulation and transcription, (7) cell metabolism and (8) hypothetical proteins. Based on their putative function, seven ESTs have been selected in (1) detoxification, (2) cell communication, membrane receptor and immune system (extracellular superoxide dismutase (OeEcSOD), inhibitor of apoptosis (OeIAP), OeFas-ligand, cathepsin B and cyclophilin, ferritin and OeC1q) (Table 3a and 3b). Extracellular superoxide dismutase (OeEcSOD), inhibitor of apoptosis (OeIAP), OeFas-ligand, cathepsin B and ferritin have been selected in forward bank and Oe-C1q and cyclophylin were selected in reverse bank.

3.2 Characterization of Ostrea edulis immune-related genes

3.2.1 Ostrea edulis Fas ligand (OeFas-ligand)

OeFas-ligand cDNA showed a 1097 nucleotide (nt) sequence. The sequence contained a short 5'-untranslated region (1-29 nt) followed by an ORF of 863 nt (287 codons: 30-893 nt) and finished by a 3'-untranslated region (894-1097 nt) (Fig. 2). The complete ORF product had a putative molecular mass at 32 442.70 Da and a predicted isoelectric point at 6.75. The analysis of the ORF product revealed the presence of the transmembrane domain (MIFSCAVNVVLAIVFGVLVGLNW) and a TNF domain (154-257). Moreover, tree N-glycosylation sites at ^{57}NATV^{60}, ^{85}NETL^{88} and ^{125}NSSS^{128} could be identified. Signal peptide prediction results showed that there was a putative signal peptide in the OeFas-ligand (MSAPSSRNCKCFMIFSCAVNVVLAIVFGVLVG).

Phylogenic tree analysis of Fas ligand, TNF alpha and lymphotoxin alpha grouped OeFas-ligand within the Fas ligand clade and showed closest affinity with its homologous in abalone *Haliotis discus hannai* (Fig. 3).

3.2.2 Ostrea edulis Inhibitor of Apopotosis (OeIAP)

OeIAP cDNA was composed of 1241 nucleotides (nt) in length. The sequence contained a short 5'-untranslated region (1-16 nt) followed by an ORF of 1076 nt (377 codons: 17-1150 nt) and finished by a 3' -untranslated region (1151-1241 nt) (Fig. 4). The complete ORF product showed a molecular mass at 41646.89 Da and predicted isoelectric point at 5.63. ScanProsite analysis revealed that the predicted *OeIAP* protein contained one BIR domain (93-158 aa), followed by a RING domain (330-365) at its C terminus. Moreover, it was identified one N-glycosylation sequence at ^{51}NETD^{54}. The BIR domain of *OeIAP* had the conserved BIR motif of $CX_2CX_6WX_3DX_5HX_6C$ [18]. OeIAP is more similar to the ML-IAP (livin), a human IAP that contains a single BIR and RING finger domain than to IAP identified in invertebrates. The multiple alignment of BIR domains from *Penaeus monodon* IAP (*PmIAP* ABO38431.1), *Drosophila melanogaster* (*DIAP1* Q24306.2) and (*DIAP2* AAF58095.1) and *Homo sapiens sapiens ML-IAP* (*livin* NP_647478.1) showed a maximum of 62% identity with the BIR3 from *PmIAP* (Fig. 5). The multiple alignment of RING domain between *Oe-IAP* RING domain and the DIAP1, DIAP2, PmIAP and livin RING domain showed 61% of identity with livin RING domain (Fig. 5).

3.3 Haemocyte expression of selected ESTs

In order to verify that differential expression of genes identified by SSH existed between resitant and wild oysters, qRT-PCR was carried out to determine the relative expression of the target genes. SSH libraries allowed identifying seven ESTs potentially involved in the resistance or susceptibility to bonamiosis. Significant differences in expression were observed between resistant and wild oysters for five of the seven transcripts. OeEcSOD, OeIAP, OeFas-ligand and Cathepsin B were found to be 2.4, 6.5, 3.8 and 3.6-fold over expressed respectively in resistant oysters compared to wild ones (Fig. 6). Cyclophylin was 0.07 fold less expressed in resistant oysters (Fig. 6). No significant difference was found in the expression of Oe-C1q and ferritin between resistant and wild oysters by qRT-PCR (Fig. 6).

4. Discussion

In the present study, we compared the molecular response of haemocytes from two populations (resistant and wild) to the parasite *B. ostreae*. Molecular responses were studied using suppression subtractive hybridization method (SSH) and real time PCR method. SSH has already been used to better understand host/pathogen interactions at a molecular level in different bivalve species including *Crassostrea gigas* [19], *Crassostrea virginica* [20], *Saccostrea glomerata* [10], *O. edulis* [21], *Ruditapes phillipinarum* [22] and *Ruditapes decussatus* [23].

In our study, this method allowed the identification of 1920 ESTs differentially expressed between infected haemocytes from resistant and wild oysters.

A total of 382 ESTs showed similarities with genes available in databases with an E value< 10-4. These ESTs were classified in 8 main categories according to their putative functions predicted by NCBI using GO.

Seven ESTs were selected according to their putative biological function. We have focused on genes involved in detoxification and stress and in cell communication, membrane receptor, and immune system.

The expression pattern of these ESTs was measured in an *in vitro* infection experiment. Real-time PCR analysis confirmed the differential expression of five out of the seven ESTs (more than one fold difference) while Oe-C1q and Ferritin showed similar expression levels between tested conditions.

A gene presenting homology with C1q was previously identified in clam species like *Mercenaria mercenaria*, *Mytilus galloprovincialis* and *Ruditapes decussatus* [23], [24] and [25]. C1q is the first subcomponent of the C1 complex of the classical pathway of complement activation which is crucial for the clearance of pathogens in vertebrates [26] and invertebrates [27]. The C1q gene was significantly up regulated in *Mercenaria mercenaria* and *R. decussatus* in response to QPX and *Perkinsus olseni,* respectively [24] and [23] while in our study the expression of this gene was not affected by the status of the oyster resistant or wild Although C1q gene was identified in *S. glomerata* resistant population to the parasite *M. sydneyi*, the expression of this gene appeared not modulated in resistant population compared to wild oyster population [10].

Ferritin has been classified as a stress protein [19] and has also been associated with defence mechanisms because of its role in the regulation of iron availability to infectious agents [28]. Previous studies showed an increase of ferritin expression in invertebrates following exposures to pathogen-associated molecular patterns (PAMPs) or bacterial challenge [19] and [29]. [24] Perrigault et al. have reported an increase of ferritin expression in clams challenged by Quahaog parasite X (QPX) after 28 and 48 days. In our study, we did not observe difference between wild and resistant oysters suggesting that this mechanism is not involve in resistance or susceptibility to *B. ostreae.*

The putative cyclophilin gene was significantly down regulated in infected haemocytes from resistant oysters. On the contrary, cyclophilin was found up-regulated in response to the bacteria *Vibrio splendidus* 10 hours after inoculation in resistant cupped oyster *Crassostrea gigas* [30]. Cyclophilin, a peptidylprolyl isomerase is known in mammals to mediate signalling events leading to T-cell activation [31].

A decrease of phagocytosis activity has been observed when comparing activities of hameocytes from *B. ostreae* infected and no infected resistant oysters (morga pers. com.). This apparent phagocytosis inhibition could be related to the down regulation of the putative cyclophilin observed in the present study.

The over expression of OeEcSOD gene in resistant flat oysters might be related to an increased production of cytotoxic components generated during an immune response as shown by [10] Green et al. in *S. glomerata*. SOD is known to be involved in the regulation of oxidative stress response. [21] Morga et al. have shown that Oe-EcSOD presented a high sequence homology with other bivalve ecSODs, notably Dominin from the eastern oyster, *C. virginica* [20] with Carvortin from the Pacific oyster, *C. gigas* [32] and with ecSOD from *Saccostrea glomerata* [10]. The ability of resistant *O. edulis* to generate hydrogen peroxide at a faster rate and higher concentration than wild flat oysters during respiratory burst could explain their better survival to infection with *B. ostreae*. In a previous study, we showed a significant decrease of reactive oxygen species (ROS) production in wild flat oyster haemocytes after an *in vitro* infection of *O. edulis* haemocytes with live parasites versus haemocytes alone [33]. Moreover a recent study on the relative expression of this gene after an *in vitro* infection of haemocytes from wild flat oyster with the parasite *Bonamia ostreae* revealed a down regulation of OeEcSOD after 1h of contact [21]. An increased production of

SOD in resistant oysters may be regarded as an advantage in controlling negative effects of ROS production on host cells.

Three genes involved in apoptosis (programmed cell death) have been found over expressed in resistant oysters in response to the infection with the parasite *B. ostreae*: genes encoding Fas Ligand, an IAP and cathepsin B. Apoptosis is a common physiological process that eliminates unwanted or diseased cells, and it plays important roles in embryogenesis, homeostasis, metamorphosis and immunity [34] and [35]. The mammalian extrinsic apoptosis pathway is regulated by various genes and factors, including Fas (APO-1 or CD95), Fas ligand (CD95 ligand), tumour necrosis factor (TNF) and related apoptosis-inducing ligand (Apo2L/TRAIL). It is well known that the Fas and Fas ligand (FasL) system plays a central role in apoptosis [35]. Fas is a type I transmembrane glycoprotein that mediates apoptosis and its biological ligand (Fas ligand) induces apoptosis through the binding to Fas [36]. Phylogenic tree analysis of OeFas ligand has shown closest affinity with its homologous in abalone *Haliotis discus hannai*.

The over expression of the OeFas ligand in infected haemocytes from resistant oysters suggested that the product of this gene is involved in the immune response against the parasite *B. ostreae*.

Inhibitors of apoptosis proteins (IAPs) are a conserved group of proteins that regulate apoptosis in both vertebrates and invertebrates. Since the first discovery of an IAP gene in a baculovirus [37], numerous cellular IAP homologues have been identified [38] The hallmark of an IAP protein is the presence of one to three copies of a zinc-binding baculoviral IAP repeat (BIR) domain. These BIR domains are essential for the anti-apoptotic properties of the IAPs and directly bind to the caspases in order to inhibit them [39] and [40]. The structure of the OeIAP is more similar to the ML-IAP (livin) from human [41] and [42] but the multiple alignment of the BIR domain from OeIAP with BIR domains from other species showed a high identity with the BIR3 domain from *Penaeus monodon*.

Cathepsins have often been considered as intracellular proteases capable of mediating caspase-independent cell death [43]. Cathepsins have recently been shown to be associated with cell death regulation [44] and [45]. The over expression of the cathepsin B in resistant infected haemocytes leave suspect an implication of this gene in the apoptosis pathway according the previous results notably the over expression of the OeFas-ligand and the OeIAP.

All these results suggest that apopotosis plays a key role in the resistance of flat oysters to bonamiosis. Both the parasite and the host might influence this mechanism. By inducing the expression of the anti-apoptotic gene OeIAP, *B. ostreae* seems to inhibit apoptosis in order to survive inside the haemocytes. As for the host, our results seem to indicate that resistant oysters eliminate or limit the development of the parasite by inducing apoptosis. This hypothesis is supported by the over expression of Oe-Fas-ligand observed in resistant oysters versus wild oysters. The manipulation of the host apoptosis by intracellular parasites is currently described in vertebrate and invertebrate hosts [39]. Mammalian intracellular parasites such as *Toxoplama gondii*, *Trypanosoma cruzi, Plasmodium sp.*, *Cryptosporidium parvum* are able to trafficking the apoptosis, [46]. Macrophages and T cells infected by the parasite *Toxoplasma gondii* are able to induce apoptosis by the Fas ligand. However, an inhibition of the apoptosis has also been reported in the macrophage and different cell lines by the up regulation of the anti-apototic homologue such as Bcl-2 [47]. In invertebrates, apopotosis seems to play also a major role in the response to pathogens like in the oyster *C. virginica* against *Perkinsus marinus* [48] or in the clam *R. decussatus* against *P. olseni* [23]. A large number of genes involved in apoptosis has recently been identified in *C. gigas* and was found overexpressed in the context of an infection with *Vibrio anguillarum* [49].

Our study is the first one investigating resistance of a marine bivalve to an infection with an obligate intracellular parasite. It has not only generated new data concerning the flat oyster genome but has also contributed to a better understanding of the molecular basis involved in the resistance to *B. ostreae* infection. Two mechanisms including apoptosis (cathepsin B,

OeFas-ligand and OeIAP) and oxidative detoxification (Oe-EcSOD) appear involved in the resistance to the parasitosis. Further functional studies are now required to better characterized how these mechanisms allow the host to resist to *B. ostreae*.

Acknowledgement

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5. Figure legends

Figure 1 Functional classification of the sequences identified in both libraries (ESTs). Genes were clustered into 8 categories according to their putative biological function (A Forward bank and B reverse bank).

Figure 2 Complete ORF nucleotide and deduced amino acid sequences of the flat oyster OeFas-ligand. ORF contains a peptid signal (boxed in black), a transmembrane domain (uderlined in black) and a TNF domain (dotted in black). N-glycosylation sites were surrounded.

Figure 3 Neighbour-joining tree showing phylogenetic analysis of Fas ligand, TNF alpha and lymphotoxin sequences from various species (Fas ligand: *Homo sapiens sapiens* NP000630, *Mus musculus* NP034307, *Rattus norvegicus* NP037040, *Macaca mulatta* AB035139.1, *Sus scorfa* AB069764.1, *Haliotis discus discus* ASJ12607; TNF alpha: *Homo sapiens sapiens* M10988.1, *Mus musculus* U68414.1, *Rattus norvegicus* X66539.1, *Sus scorfa* X57321.1, *Bos Taurus* NP776391, *Xenopus tropicalis* AM041993.1, *Oreochromis niloticus* AY428948.1, *Paralichtys olivaetus* AB040448.1, *Ictalurus punctatus* AJ417565.2, *Salmo salar* NP001117061; Lymphotoxin alpha: *Homo sapiens sapiens* X01393.1, *Rattus norvegicus* NP542947, *Sus scorfa* NP999618, *Bos Taurus* Z14137.1, *Xenopus tropicalis* AM041994.1). The tree is based on 1000 bootstrap replications. The scale for branch length (0.1 substitutions/site) is shown below the tree.

Figure 4 Complete ORF nucleotide and deduced amino acid sequences of the flat oyster OeIAP. ORF contains a BIR domain (uderlined in black) and a RING finger domain (dotted in black). Signature amino acids were boxed in red. N-glycosylation sites were surrounded.

Figure 5 Multiple sequence alignments of the BIR and RING domains of OeIAP with the corresponding domains of other IAP family members : *Penaeus monodon* IAP (*PmIAP* ABO38431.1), *Drosophila melanogaster* IAPs (*DIAP1* Q24306.2) and (*DIAP2* AAF58095.1) and *Homo sapiens sapiens ML-IAP* (*livin* NP_647478.1). Identical residues are indicated in black and similar residues are indicated in grey.

Figure 6 Relative expression by quantitative PCR of selected transcripts from SSH libraries (OeIAP, OeFas-ligand, OeEc-SOD, OeC1q, ferritin, cathepsin B and cyclophillin). Expression levels were normalized to EF1-a and presented as relative expression to controls (mean ± SD, n = 2). * Indicates significant differences of gene expression compared to controls.
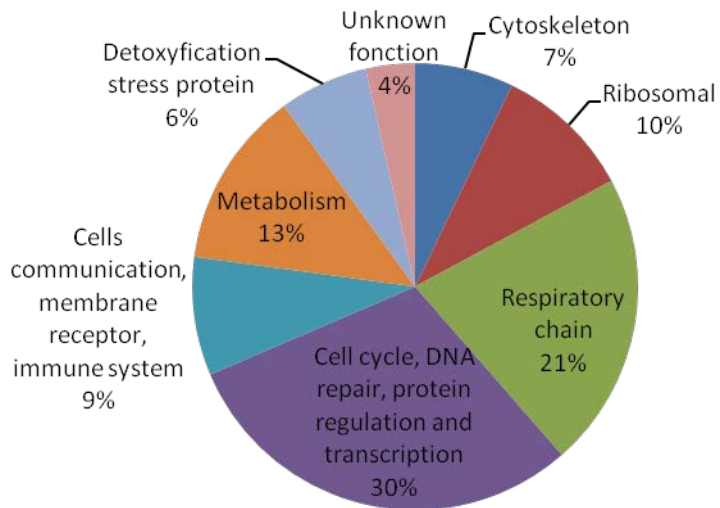
A
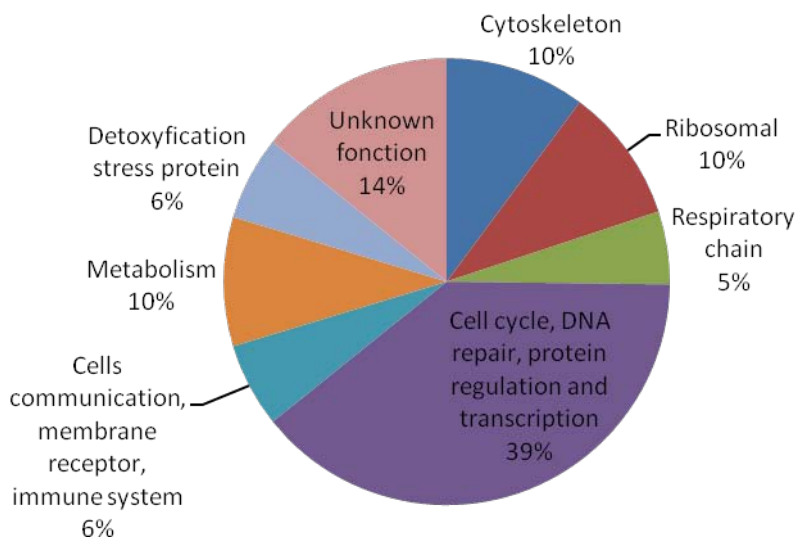
B

Figure 1

```
           10         20         30         40         50         60         70         80
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
1   TA CG CGGGA GA GA T CAAAA CA CA T T CA GGA TGA GTG CT C CA T CGA GT CGA AA T TG CA AG TG CT T CA TGA TA T T T T CCT GT  80
                             M  S  A  P  S  S  R  N  C  K  C  F  M  I  F  S  C
```
```
           90        100        110        120        130        140        150        160
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
51  GCA GTA AA T GTG GTA CT T GCGA TA GTA T T T GGC GT GT T GGTA GGA CTGA A T T GGGA CAAA T TA CT TGA CAA CTA T T T T CG  160
       A  V  N  V  V  L  A  I  V  F  G  V  L  V  G  L  N  W  D  K  L  L  D  N  Y  F  R
```
```
          170        180        190        200        210        220        230        240
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
161 GT T T T CAAA CT CGGA GA CT CCA CAA T CA GTGA T CGGAAA TG CAA CTGTAA GCGGGA AA GCCA TAA CGA T TA GA GA CT CTG  240
      F  S  N  S  E  T  P  Q  S  V  I  G  N  A  T  V  S  G  K  A  I  T  I  R  D  S
```
```
          250        260        270        280        290        300        310        320
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
241 T T T GT T T T T CT T GTGA CTA T T T GGG CA GTG GT GTA A GTGTAAA TGAAA CA CTA TA CGA GGAAA T CGT TA CAA CAGA T TGT  320
      V  C  F  S  C  D  Y  L  G  S  G  V  S  V  N  E  T  L  Y  E  E  I  V  T  T  D  C
```
```
          330        340        350        360        370        380        390        400
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
321 AAA CA CAAA CTGTG CTG CA TAAAA GA CAAA GTGA T CCA TGA T T T T GTGA TA GCGA CT CAAA TGAAAA TAA CGAAAA TGA  400
      K  H  K  L  C  C  I  K  D  K  V  I  H  D  F  V  I  A  T  Q  N  E  N  N  E  N  E
```
```
          410        420        430        440        450        460        470        480
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
401 AAA CA GT T CA T CAA T TA GGGA T T CCT T CGCCT GGT GGA GAAA CA GGG CTAA CT CA GCCCA T T T CTA T CTGGA CCGGGAAA  480
      N  S  S  I  R  D  S  F  A  W  W  R  N  R  A  N  S  A  H  F  Y  L  D  R  E
```
```
          490        500        510        520        530        540        550        560
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
481 T CCCCGTA T CAAA GAAAA CAAA CT T CGT TGGAAA GCTGA CGA TA CA TA TGGGA CTG CA T T T GCA CAA GA TA TA GT T CT T  560
      I  P  V  S  K  E  N  K  L  R  W  K  A  D  D  T  Y  G  T  A  F  A  Q  D  I  V  L
```
```
          570        580        590        600        610        620        630        640
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
561 GT CAA TGGAA CT CA CA TGA GAA T CAAA CAA GA TGGAA T CTA CT T CA T CTA CA CT T CCA CCA CCT T CGA CCA TA GTA GGCA  640
      V  N  G  T  H  M  R  I  K  Q  D  G  I  Y  F  I  Y  T  S  T  T  F  D  H  S  R  Q
```
```
          650        660        670        680        690        700        710        720
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
541 GGA CA CGGTGAAA CCA T CTA T CAAA GT CTGA T CAA GTA CCA CCCT TA T T TA CCAAA GA CA GGCGA CGT GGA CCT CGT T T  720
      D  T  V  E  T  I  Y  Q  S  L  I  K  Y  H  P  Y  L  P  K  T  G  D  V  D  L  V
```
```
          730        740        750        760        770        780        790        800
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
721 T CA GCAA GT T GGT GGA T CCCGT GAA T CTGA CA GA CA T CA CA CAAA T T T T T T GGCA GGA GT GT T CAA GCTGGAA CCCGGA  800
      F  S  K  F  G  G  S  R  E  S  D  R  H  H  T  N  F  L  A  G  V  F  K  L  E  P  G
```
```
          810        820        830        840        850        860        870        880
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
801 T T CCT CA T CGCAA GT T CCCT T T CT CA GA GA GGA CA CGTGT T T CT TGA TG CA T CT T CGCCCA TAAAAAA T TA TA T CGGG CT  880
      F  L  I  A  S  S  L  S  Q  R  G  H  V  F  L  D  A  S  S  P  I  K  N  Y  I  G  L
```
```
          890        900        910        920        930        940        950        960
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
881 A TA TAA GT TA TA GCTA CCA GA TA CAA TA GT TGAAA CTGAAA TGA T T TGA TA CTGA GA GTA CA CTA GCTGT GTAAA TG CCA  960
      Y  K  L  *  L  P  D  T  I  V  E  T  E  M  I  *  Y  *  E  Y  T  S  C  V  N  A
```
```
          970        980        990       1000       1010       1020       1030       1040
    ····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|····|
961 GT TGA TAA TA T T T CT T TA CA GA CCGA T CGAA GA T CA GAA CA CTA AGA GTGTAAA T CA T T T T GT CAAA CA GTGA T T T T T CT C  1040
      S  *  *  Y  F  F  T  D  R  S  K  I  R  T  L  R  C  K  S  F  C  Q  T  V  I  F  L
```

Figure 2

15

Figure 3

Figure 4

TNF alpha

Lymphotoxin alpha

Xenopus tropicalis lymphotoxin alpha

Paralichthys olivaceus TNF alpha

Xenopus tropicalis TNF alpha

Oreochromis niloticus TNF alpha

Salmo salar TNF alpha

Rattus norvegicus lymphotoxin alpha I

Ictalurus punctatus TNF alpha

Homo sapiens lymphotoxin

Sus scrofa lymphotoxin alpha (L

Mus musculus TNF alpha

Bos taurus lymphotoxin alpha

Rattus norvegicus TNF alpha

Homo sapiens TNF alpha

Haliotis discus discus Fas ligand

Bos taurus TNF alpha

Sus scrofa TNF alpha

Ostrea edulis Fas ligand

Sus scrofa Fas ligand

Rattus norvegicus Fas ligand

Mus musculus Fas ligand

Macaca mulatta Fas ligand

Homo sapiens Fas ligand

Fas ligand

0.1

	Identity	Similarity
OeIAP-BIR	51%	70%
HslvIn-BIR	25%	48%
PmIAP-BIR1	36%	66%
DmIAP1-BIR1	30%	48%
DmIAP2-BIR1		
	51%	70%
	31%	50%
	53%	74%
	34%	61%
	51%	70%
	62%	72%
	46%	65%
	61%	80%
	50%	75%
	44%	69%
	58%	80%

Figure 5

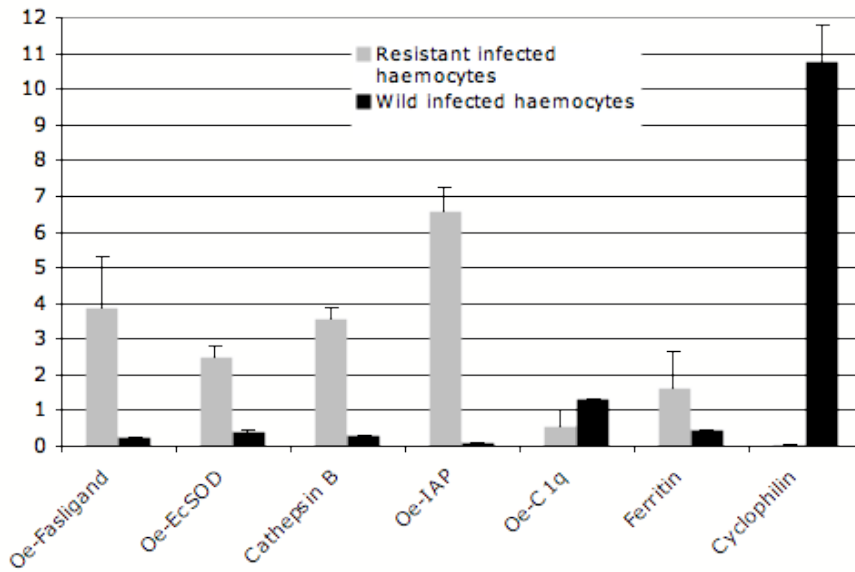Figure 6

Tables

Name	Oligonucleotide sequence (5'-3')	Concentration of Forward primer (µM)	Concentration of Reverse primer (µM)
OeIAP	Forward: CTACCTCCCCAGGATTGTCA Reverse: CACCACTCTCCTCCATGTCA	3	3
OeFas	Forward: TTTGGGCAGTGGTGTAAGTG Reverse: TAGCCCTGTTTCTCCACCAG	1.5	3
Cathep	Forward: CTGCACTGATCTGGGACTCA Reverse: ATTACYGGCTGGTGGCAAAC	3	3
OeEc-SOD	Forward:GAGGAGGAAGAGGACCATCC Reverse: ATTTTCCTCCGCTTTGTGTG	2,5	2,5
Oefer	Forward: GATGTGATCGGACACCTCCT Reverse: TCACAAAGTTGCAGGCAGTC	3	3
OeC1q	Forward: CAGTCCCTCAGAGCCTGTTC Reverse: ACAGGTATACGCCGGTTTTG	3	3
Oepepti	Forward: ATGAGCCAGGTCATCAAAGG Reverse: GGCCTAGGAGTTCCACATCA	3	3
ElongPCRQ5m	Forward:GTCGCTCACAGAAGCTGTACC Reverse: CCAGGGTGGTTCAAGATGAT	3	3
TOPO	Forward: GACCATGATTACGCCAAGC Reverse: CCCAGTCACGACGTTG		

Table 1: Combinations of primers used in quantitative PCR assays and for amplification of plasmid TOPO TA Cloning (Invitrogen).

Total clones sequenced	1920
Sequences analysed	1831
Total of sequences with significant match	382
Contigs	333
Singletons	606
Redundancy	48%
Average size number pb	450

Table 2: General characteristics of the subtracted library and cDNA sequences from *Ostrea edulis* haemocyte.

Putative match	*Species match*	E value	Lenght	Accession number
Detoxification and stress protein				
MAP-kinase activating death domain	*Xenopus tropicalis*	4e-04	397	NP_001072717
	Xenopus tropicalis	4e-04	397	NP_001072717
Ferritin	*Crassostrea ariakensis*	2e-31	360	ABE99842
	Crassostrea ariakensis	4e-42	516	ABE99842
Universal stress protein family	*Nematostella vectensis*	4e-05	384	XP_001641771
Metallothionein	*Ostrea edulis*	4e-25	302	CAC83770
heat shock protein 90 putative	*Albugo laibachii Nc14*	1e-39	419	CCA24455
	Albugo laibachii Nc14	2e-46	419	CCA24455
	Albugo laibachii Nc14	1e-48	482	CCA24455
	Albugo laibachii Nc14	4e-52	518	CCA24455
	Albugo laibachii Nc14	2e-39	416	CCA24455
HSP90 family member	*Thalassiosira pseudonana*	1e-52	633	XP_002291118
HSP70	*Acanthamoeba culbertsoni*	6e-21	216	AAU94664
dominin precursor	*Crassostrea virginica*	1e-39	589	BAF30874
heat shock protein 70B	*Crassostrea gigas*	1e-53	441	BAJ83619
Cell communication, receptor and immune system protein				
carboxypeptidase A5-like	*Saccoglossus kowalevskii*	2e-08	420	XP_002731444
inhibitor of apoptosis 2 protein	*Glossina morsitans morsitans*	4e-13	363	ADD19534
inhibitor of apoptosis protein	*Crassostrea gigas*	5e-31	540	AEB54800
Senescence-associated protein	*Brugia malayi*	3e-09	176	XP_001900327
	Brugia malayi	3e-09	176	XP_001900327
Similar to G-protein-coupled receptor induced protein	*Canis familiaris*	3e-07	525	XP_539160
cAMP-responsive element binding protein	*Crassostrea ariakensis*	5e-56	542	ABQ59345
E-selectin	*Canis lupus familiaris*	0.031	332	AAG10039
peptidyl-prolyl cis-trans isomerase	*Metarhizium anisopliae*	1e-39	552	EFZ01921
cyclophilin 1	*Theileria parva strain Muguga*	2e-36	482	XP_765771
putative peptidyl-prolyl cis-trans isomerase	*Maconellicoccus hirsutus*	1e-49	420	ABM55516
	Maconellicoccus hirsutus	6e-51	404	ABM55516
cathepsin B	*Pinctada fucata*	1e-30	411	ADX32985
Fas ligand-like protein	*Haliotis discus discus*	6e-06	482	ACJ12607
cystathionase	*Rattus norvegicus*	2e-15	298	EDL82568

Table 3A: Identified SSH up-regulated clones in categories 1 and 2 in *Bonamia ostreae* infected haemocytes from resistant flat oysters with significant database match (Forward bank).

Putative match	Species match	E value	Lenght	Accession number
Detoxification and stress protein				
Stress-associated endoplasmic reticulum protein 1	*Salmo salar*	1e-20	331	ACM08529
Similar to cytochrome P450, family 2, subfamily j, polypeptide 6	*Ciona intestinalis*	2e-11	328	XP_002124100
Ferritin GF2	*Crassostrea gigas*	1e-38	472	AAP83794
	Crassostrea gigas	5e-15	284	AAP83794
Heat shock protein 90	*Crassostrea hongkongensis*	2e-89	532	ADL59936
Similar to MEK5alpha-1	*Strongylocentrotus purpuratus*	2-16	326	XP_786275
Heat shock protein 70	*Crassostrea virginica*	1e-42	295	CAB89802
Universal stress protein family	*Nematostella vectensis*	4e-04	266	XP_001626758
Metallothionein	*Ostrea edulis*	1e-08	207	CAC83770
Cell communication, receptor and immune system protein				
cathepsin B sequence	*Pinctada fucata*	9e-80	511	ADX32985
Hypothetical protein isoform 1 containing immunoglobulin domain	*Strongylocentrotus purpuratus*	2e-11	301	XP_782924
	Strongylocentrotus purpuratus	4e-12	319	XP_782924
Peptidyl-prolyl cis-trans isomerase (cyclophilin)	*Haliotis discus discus*	8e-29	279	ABO26659
	Haliotis discus discus	4e-09	244	ABO26659
Cystatin B-like protein	*Crassostrea gigas*	3e-38	439	ADI33157
Peptidyl-prolyl cis-trans isomerase B	*Danio rerio*	4e-09	244	NP_998184
Very low-density lipoprotein receptor	*Culex quinquefasciatus*	4e-04	592	XP_001845800
Prostaglandin E2 receptor EP4 subtype-like	*Pongo abelii*	3e-05	148	XP_002815561
PREDICTED: similar to Neurogenic locus Notch protein precursor	*Ciona intestinalis*	4e-07	359	XP_002123017
Putative C1q domain containing protein MgC1q78	*Mytilus galloprovincialis*	4e-04	344	CBX41727
Senescence-associated protein	*Brugia malayi*	7e-44	453	XP_001900327

Table 3B: Identified SSH up-regulated clones in categories 1 and 2 in *Bonamia ostreae* infected haemocytes from wild flat oysters with significant database match (Reverse bank).