Fish & Shellfish Immunology 32 (2012) 958–968

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

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

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ARTICLE INFO

Article history:
Received 8 December 2011
Received in revised form
20 January 2012
Accepted 29 January 2012
Available online 2 March 2012

Keywords:
Protozoan
Ostrea edulis
Flat oyster
Transcriptomic
Gene expression

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.

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

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doi:10.1016/j.fsi.2012.01.026

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 *O. 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 1 mL syringe equipped with a needle (0.40 × 90 mm). 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 2 h 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 h, supernatant previously filtered at 0.22 μm to eliminate bacteria, was introduced again in flasks. After 12-h 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 macroarrays. Inserts were PCR amplified using TOPO F and R primers (Table 1) 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

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

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		