



Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas*

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Abstract

An expressed sequence tag program was undertaken to isolate genes involved in defense mechanisms of the Pacific oyster, *Crassostrea gigas*. Putative function could be assigned to 54% of the 1142 sequenced cDNAs. We built a public database where all EST information are accessible through numerous search profiles (<http://www.ifremer.fr/GigasBase>). Based on sequence similarities we identified 20 genes that may be implicated in immune function. We investigated the expression of four of these genes during bacterial challenge of oysters. Three of them were induced in response to challenge lending support to their involvement in oyster immunity. Moreover, four other genes were highly homologous to components of the NF- κ B signaling pathway which is involved in innate immune response in *Drosophila* and mammals. Altogether, our results open a new way to investigate the immune response in mollusks.

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1. Introduction

Repeat partial sequencing of expressed sequences, so-called expressed sequence tags (ESTs) have proven to be a powerful means for identification of genetic polymorphisms and for determination of differential gene expression. Now, EST analysis has become a commonly used approach to identify genes involved in specific biological functions, and especially in organisms where genomic data are not available, like for instance, tolerance to osmotic stress in plant (Zhang et al., 2001) or gene profiling during

embryogenesis in ascidians (Satou et al., 2002). The EST approach was used to identify genetic markers in salmon (Davey et al., 2001), environmental stress indicators in American oyster (Jenny et al., 2002) or to characterize immune genes in shrimp (Gross et al., 2001). Indeed, in such marine organisms with economical interest, the access to genomic data may provide new insight into the management of aquaculture activities. This is particularly evident for the shellfish industry where the Japanese oyster, *Crassostrea gigas*, represents 97% of the worldwide oyster production.

Today, most of knowledge on oyster innate immunity is based on biological activities, and molecular features of immune effectors remain largely unknown. The molluscan internal defense mechanisms involve cell-mediated and humoral reactions that interplay to recognize and eliminate pathogens. It has become increasingly evident that the hemocytes play a central role in the bivalve internal defense through chemotaxis, recognition involving opsonins such as

Abbreviations: EST, expressed sequence tag; cDNA, complementary DNA, NF- κ B, nuclear factor κ B; PCR, polymerase chain reaction; DD-RT-PCR, differential display reverse transcription-PCR.

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lectins (Vasta et al., 1984) and phagocytosis including intracellular non-oxidative (Pipe, 1990) and oxidative cytotoxic mechanisms (Bachère et al., 1991). Recently, oyster hemocytes have also been shown to participate in homeostatic mechanisms, including apoptosis and neuro-endocrine signaling (Lacoste et al., 2001, 2002).

In the oyster *C. gigas*, less than 52 gene sequences were characterized (including 15 mitochondrial and 11 ribosomal sequences) and 120 ESTs. The use of the mRNA differential display method (DD-RT-PCR) allowed the characterization of genes involved in cell signaling (Escoubas et al., 1999) and defense mechanisms (Montagnani et al., 2001). Thus, to progress in immune gene characterization, we generated expressed sequence tags (ESTs) from a hemocyte cDNA library originating from oysters subjected to microbial challenge. Among the 1142 ESTs generated, this approach allowed the characterization of 20 genes (among the 1142 ESTs generated) that may be implicated in immunity. In addition, more information on every EST (Blastx results, functional classification, contig assembly) is available using the public and interactive *C. gigas* database available on a web site (<http://www.ifremer.fr/GigasBase>).

2. Materials and methods

2.1. Animals, immune challenge and hemolymph withdrawal

Three- to four-year-old oysters (*Crassostrea gigas* Thunberg) were collected from a commercial farm (Palavas, Gulf of Lion, France) and kept in sea water at 15 °C. To minimize individual variability, at least ten oysters were used in each experimental condition. Oysters were challenged by filing the shell and injecting into the adductor muscle 100 µl of a mixture of four pathogenic *Vibrio* strains (*V. anguillarum*, *V. metshnikovii*, *V. tubiashii* and *V. S322* grown separately overnight at 24 °C in marine broth) (Lambert and Nicolas, 1998). Concentration of bacterial cultures was calculated from the optical density at 550 nm (1 unit OD₅₅₀ corresponds to 5 × 10⁸ bacteria/ml). The bacterial cells were collected by centrifugation (10,000 × g, 5 min), washed twice and resuspended in fresh medium. After injection, oysters were returned to sea water tanks for 12 h. The choice of immune challenge duration was based on previous works realized in our laboratory (Montagnani et al., 2001) as well as studies concerning time course analysis of antimicrobial gene expression in *Drosophila* (Lemaitre et al., 1996, 1997). Hemolymph from unchallenged and challenged oysters was collected from the pericardial cavity through the adductor muscle and immediately centrifuged at 1000 × g for 10 min at 4 °C. Hemocyte pellets were used for RNA extraction.

2.2. cDNA library construction and clone arrangement

Total RNA from challenged-oyster hemocytes was

isolated using Trizol reagent (Gibco BRL) (1 ml/10⁷ cells). Poly (A)⁺ RNA was purified twice using Dynabeads Oligo (dT)₂₅ (Dynal). mRNA (7.5 µg) was reverse transcribed and directionally cloned in the λZAP Express vector (Stratagene). The primary library (1.6 × 10⁵ pfu/ml) was amplified to a titer of 1.2 × 10⁹ pfu/ml. Mass excision was performed and the cDNA inserts from the amplified λZAP library were rescued as pBK-CMV phagemids in XL0LR *Escherichia coli*. A total of 55,000 colonies were randomly picked and rearranged in 384-well plates.

2.3. Northern blot

Phagemids containing cDNAs used as probes in the Northern blot experiments were hydrolysed by restriction enzymes *Eco*RI and *Xho*I. Inserts were then purified from agarose gel using the GeneClean II Kit (Bio101) and radio-labeled by [α -³²P]dCTP using the random priming kit from Invitrogen.

Total RNAs (20 µg) were fractionated by denaturing 1.2% agarose/formaldehyde gel electrophoresis and blotted onto Hybond-N membrane (Amersham). Membranes were prehybridized for 2 h at 65 °C in 50% formamide, 5× SSC, 8× Denhardt's, 50 mM NaH₂PO₄ (pH 6.5), 0.1% SDS and 100 µg/ml denatured salmon sperm DNA. Hybridizations were performed overnight at 42 °C in prehybridization buffer containing the radio-labeled probe. After hybridization, membranes were washed twice for 15 min at room temperature in a solution of 2× SSC containing 0.1% SDS, and twice for 20 min at 60°C in a solution of 1× SSC containing 0.1% SDS. Finally, membranes were exposed to autoradiographic film and the obtained signal intensity was quantified using the STORM system technology from Molecular Dynamics.

2.4. Sequence analysis

Vector sequences were removed and database search were limited to ESTs > 100 bp in length. Database searches were carried out using the WU BlastX program (Altschul et al., 1997). Contigs were built using the CAP3 assembly program (Huang and Madan, 1999). EST sequences have been submitted to the dbEST and GenBank databases (accession numbers from BQ426227 to BQ427368).

3. Results and discussion

3.1. EST sequencing and general characteristics

A total of 1248 randomly selected clones, in phagemid form, were single-pass sequenced from the 5' end, resulting in the characterization of 1142 ESTs that were longer than 100 bp after elimination of vector sequence (Table 1). The average insert size was estimated to be 810 bp by PCR amplification of inserts from 50 randomly selected clones.

Table 1
General characteristics of *Crassostrea gigas* hemocyte ESTs

Total number of cDNA sequenced	1248
Total number of cDNA analysed ^a	1142
Average insert size ^b (range)	810 bp (250–3230)
Average EST length	557 bp
EST clusters ^c	115
Singletons ^d	710
Redundancy ^e	38%

^a Length of sequence used for comparison after editing (inserts < 100 base pairs were excluded).

^b The average insert size was calculated for 50 randomly selected cDNA clones.

^c ESTs with 90% or greater identity over a 100 bp region were clustered together forming 115 EST clusters.

^d 710 sequences did not sufficiently match any sequence in the data set to allow assembly.

^e Redundancy = number of ESTs assembled in clusters/total ESTs.

We used the assembly program CAP3 to organize the redundant ESTs into overlapping contigs. These ESTs coalesced into 115 contigs and 710 singletons, suggesting that the overall redundancy of the library was 38%. On average, each contig was composed of 3 ESTs and spanned 776 bp.

3.2. Functional groups of ESTs

BlastX analysis was performed using the substitution matrix BLOSUM62 (Henikoff and Henikoff, 1992). Comparison of 1142 ESTs against nonredundant SwissProt and GenBank databases revealed that 615 clones (53.9%) were significantly similar (E value < 10^{-3}) to isolated genes. Of these, 95 showed the highest similarities to previously identified oyster genes (*C. gigas* or *C. virginica*).

The ESTs were clustered into ten categories (Fig. 1). These were: (1) sequences which yielded no hits or had poor similarity (E-values > 10^{-3}) to genes in the nonredundant databases; (2) sequences with significant homologies to genes of unknown function; ESTs similar to genes encoding proteins involved in: (3) DNA replication and repair as well as transcription and translation including mitochondrial sequences; (4) immune functions; (5) general metabolism including glycolysis; (6) cell structure and motility; (7) ribosome composition; (8) cell signaling within cell but also across the plasma membrane; (9) cell division and proliferation; and finally, (10) ESTs that did not fit into the categories listed above.

In addition, we built a database (<http://www.ifremer.fr/GigasBase>), where all the EST and their corresponding functional classification are available as well as a complete list of BlastX matches, and contig assemblage.

3.3. Genes potentially involved in defense mechanisms

Twenty clone clusters (including 55 ESTs) discussed below showed significant similarities to genes potentially involved in immunity. These clusters were classified into

four broad groups according to predicted functions (Table 2).

3.3.1. Group I: Proteases and protease inhibitors

Proteases and protease inhibitors are important in many extracellular processes in addition to their role in nutrient digestion. It is becoming clear that proteases and their inhibitors are important in invertebrate anti-infectious response. They may be expressed during humoral immune response to inactivate proteases that are produced by invading pathogens (Kanost, 1999). This is well illustrated by the humoral immune response of the great wax moth *Galleria mellonella* in which inhibitor of metalloprotease, metalloprotease as well as serine protease are released into the larva hemolymph after infection (Wedde et al., 1998; Griesch et al., 2000). The implication of protease inhibitors in defense mechanisms is also illustrated by the role of the *Drosophila* serpin (serine protease inhibitor) in the control of the antifungal response (Levashina et al., 1999). Owing to the characterization of a protease inhibitor (*Cg*-TIMP) involved in *C. gigas* defense mechanisms (Montagnani et al., 2001), we assume that the two proteases (metalloproteinase, cysteine proteinase) and protease inhibitors, as well as their receptors (serpin, α_2 -macroglobulin, α_2 -macroglobulin receptor) identified during the EST program, may be involved in oyster immune response.

3.3.2. Group II: Adhesive proteins

Six EST clusters were grouped in adhesive proteins due to their homologies with proteins involved in non self recognition, opsonization or encapsulation.

Ficolins are animal lectins with collagen-like and fibrinogen-like domains that are involved in the first line

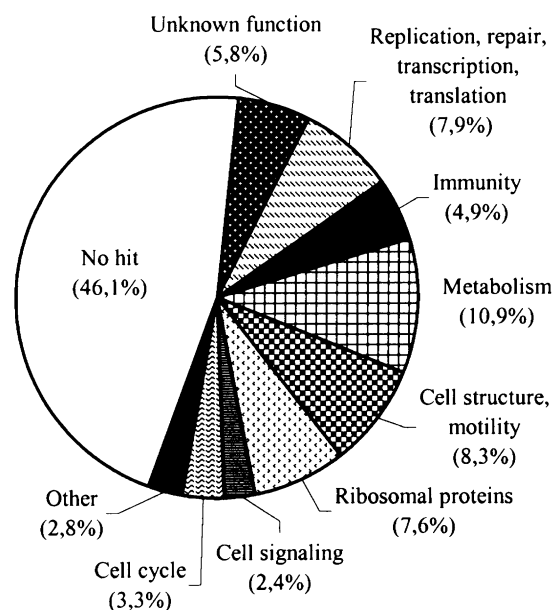


Fig. 1. Functional classification of *Crassostrea gigas* hemocyte ESTs. The 1142 ESTs were clustered into ten categories according to their putative biological function.

Table 2
ESTs similar to genes potentially involved in defense mechanisms

Putative function	Redundancy	GenBank accession no.	E-value	Organism with highest similar sequence
<i>Proteases and protease inhibitors</i>				
Metalloproteinase	3	BQ426653	5 e-43	Sea urchin
Cysteine proteinase (cathepsin)	3	BQ426622	1 e-37	Maize weevil
Tissue inhibitor of metalloproteinase	19	BQ427105	1e-119	Pacific oyster
Serine proteinase inhibitor (serpin)	1	BQ426586	2 e-13	Barley
α_2 -Macroglobulin	1	BQ426790	4 e-20	Horseshoe crab
α_2 -Macroglobulin receptor	1	BQ427140	3 e-04	Mouse
<i>Adhesive proteins</i>				
Ficolin	1	BQ426875	5 e-11	Macaque
Galectin	4	BQ426390	8 e-18	<i>Xenopus</i>
Ig superfamily protein: lachesin	1	BQ426769	5 e-09	Grasshopper
Integrin	1	BQ426737	8 e-05	jellyfish
Coagulation factor v	1	BQ426657	3 e-19	Mouse
Transglutaminase	2	BQ426592	6 e-65	Ascidian
<i>Stress proteins</i>				
Ferritin	10	BQ426741	3 e-76	Great pond snail
Metallothionein	1	BQ427137	6 e-10	Eastern oyster
Superoxide dismutase	1	BQ426796	9 e-60	Mouse
<i>Signal transduction</i>				
vav 2 oncogene	1	BQ427355	1 e-39	Human
MyD88	1	BQ426631	7 e-07	Mouse
TRAF	1	BQ426742	4 e-53	Human
ECSIT	1	BQ427193	8 e-20	Fruit fly
Cactus protein	1	BQ427181	9 e-22	Fruit fly

When more than one clone matched the same gene, only the highest scoring clone is listed and the total number of clone (i.e. including the listed one) is indicated in the 'redundancy' column.

of host defense against pathogens. It has been suggested that ficolins might act as a phagocytic receptor for microorganism recognition and thus play a role in innate immunity by acting as an opsonin (Teh et al., 2000). Another family of lectins, galectins, found both in vertebrates and invertebrates, is characterized by a specific affinity for β -galactoside sugar structures. Several studies indicate that galectins may be involved in various immune-related phenomena including adhesion, wound repair and encapsulation reactions (Vasta et al., 1999; Sato et al., 2002).

One EST clone matches to lachesin, a protein of the immunoglobulin superfamily (IgSF), characterized in the grasshopper and *Drosophila*, that is hypothesized to be involved in neurogenesis (Karlstrom et al., 1993). This clone is also homologous to hemolin (E-value 3 e-06), an insect-immune protein belonging to the IgSF (Sun et al., 1990). It has been suggested that hemolin is involved in the recognition process as well as in hemocyte adhesion during recognition and response to bacterial infections (Schmidt et al., 1993). Complete sequencing of this clone will help us to determine if we have characterized a hemolin-like molecule in oyster.

Unlike the molecules previously described, integrin (a transmembrane receptor) is implicated in promoting blood cell adhesion and immune reactions such as phagocytosis and encapsulation. The integrin ligands belong to many

different protein families including extracellular matrix proteins (e.g. collagen) and plasma proteins such as coagulation factors (Johansson, 1999). Although clotting processes have not been reported in oysters, the isolation of an EST clone presenting homologies to coagulation factor raises the question of the function of this gene in *C. gigas*. Moreover, the last EST cluster of this group shows homologies to transglutaminase (TGase). In crayfish, TGase catalyses crosslinking reaction between soluble proteins from the plasma and results in the formation of stable and insoluble clots (Wang et al., 2001).

3.3.3. Group III: Stress proteins

Three other clusters were classified as stress proteins due to their homologies with proteins involved in detoxification processes triggered by stress. Two of them encoded metal binding proteins, ferritin and metallothionein, involved in iron metabolism and regulation. Recently, it has been suggested that ferritin may also be considered as an acute phase protein (Beck et al., 2002). Metallothionein is a metal binding protein thought to be involved in the detoxification of heavy metals, in free radicals scavenging and also in the inflammatory response (Kanekiyo et al., 2002). The third EST was homologous to superoxide dismutase (SOD) an antioxidant enzyme that protects cells against cytotoxic effects of reactive oxygen intermediates (ROI) produced

during phagocytosis. It has been proposed that the extracellular SOD may also participate in immunity by mediating or regulating hemocyte adhesion and phagocytosis (Johansson et al., 1999).

3.3.4. Group IV: Signal transduction

Five ESTs were classified in the signal transduction group. The first was homologous to the *vav-2* oncogene. Vav proteins participate in the antigen receptor signaling cascade which, in mammals, regulates the survival, proliferation and differentiation of lymphocytes (Doody et al., 2001). Vav proteins also transmit signals to the Rel/NF- κ B cascade, a phylogenetically conserved signal transduction pathway. In mammals, this cascade is considered as the central mediator of the immune response. Indeed a large number of genes, regulated by NF- κ B, encodes proteins involved in the host immune response, such as cytokines, chemokines, MHC molecules or proteins involved in antigen presentation (Pahl, 1999). In *Drosophila*, this pathway plays a significant role in cell mediated immunity by regulating hemocyte proliferation in larvae (Qiu et al., 1998) and controls the humoral immune response through the production of antimicrobial peptides (Hoffmann and Reichhart, 2002). Four other ESTs classified in this group were highly homologous to components of the Rel/NF- κ B pathway including three adaptor proteins MyD88, TRAF and ECSIT in addition to cactus, the *Drosophila* homologue of I κ B (Aderem and Ulevitch, 2000; Hoffmann and Reichhart, 2002). Interestingly, we have already characterized a protein kinase (σ IKK) in *C. gigas* that possesses the characteristic organization as well as the functional activities of mammalian I κ B kinase proteins (IKK) involved in Rel/NF- κ B cascade (Escoubas et al., 1999). More recently, we isolated a cDNA encoding a Rel/NF- κ B like protein (*Cg-Rel*) highly homologous to *Drosophila* transcription factors Dorsal and Dif. Transient expression experiments in *Drosophila* embryonic cells showed that *Cg-Rel* was a transcription factor of the NF- κ B/Rel family (unpublished results). Altogether, these data sustain the concept of an evolutionary conserved induction system of the innate immune response in *C. gigas* similar to the Rel/NF- κ B pathway described in insects and mammals.

3.4. Genes potentially involved in blood cell proliferation

Cell proliferation is an important component of the immune response in vertebrates and in invertebrates. Two EST clones classified as regulator of the 'cell cycle' gave matches to genes specifically expressed in hematopoietic cells. The first one is homologous to LckBP1 (E-value 3 e-81, accession number BQ427191), a mouse protein only produced in hematopoietic cell lineages. LckBP1 is involved in the T-cell receptor signaling pathway leading to proliferation and differentiation of blood cells (Takemoto et al., 1995). A second one is homologous to the *Drosophila* protein Drac3 (E-value 4 e-09, accession number

BQ427023), a small GTPase of the Rho family known to be involved in the regulation of cell proliferation and apoptosis. Drac3 is highly expressed in hemocyte precursors and essential to their development into mature cells (Sasamura et al., 1997).

3.5. Infection responsiveness

Northern blot analyses were carried out using total RNAs extracted from hemocytes withdrawn from challenged and unchallenged oysters. Two cDNAs were used as control. The first, *s6*, encodes a ribosomal protein which is constitutively expressed and the second, *Cg-timp*, encodes a tissue inhibitor of metalloproteinase that is induced by bacterial challenge (Montagnani et al., 2001). Four EST clusters potentially involved in defense mechanisms were experimentally tested for their response to immune challenge. Three of the four genes appeared to be immune induced including metalloproteinase (*mmp*), transglutaminase (*tgase*) and galectin (*gal*), whereas the fourth one ferritin (*ferri*) was not (Fig. 2).

We previously hypothesized that *Cg-TIMP* is involved in oyster immune response by inhibiting proteases produced by pathogens (Montagnani et al., 2001). Here, we observe that in the same challenge conditions a metalloprotease was also induced signifying that it may participate to defense mechanism by degrading secretory/excretory products released by pathogens during infection. The up-regulation

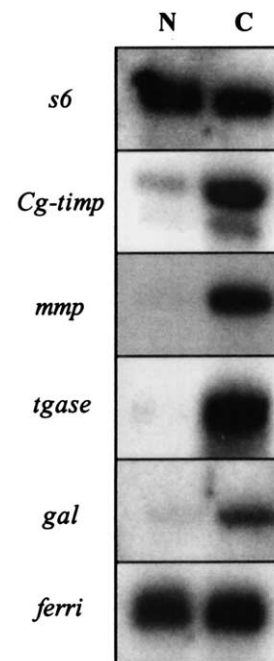


Fig. 2. Expression level of four putative immune genes, metalloproteinase (*mmp*), transglutaminase (*tgase*), galectin (*gal*) and ferritin (*ferri*), was analysed by Northern blot on total RNA (20 μ g/lanes) extracted from naïve (N) and challenged (C) oysters. The ribosomal protein S6 gene (*s6*) was used as control to verify RNA loading, and the tissue inhibitor of metalloproteinase gene (*Cg-timp*) was used to control the effectiveness of the challenge condition.

of TGase and galectin during bacterial infection also suggests the involvement of these two molecules in oyster defense mechanisms. Each may participate in non self recognition and aggregation in synergy with other adhesive proteins to facilitate the clearance of pathogens from the host.

The ferritin family is a widespread group of proteins that maintain iron in a soluble form and protect against the toxic effects of excess iron. Recently, Beck and coworkers suggested that echinoderm ferritin is an acute phase protein and that iron sequestration may be a component of the invertebrate immune response (Beck et al., 2002). We observed that ferritin mRNA concentration does not change during bacterial challenge, which seems contradictory to results of Beck and coworkers. Nevertheless, regulation of ferritin expression is complex and subjected to transcriptional (Pham et al., 1999) and/or translational control (Rouault and Klausner, 1997).

4. Concluding remarks

The primary goal of the EST project was to identify genes involved in defense mechanisms of the Pacific oyster *Crassostrea gigas*. Fifty-five sequences (grouped in 20 clone clusters) out of 1142 ESTs analysed were classified in immune function based on sequence homologies with immune genes characterized in other organisms. Expression analysis by Northern blot of three EST clusters potentially involved in defense mechanism revealed that they are up-regulated in challenged oysters.

Among the ESTs similar to genes potentially involved in defense mechanisms, the tissue inhibitor of metalloproteinase (*Cg-timp*) is highly represented (19 ESTs out of 55), suggesting its involvement in immune response. In the EST program realized on *C. virginica* hemocytes, none of the cDNAs analysed matched with this metalloproteinase inhibitor (Jenny et al., 2002). Moreover, using *Cg-timp* probe, we were unable to visualize, in *C. virginica*, *timp* expression by Northern blot analysis (data not shown). Interestingly, Faisal and collaborators showed that proteinase inhibitors in *C. gigas*, which is refractory to the oyster parasite *Perkinsus marinus*, possess a greater activity than the ones in *C. virginica* (Faisal et al., 1998). These observations together with our EST results suggest that *Cg-timp* may participate in the *C. gigas* resistance to *P. marinus*.

No genes encoding antimicrobial peptides (AMPs) have been evidenced based on sequence similarities search. This surprising result could reveal that the innate immune response in *C. gigas* does not rely on the production of AMPs, which however is likely improbable since such immune effectors are widespread in almost all living organisms. A more suitable possibility is that *C. gigas* may produce original AMPs which are different from AMPs hitherto characterized in other organisms. Nevertheless, and

complementary studies based on isolation and biochemical characterization of AMPs would permit to verify the presence and involvement of such effectors in the oyster immune system.

The most striking finding is probably the identification of four cDNAs homologous to molecules of the Rel/NF- κ B signal transduction pathway. These data, in addition to the genes previously characterized in our laboratory (oIKK and *Cg-Rel*), confirm the existence of a Rel/NF- κ B cascade in oyster. To our knowledge, it is the first time that six genes related to the Rel/NF- κ B pathway are characterized in an invertebrate other than *Diptera*. Moreover, mollusks constitute a group more ancient than insects; consequently, phylogenetic analysis of the genes characterized in oyster will probably help to understand the evolution of this signaling pathway.

Finally this work initiates a genomic scale investigation of oyster immune functions. Indeed ESTs now provide useful materials to develop cDNA microarrays for high-throughput expression profiling of oyster defense reactions. This investigation process will be extended to explore other physiological functions such as reproduction or embryonic development, which have to be better understood in order to improve oyster domestication.

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