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# Discovery of genes expressed in response to *Perkinsus marinus* challenge in Eastern (*Crassostrea virginica*) and Pacific (*C. gigas*) oysters

Arnaud Tanguy[a,b], Ximing Guo[a,*], Susan E. Ford[a]

[a] *Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA*
[b] *Laboratoire des Sciences de l'Environnement Marin (LEMAR), UMR-CNRS 6539, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, Place Nicolas Copernic, 29280, Plouzané, France*

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**Abstract**

The protozoan pathogen *Perkinsus marinus* is the causative agent of Dermo, a lethal disease of the eastern oyster *Crassostrea virginica*, but not the Pacific oyster *Crassostrea gigas*. To understand the response of these two oysters to parasite exposure, a suppression subtractive hybridization (SSH) method was employed to characterize genes up-regulated during parasite challenge in both hemocytes and gills. The number of differentially expressed gene sequences obtained was 107 for *C. virginica* and 69 for *C. gigas*, including 46 and 37 sequences, respectively, that matched known genes in GenBank. Most of the sequences have not been characterized in other molluscs. Nineteen genes involved in immune system and cell communication, protein regulation and transcription, cell cycle, respiratory chain and cytoskeleton were selected for expression analysis by semi-quantitative PCR. Although varying in magnitude and timing post exposure, all genes screened showed over-expression in challenged oysters in both species, validating the SSH method. Results of this study highlighted some differences in gene expression between the two oysters in response to *P. marinus* infection, providing candidate genes and pathways for further analysis.
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*Keywords:* Substractive libraries; Parasite; Gene expression; Immune system; Mollusca

## 1. Introduction

*Perkinsus marinus* is a protozoan pathogen (Levine, 1978) that is the causative agent of Dermo, a lethal disease of the eastern oyster (*Crassostrea virginica*). Effects of *P. marinus* infection in *C. virginica* range from reduction in shell growth, haemolymph protein concentrations and lysozyme activity, to severe emaciation, inhibition of gonadal development and reproductive output, and ultimately death of the oyster (Ford and Tripp, 1996). Although *P. marinus* has devastated *C. virginica* populations on the East Coast of the United States for many years (Burreson and Ragone Calvo, 1996), relatively little is known about interactions between this parasite and the defense reactions of its host. Numerous biochemical analyses have been conducted showing the involvement of parasite-associated extra-cellular proteins (ECP), especially proteases, in the pathogenicity of *P. marinus* (Oliver et al., 1999; La Peyre and Vollety, 1999). Antimicrobial activity against *P. marinus* occurs in plasma of oysters, and some low molecular weight protein inhibitors and high antiproteolytic activity in the haemolymph may play a role in oyster defense (Elsayed and Faisal, 1999). However, it is clear that *C. virginica* has little ability to control the multiplication and progression of *P. marinus* after infection. Mechanisms for the pathogenicity of *P. marinus* and the physiological response of the host are still poorly understood (Anderson, 1996).

The Pacific oyster, *Crassostrea gigas*, a species native to Asia, which has been introduced to many other regions for aquaculture, is less susceptible to infection and disease caused by *P. marinus* (Meyers et al., 1991). La Peyre et

*Abbreviations:* RT-PCR, reverse transcription poly chain reaction; EST, expressed sequence tag; FSW-PS, filtered sea water with penicillin and streptomycin; RFTM, Ray's Fluid Thioglycollate Medium; MAPK, mitogen activated protein kinase; TRAP, Tumor Necrosis Factor receptor associated protein; PAK, protein activated kinase; SSH, suppression subtractive hybridisation.

* Corresponding author. Tel.: +1-856-785-0074; fax: +1-856-785-1544.

*E-mail address:* xguo@hsrl.rutgers.edu (X. Guo).

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al. (1995) proposed that the greater resistance to infection and disease in *C. gigas* compared to *C. virginica* could be attributed to elevated cellular and humoral activities that may degrade the parasite more effectively, and/or to lower plasma protein levels that may limit parasite proliferation. At present, almost no information about the molecular response of oysters to *P. marinus* is available in the literature. Recently, some expressed sequence tags (ESTs) were obtained from cDNA libraries and used to identify genes involved in the response to environmental stressors in *C. virginica* (Jenny et al., 2002) and in *C. gigas* (Gueguen et al., 2003). In the present study, we employed a PCR-based technique, namely suppression-subtractive hybridization (SSH), to identify specific genes expressed in both *C. virginica* and *C. gigas* in response to *P. marinus* challenge, in an attempt to understand the molecular processes involved. Our approach allowed the identification of new genes and highlighted the differential response to *P. marinus* between the two species.

## 2. Materials and methods

### 2.1. Isolation of natural P. marinus

*P. marinus* were isolated from naturally infected Delaware Bay oysters using the following procedure. Oyster tissues ($N=60$) were minced and placed in 0.2-μm filtered seawater (FSW) laced with penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml). Approximately 25 ml of this solution was used for every gram of wet oyster tissue. The tissues were then homogenized in a blender and filtered through a series of Nytex screens: 230, 183, 130, 41 and 15 μm. The filtrate was placed in 50-ml tubes and centrifuged at $500\times g$ for 10 min. The supernatant, containing the parasites, was removed into another set of 50-ml tubes, then centrifuged at $800\times g$ for 20 min and washed with FSW-PS (penicillin and streptomycin) five times. The pellet, now containing the parasites, was resuspended in 10 ml FSW-PS. One-milliliter samples of this suspension were placed in sterile glass tubes containing 5 ml of Ray's Fluid Thioglycollate Medium (RFTM) and incubated at 28 °C overnight to promote parasite enlargement for easier counting. The remaining sample was refrigerated at 4 °C to prevent parasite multiplication. The 28 °C incubated cells were counted using a counting cell, and the *P. marinus* concentration in the refrigerated sample was adjusted to the desired concentration using FSW-PS.

### 2.2. Maintenance and oyster inoculation

Presumed uninfected *C. virginica* ($N=80$) and *C. gigas* ($N=80$) were obtained from Damariscotta (Maine, USA) and Taylor Shellfish Farms, (Washington, USA), respectively. Forty oysters of each species were maintained in each of four separate 700-l tanks filled with 1-μm filtered seawater at 26 °C for 1 week before the beginning of the experiment. All oysters were notched at the ventral edge of the shell adjacent to the gills. Forty oysters of each species were each injected into the shell cavity with 100 μl of inoculum containing $10^6$ *P. marinus* cells. The remaining 40 oysters of each species were injected with 100 μl FSW-PS as controls. After injection, a wide rubber band was placed around each oyster to cover the notch and to minimize ejection of the inoculum. A damp paper towel was placed over the oysters, which were held overnight at 10 °C. The oysters were returned to their tanks and fed every day with cultured algae. Water was changed every week.

### 2.3. Sample collection

Ten days after inoculation, 20 challenged and 20 control *C. virginica* and *C. gigas* were sampled. A piece of gill was collected from each oyster. Hemolymph of sampled oysters was collected, pooled and centrifuged to provide a single sample each for challenged and for control oysters from both species. The hemocyte pellets were frozen in liquid nitrogen and kept at 80 °C until RNA extraction. Forty-five days after inoculation, the remaining 20 challenged and 20 control oysters were sampled using the same protocol. For each oyster sampled at 10 and 45 days post-inoculation, the rectum and pieces of the mantle and the palps were also collected and put in 5 ml RFTM for 1 to 2 weeks until *P. marinus* infection level determination using standard procedures (Ray, 1954). Infection intensities were scored from 0 (no parasites detected) to 5 (very heavy infection) based on the scale of Choi et al. (1989).

### 2.4. RNA preparation

Total RNA was isolated using the RNAgents total RNA Isolation System (Promega, Madison, USA), according to the manufacturer's protocol. RNA was resuspended in nuclease-free water and quantified with a spectrophotometer at 260 nm. For SSH library construction, mRNA was isolated from total RNA using the Polytract RNA mRNA Isolation System (Promega) according to the manufacturer's protocol.

### 2.5. Suppression-subtractive hybridization

To target up-regulated genes, SSH libraries were constructed for each species by subtracting mRNA extracted from hemocytes and gill of the control oysters from mRNA of the challenged oysters. For each species, one SSH library was constructed from hemocytes and one from gill tissue collected at Day 10; two similar libraries were constructed at Day 45, for a total of four libraries. SSH libraries were constructed with the PCR-SELECT cDNA Subtraction Kit (Clontech) according to the manufacturer's protocol. PCR products generated by SSH were cloned into the pGEM-T vector (Promega). For each library, 250 colonies were

Table 1
Identified SSH up-regulated clones in *P. marinus*-exposed *C. virginica* (after 10 and 45 days of exposure) with significant database matches

| Homolog (protein); BlastX value | Homolog species | Insert size (bp) | Accession number | SSH library |
|---|---|---|---|---|
| *Detoxification and stress proteins* | | | | |
| Adaptor related complex; 1e − 12 | *H. sapiens* | 379 | CD526736 | H (45 days) |
| Deoxyribonuclease II; 6e − 10 | *H. sapiens* | 444 | CD526732 | H and G (45 days) |
| Alpha/beta hydrolase; 3e − 7 | *M. musculus* | 156 | CD526713 | H (10 and 45 days) |
| Heat Shock factor 2; 4e − 11 | *D. renio* | 524 | CD526741 | H (10 days) |
| Glutathione *S*-transferase YC alpha II; 8e − 5 | *O. cuniculus* | 199 | CD526720 | H (45 days) |
| *Cell cycle, DNA repair, protein regulation and transcription* | | | | |
| Pescadillo homolog; 6e − 51 | *M. musculus* | 779 | CD526714 | H (45 days) |
| Nuclear corepressor KAP-1; 8e − 7 | *H. sapiens* | 616 | CD526733 | H and G (10 and 45 days) |
| Ring finger 10 protein; 5e − 8 | *H. sapiens* | 433 | CD526710 | H (45 days) |
| MAPK 8 interacting protein; 4e − 27 | *H. sapiens* | 272 | CD526707 | H and G (10 and 45 days) |
| Beige protein homolog; 5e − 14 | *M. musculus* | 284 | CD526728 | H (45 days) |
| Elongation factor 1; 2e − 65 | *M. edulis* | 666 | CD526731 | H (10 and 45 days) |
| G protein a subunit q class; 2e − 80 | *H. sapiens* | 801 | CD526752 | H (10 and 45 days) |
| RHO GTPase, 5e − 100 | *A. californica* | 819 | CD526744 | H and G (10 and 45 days) |
| GTP binding protein alpha sub-unit; 7e − 70 | *P. yessoensis* | 801 | CD526737 | H (10 and 45 days) |
| *Respiratory chain* | | | | |
| Cytochrome C1; 9e − 23 | *X. laevis* | 229 | CD526711 | H and G (10 and 45 days) |
| NADH oxidoreductase; 9e − 44 | *B. taurus* | 325 | CD526722 | H and G (10 days) |
| *Metabolism* | | | | |
| Phosphoenolpyuruvate carboxykinase; 3e − 33 | *H. sapiens* | 277 | CD526738 | G (10 and 45 days) |
| 5′ Methylthioadenosine phosphorylase; 3e − 14 | *D. renio* | 307 | CD526712 | H (45 days) |
| GCN5-like protein; 5e − 8 | *L. hardwickii* | 432 | CD526725 | H and G (45 days) |
| *Cell communication, membrane receptor, immune system* | | | | |
| Tetraspanin 66E; 2e − 4 | *D. melanogaster* | 746 | CD526719 | H and G (10 and 45 days) |
| Toll-like receptor 4; 1e − 13 | *G. gallus* | 524 | CD526746 | H (45 days) |
| T-cell receptor chain alpha c6.1 fusion protein; 2e − 40 | *H. sapiens* | 342 | CD526740 | H (10 and 45 days) |
| SREC receptor class F; 5e − 12 | *H. sapiens* | 509 | CD526723 | H (45 days) |
| Beta galactoside binding lectin; 8e − 9 | *X. laevis* | 420 | CD526748 | H (10 and 45 days) |
| Lanthionin synthetase C-like protein; 3e − 27 | *H. sapiens* | 421 | CD526726 | H (10 and 45 days) |
| Pernin precursor; 2e − 5 | *P. veridis* | 294 | CD526735 | H and G (10 and 45 days) |
| Pak interacting exchange factor; 8e − 8 | *R. norvegicus* | 638 | CD526734 | H (45 days) |
| TNF type 1 receptor associated protein; 3e − 12 | *H. sapiens* | 312 | CD526742 | H and G (45 days) |
| Calmodulin, 9e − 25 | *A. punctulata* | 412 | CD526718 | H and G (10 and 45 days) |
| RAB21; 1e − 28 | *H. sapiens* | 289 | CD526747 | H and G (10 days) |
| Metal binding protein T11B7; 2e − 20 | *C. elegans* | 396 | CD526717 | H (45 days) |
| *Cytoskeleton* | | | | |
| RalB binding protein; 1e − 27 | *R. norvegicus* | 362 | CD526730 | G (45 days) |
| RalA binding protein; 4e − 24 | *X. laevis* | 361 | CD526729 | H and G (45 days) |
| β-thymosin; 7e − 4 | *H. sapiens* | 178 | CD526708 | H and G (10 and 45 days) |
| Actin 3; 6e − 45 | *P. carnea* | 394 | CF369133 | H and G (10 and 45 days) |
| *Ribosomal proteins* | | | | |
| Ribosomal protein L17; 6e − 34 | *D. melanogaster* | 254 | CD526715 | H and G (10 and 45 days) |
| Ribosomal protein L37; 6e − 7 | *E. nidulans* | 141 | CD526724 | H and G (10 and 45 days) |
| Ribosomal protein L35; 5e − 25 | *M. musculus* | 297 | CD526721 | H (10 days) |
| Ribosomal protein S30; 2e − 4 | *S. crofa* | 679 | CD526709 | H and G (10 and 45 days) |
| Ribosomal protein L6; 6e − 44 | *I. punctatus* | 730 | CD526745 | H and G (10 and 45 days) |
| Ribosomal protein L23; 5e − 21 | *M. musculus* | 145 | CD526716 | H and G (10 and 45 days) |
| Ribosomal protein S14; 4e − 32 | *I. punctatus* | 304 | CD526739 | H (10 days) |
| *Unknown function* | | | | |
| Putative senescente associated protein; 5e − 33 | *P. sativum* | 297 | CD526743 | H and G (10 and 45 days) |
| Protein FLJ10342; 2e − 20 | *M. musculus* | 788 | CD526749 | G (45 days) |
| ENSANGP00000011290; 5e − 6 | *A. gambiae* | 249 | CD526751 | G (45 days) |
| Riken 2510006C20; 4e − 44 | *H. sapiens* | 698 | CD526750 | H (45 days) |
| Unidentified sequences[a] | | | CD526753 – CD526813 | |

[a]Sequences presented no significant alignment ($<0.01$) or significant alignment with an unknown protein. H: hemocyte SSH library, G gill SSH library.

cultured in tubes containing LB growth medium with 100 mg/l ampicillin and were screened by *Apa*I and *Pst*I restriction of alkaline lysis plasmid minipreparations. Plasmid samples were sequenced at the Arizona Research Laboratory (University of Arizona) using an ABI prism, Model 3700 DNA analyzer, and Big Dye terminator reactions.

### 2.6. Sequence analysis

Vector sequences were removed and database searches were carried out using the WU BlastX program (Altschul et al., 1997) and EST sequences have been submitted to the GenBank databases.

Table 2
Identified SSH up-regulated clones in *P. marinus*-exposed *C. gigas* (after 10 and 45 days of exposure) with significant database matches

| Homolog (protein); BlastX value | Homolog species | Insert size (bp) | Accession number | SSH library |
|---|---|---|---|---|
| *Detoxification and stress proteins* | | | | |
| Lysosomal associated transmembrane protein; 4e – 7 | *H. sapiens* | 603 | CD526837 | H (10 and 45 days) |
| Proteasome iota chain; 2e – 58 | *M. musculus* | 509 | CD526846 | H and G (45 days) |
| Cytochrome P450-like TBP; 4e – 11 | *N. tabacum* | 237 | CD526847 | H and G (45 days) |
| *Cell cycle, DNA repair, protein regulation and transcription* | | | | |
| Zinc finger protein; 4e – 8 | *R. norvegicus* | 443 | CD526815 | G (45 days) |
| Nuclear corepressor KAP-1; 2e – 6 | *H. sapiens* | 532 | CD526819 | H and G (10 and 45 days) |
| Splicing factor U2AF; 5e – 23 | *M. musculus* | 310 | CD526826 | H (45 days) |
| Solute carrier family 3 protein; 1e – 15 | *O. cuniculus* | 358 | CD526827 | H (45 days) |
| Guanine nucleotide binding protein; 2e – 5 | *H. americanus* | 406 | CD526828 | H and G (10 days) |
| Small RHO1 GTPase; 6e – 68 | *H. pulcherrinus* | 823 | CD526835 | H and G (10 and 45 days) |
| *Respiratory chain* | | | | |
| Cytochrome *c* oxidase subunit I; e – 143 | *C. gigas* | 513 | AF177226 | H and G (10 and 45 days) |
| NADH dehydrogenase subunit 5; 2e – 49 | *C. gigas* | 325 | AF177226 | H and G (10 and 45 days) |
| *Metabolism* | | | | |
| Glycogen phosphorylase; 3e – 26 | *R. norvegicus* | 226 | CD526825 | H and G (45 days) |
| *Cell communication, membrane receptor, immune system* | | | | |
| Tetraspanin 66E; 2e – 6 | *D. melanogaster* | 778 | CD526816 | H and G (10 and 45 days) |
| Glucocorticoïd sensitive T-cell protein; 4e – 21 | *M. musculus* | 343 | CD526820 | H (45 days) |
| Vav-3 protein; 7e – 8 | *H. sapiens* | 631 | CD526818 | H (10 days) |
| Rab5 GDP/GTP exchange factor; 5e – 32 | *H. sapiens* | 850 | CD526823 | H (10 and 45 days) |
| Rab9 effector p40; 1e – 65 | *H. sapiens* | 816 | CD526824 | H and G (45 days) |
| Alpha NAC; 4e – 38 | *M. musculus* | 688 | CD526829 | H (10 and 45 days) |
| RalBP1 protein; 2e – 21 | *X. laevis* | 360 | CD526836 | H (10 and 45 days) |
| Beta-1,3-glucan binding protein; 3e – 6 | *P. vanname* | 600 | CF369126 | H (10 and 45 days) |
| Blood–brain barrier transporter heavy chain; 5e – 16 | *H. sapiens* | 358 | CD526845 | H (45 days) |
| Calmodulin, 8e – 31 | *H. sapiens* | 412 | CD526833 | H and G (10 and 45 days) |
| *Cytoskeleton* | | | | |
| Coactosin-like protein, 3e – 25 | *X. laevis* | 492 | CD526821 | H (45 days) |
| Beta tubulin; 3e – 19 | *E. multilocularis* | 508 | CD526843 | H and G (10 and 45 days) |
| Actin; e – 0 | *C. gigas* | 475 | AF026063 | H and G (10 and 45 days) |
| Alpa tubulin; 4e – 21 | *M. musculus* | 336 | CD526838 | H and G (10 and 45 days) |
| *Ribosomal proteins* | | | | |
| Ribosomal protein L14, 3e – 17 | *G. gallus* | 218 | CD526817 | H (10 days) |
| Ribosomal protein S23, 5e – 25 | *M. musculus* | 417 | CD526842 | H and G (10 and 45 days) |
| Ribosomal protein L17, 5e – 24 | *D. labrax* | 420 | CD526830 | G (45 days) |
| Ribosomal protein S20, 4e – 23 | *C. farreri* | 556 | CD526844 | H and G (10 and 45 days) |
| Ribosomal protein S30, 5e – 25 | *A. irradians* | 308 | CD526841 | H and G (10 and 45 days) |
| Ribosomal protein S40, 3e – 35 | *D. melanogaster* | 304 | CD526839 | H and G (10 and 45 days) |
| Ribosomal protein L9, 5e – 27 | *H. sapiens* | 246 | CD526832 | H and G (45 days) |
| *Unknown function* | | | | |
| Hypothetical protein; 4e – 6 | *C. elegans* | 521 | CD526822 | H (10 days) |
| Putative senescente associated protein; 1e – 33 | *P. sativum* | 476 | CD526814 | H and G (10 and 45 days) |
| Arha 2; 3e – 14 | *R. norvegicus* | 832 | CD526834 | H and G (45 days) |
| CG6891; 8e – 31 | *D. melanogaster* | 441 | CD526831 | G (45 days) |
| Unidentified sequences[a] | CD526848–CD5268883 | | | |

[a]Sequences presented no significant alignment (< 0.01) or significant alignment with an unknown protein. H: hemocyte SSH library, G gill SSH library.

### 2.6.1. Reverse transcription-PCR and expression study

To further investigate and quantify differential expression of the isolated genes, total RNA isolated from hemocytes and gills of *P. marinus*-challenged and control oysters was used for expression analysis using semi-quantitative reverse transcription poly chain reaction (RT-PCR). First-strand synthesis was performed on 200 μg of total RNA using oligo-dT primer and M-MLV reverse transcriptase. Twenty-three genes isolated from sequences obtained in *C. gigas* and *C. virginica* SSH libraries were studied. Primer sequences were designed to amplify products in the size range of 150 to 500 bp. Six of the genes studied are common in the two species and common primers were designed. Polymerase chain reactions were performed using buffer (2 mM magnesium ions), 0.1 U of Taq polymerase (Promega), primers (10 pM each), reverse transcription product (100 ng), dNTPs (2.5 mM of each), and water. A PCR positive control using the 28S ribosomal gene was performed on each sample. All reactions were performed in a 25-μl volume in a GeneAmp PCR System 9700 (Perkins Elmer). The thermal cycling program used was an initial 2-min denaturation at 94 °C, then 20 to 35 amplification cycles were performed as follow, 94 °C for 30 s, 58 °C for 1 min and 72 °C for 40 s, then a final 10 min at 72 °C. Five microliters of the reaction products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. All PCR reactions were performed from 2 μg of the same reverse transcribed product to minimize differences in RT efficiency. The number of PCR cycles needed for amplification was optimized for each gene and quantification of band intensities was determined using Gene Profiler 4.03 software (Scanalytics). The relative degree of upregulation in response to challenge was estimated for each gene as the ratio of band intensity of inoculated to control oysters.

## 3. Results

### 3.1. P. marinus infection levels

No *P. marinus* was detected in any control oyster from either species collected at either sample date. Conversely, all challenged *C. virginica* were positive, with infection intensities ranging from 0.33 to 1.67 at Day 10 and from 1.00 to

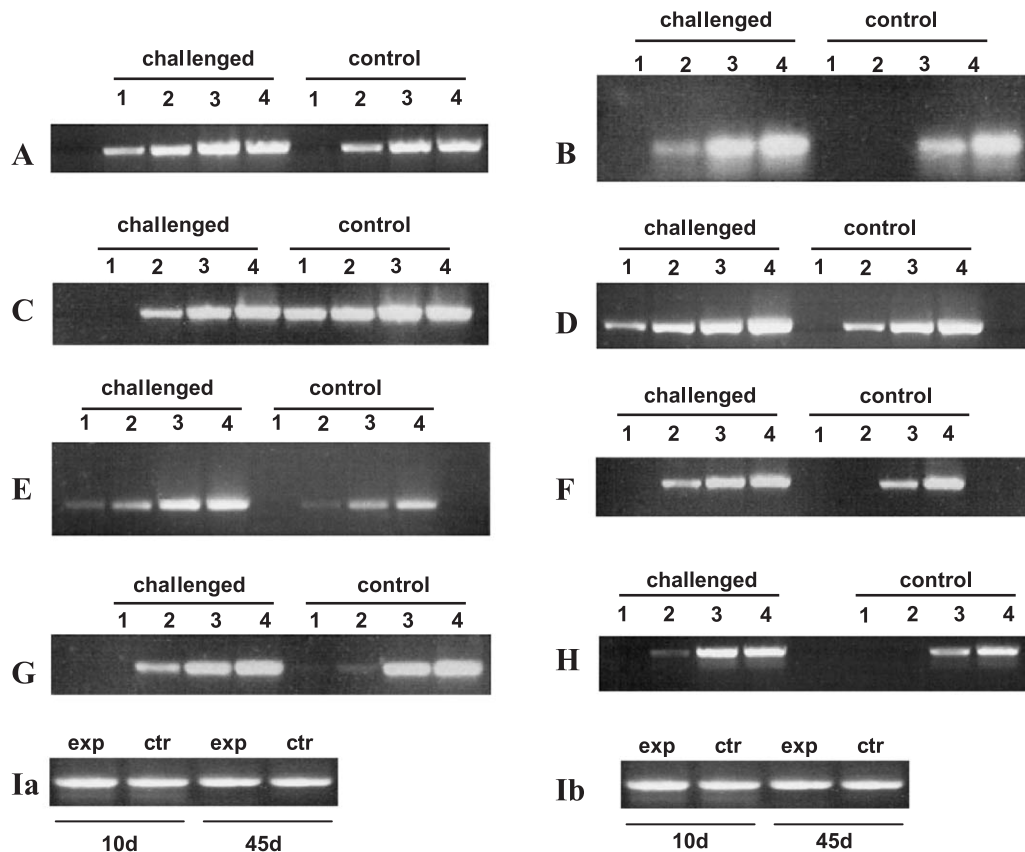


Fig. 1. Analysis of differential expression of up-regulated genes in *C. virginica* and *C. gigas* exposed to *P. marinus*. Differential expression between libraries was assessed using 100 ng of cDNA from each library as template. PCR products were collected after 10 (1), 20 (2), 30 (3) and 40 (4) cycles, for analysis of relative expression by agarose gel electrophoresis and densitometry. (A) MAPK 8 interacting protein in *C. virginica*; (B) Toll-like receptor 4 in *C. virginica*; (C) NADH in *C. virginica*; (D) Pernin precursor in *C. gigas*; (E) TNF type 1 receptor associated protein 3 in *C. virginica*; (F) beta 1,3-glucan-binding protein in *C. gigas*; (G) T-cell receptor chain alpha c6.1 fusion protein in *C. virginica*; (H) BgB lectin in *C. virginica*; (I) 28S in *C. gigas* (a) and *C. virginica* (b) respectively at 10 and 45 days of *P. marinus* exposure and their corresponding control.

Table 3
Expression analysis of 19 genes by semi-quantitative RT-PCR and densitometry in *C. virginica* and *C. gigas* exposed to *P. marinus*

| Gene name, forward (F) and reverse (R) primer sequence | Ratio of PCR product density between *P. marinus* exposed and control oysters | | | |
|---|---|---|---|---|
| | *C. virginica* | | *C. gigas* | |
| | 10 days | 45 days | 10 days | 45 days |
| Pescadillo protein<br>F: GGGACCTGGATGACTGTCTGTCC;<br>R: TAATGAAGCCCAGTAGTGTGGTGT | 2.1 | 4.3 | | |
| Lanthionin<br>F: CTGAATGCATGCTTATGCTGT;<br>R: CATTGTTGCAGCTATTGAACAC | 1.3 | 4.5 | | |
| Calmodulin<br>F: AGAAGCATTCCGAGTATTTGAC;<br>R: TCCTCAGAGCAACACAATAAGA | 2.6 | 3.4 | 2.2 | 3.6 |
| RHO<br>F: GGAATGGTGCTCTGTTTACACT;<br>R: CCTATAATCCTGGTAGGCAACA | 4.2 | 3.8 | 3.1 | 4.5 |
| Toll receptor<br>F: TGTATGGCACTTGGTAAGATTG;<br>R: TAATTAAACGAAGCCAGTCTCG | 5.1 | 2.3 | | |
| Beige protein<br>F: AGTCATTGGTGAACGTCAGACT;<br>R: GAGCGTCTCTCTGGACAGTAGT | 1.2 | 4.5 | | |
| Nuclear corepressor<br>F: TGCAGTCATCAGTAGGGTAAAA;<br>R: CGGTACATCTGTCAAACGGTA | 1.5 | 3.7 | 1.7 | 4.2 |
| PEPCK<br>F: ACAGTGGAAGCACACTAAGGAC;<br>R: GAGTGCACACATCTGACAAAGT | 1.4 | 2.9 | | |
| Pernin precursor<br>F: CTCCTGATCATGCTGAACCT;<br>R: GATCATGTTTGTTCGGTCATC | 1.1 | 3.2 | | |
| BgB lectin<br>F: GGTTTGTGACCTACGTAAATGG;<br>R: GCGACAACTCCTTCACATATAA | 4.6 | 2.4 | | |
| MAPK-8 receptor associated protein<br>F: AGATTTACGAGAGTGGAGATGG;<br>R: AGGTTGCTGAAGAAGTTCCATA | 2.9 | 1.6 | | |
| GCN-5<br>F: ACTTAAATGGAGGGAACTTGGT;<br>R: CTCCTCAACTCAAAATCCAATC | 1.5 | 3.5 | | |
| Tetraspanin<br>F: CACCTGTAATCTCGGCTATCAT;<br>R: TACTCATCGTTGAGATGTGTGC | 1.2 | 4.2 | 1.9 | 3.8 |
| T-cell receptor chain alpha c6.1A fusion protein<br>F: CTCTTCCTCCTGACACAGAATC;<br>R: GGTATCACTCTCACCCTCACAT | 2.8 | 1.1 | | |
| Heat Shock Factor 2<br>F: GCTTTCTTGACCAAGTTGTGG;<br>R: ATGTATGCATCAAGAGCAACG | 1.4 | 3.2 | | |
| Alpha/beta hydrolase<br>F: ACTCCTTGCCTATTTCCAGC;<br>R: ACAGGAGAAGAGCTGGGTTTAC | 2.8 | 2.1 | | |
| TRAP-1<br>F: GAAAGTTTATTCCCTGCAACAG;<br>R: CCATCTCTCCAGCATAGTTCTC | 1.3 | 6.2 | | |
| NADH oxidoreductase<br>F: CAATAGGAACACATGCTCTTGA;<br>R: TGACCCAGATTCTGTTTTCTGT | 4.2 | 0.4 | 3.5 | 5.3 |
| Glycogen phosphorylase<br>F: GAGAATTATCGCGTGTCTCTTG;<br>R: TAAAAGATACCAGGCCATTTGC | | | 1.1 | 3.4 |

Table 3 (*continued*)

| Gene name, forward (F) and reverse (R) primer sequence | Ratio of PCR product density between *P. marinus* exposed and control oysters | | | |
|---|---|---|---|---|
| | *C. virginica* | | *C. gigas* | |
| | 10 days | 45 days | 10 days | 45 days |
| Arha2<br>F: CCCTTCTTCTTTTTCTTGGTTT;<br>R: TTCTCTATAGATAGCCCCGACA | | | 1.3 | 2.5 |
| Beta-1,3-glucan protein<br>F: CTCGGCAAAGAAACCGCTGGTT;<br>R: GCCCCTACCATAACATAGAGGA | | | 3.2 | 1.8 |
| Proteasome<br>F: GATACGAGATTCCTGTGGATGT;<br>R: AGCCACAAGATGCTGATCTATT | | | 1.4 | 3.2 |
| Actin<br>F: GTCACGTGGTTCTCGTTTATC;<br>R: ACCACCATGTTCCCAGGTAT | 2.1 | 4.5 | 2.3 | 3.1 |

4.67 at Day 45. No *C. gigas* was determined to be infected at Day 10, and only 5 of the 20, at Day 45, when infection intensities ranged 0.33 and 1.00. No mortality was observed for either species during the experiment.

### 3.2. EST sequencing and general characteristics

In this study, we focused our effort on the characterization of up-regulated genes in response to *P. marinus* challenge in both species. For *C. virginica*, a total of 500 clones selected from the four SSH libraries were single-pass sequenced, 200 from Day 10 and 300 from Day 45 SSH libraries (gill and hemocytes), resulting in the characterization of 105 unique sequences. BlastX analysis was performed and comparison of ESTs against nonredundant Swissprot and GenBank databases revealed that 46 clones matched known genes ($E$ value $<10^{-3}$). The genes identified from gill and hemocyte SSH libraries were clustered in nine main categories: (1) detoxification and stress; (2) cell cycle, DNA repair, protein regulation and transcription; (3) respiratory chain; (4) metabolism; (5) cell communication, membrane receptor and immune system; (6) cytoskeleton, (7) ribosomal proteins; (8) unknown function and (9) unidentified proteins (Table 1). Analysis of the gene distribution in both tissues showed that 70 of the 107 sequences are hemocyte-specific, 13 are gill-specific and 24 are common to both tissues. Among the 107 genes identified, 63 were expressed in hemocytes at Day 10 and 85 at Day 45, whereas only 29 were expressed in the gills at Day 10 and 37 at Day 45.

For *C. gigas*, 300 selected clones from Day-10 and Day-45 SSH libraries (gill and hemocytes) were single-pass sequenced, resulting in the characterization of 69 unique sequences. BlastX analysis was performed and comparison of ESTs against non-redundant Swissprot and GenBank databases revealed that 37 clones were significantly similar ($E$ value $<10^{-3}$) to known genes (Table 2). The analysis of the gene distribution in both tissues showed that 38 of the 69 sequences are specific for hemocytes, 6 are gill-specific, and 25 are common to both tissues. Among the 69 genes identified, 36 were expressed in hemocytes at Day 10 and 59 at Day 45, whereas only 19 were expressed in the gills at Day 10 and 30 at Day 45.

### 3.3. Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to evaluate the relative expression of target genes and confirm the differential expression of the genes identified in SSH libraries. For *C. virginica*, the expression of 19 genes was studied in samples collected at Days 10 and 45 after *P. marinus* inoculation and compared to non-challenged oysters sampled at the same dates. For *C. gigas*, we conducted a similar study for 10 genes present in the SSH libraries. PCR amplification of selected genes is presented in Fig. 1. Densitometric analysis of band intensity showed that, for all genes analyzed, the challenged oysters showed increased levels of expression compared with controls, despite some variation in timing and magnitude of up-regulation (Table 3). In *C. virginica*, about half of the genes showed no or little up-regulation at Day 10, but a 3–6 fold over-expression in the challenged oysters at Day 45, while other genes (pescadillo protein, calmodulin, RHO, Toll receptor, BgB lectin, alpha/beta hybrolase and actin) were over expressed in challenged oysters at both Day 10 and Day 45 post challenge. The T-cell receptor chain alpha c6.1A fusion protein and NADH oxidoreductase genes, were up-regulated at Day 10, but returned to normal or a down-regulated state, respectively, at Day 45.

Similarly in *C. gigas*, all genes studied exhibited significant over-expression (by 2–5 fold) in challenged oysters by Day 45, although for some genes the up-regulation was absent or modest at Day 10. The beta-1,3-glucan protein showed a higher level of over-expression in the challenged oysters at Day 10 than at Day 45. Four of the five genes shared by *C. viriginica* and *C. gigas*, showed the same pattern of up-regulation, i.e., greater over-expression over time. The expression of NADH oxidoreductase, however,

was different between the two species: it was over-expressed in challenged oysters of both species at Day 10, and its subsequent expression was intensified in *C. gigas* (density ratio increased from 3.5 to 5.3), but inhibited in *C. virginica* (density ratio decreased from 4.2 to 0.4).

## 4. Discussion

The mechanism by which oysters respond to *P. marinus* infection, particularly when that response successfully eliminates or suppresses the parasite, remain largely unknown. The use of the suppression subtractive hybridization (SSH) method may help to elucidate molecular components of the defense mechanism. Compared to existing EST databases for *C. gigas* generated after exposure to four strains of *Vibrio* sp. bacteria (Gueguen et al., 2003) and to environmental stressors for *C. virginica* (Jenny et al., 2002), most of the sequences characterized in our work are newly identified genes or ESTs, probably because of a longer time of exposure to the parasite (up to 45 days in our experiment versus a few hours for other studies).

The huge difference in *P. marinus* infection prevalence and intensity between the two oysters confirm the previous observation that *C. virginica* is highly susceptible to *P. marinus* infections, while *C. gigas* is strongly resistant (Meyers et al., 1991). Despite of the use of pooled tissue samples, the response we measured in terms of gene expression is correlated with a successful defense in the case of *C. gigas* and a largely unsuccessful one in the case of *C. virginica*. All identified genes show high homologies with vertebrate or invertebrate species, decreasing the probability that they were expressed by *P. marinus.* To confirm this premise, we searched all our *C. virginica* (107) and *C. gigas* (69) sequences against a database of 2500 *P. marinus* ESTs (courtesy of Dr. GR Vasta, University of Maryland Biotechnology Institute) and found no matches, which is not surprising because of the small numbers of sequences in both databases. Further, the *P. marinus* EST database was obtained from cultured parasites, which lose virulence (Ford et al., 2002), and it is possible that parasites in the host and in culture have different profiles of gene expression.

*P. marinus* is an intracellular parasite, often found in hemocytes. Consequently, it is perhaps not surprising that the preponderance of gene expression occurred in hemocytes. Further, hemocytes are abundant in the gill and we identified very few genes, mostly unknown, expressed only in gillin both species (only 13 in *C. virginica* and 6 in *C. gigas*).

Most described molluscan host defense mechanisms involve hemocytes, the cell considered primarily responsible for internal defense, but which is also implicated in numerous other functions such as nutrient transport, shell repair, and excretion. The internal defense system, however, also includes humoral components such as the prophenoloxidase cascade, antimicrobial peptides, lectins and lysosomal enzymes (Asokan et al., 1997). Some of these components, which are also found in or on hemocytes, were identified in our SSH libraries. Among them, a gene for beta galactoside binding lectin is up-regulated in *C. virginica* hemocytes in response to *P. marinus*. This lectin shows a strong similarity with galectin-3, a beta galactoside-binding lectin implicated in inflammatory responses as well as in cell adhesion in humans infected with *Streptococcus pneumoniae* (Sato et al., 2002). Lectins are known to promote cell–cell adhesion and mediate the innate immune response in mammals (Drickamer, 1999). They are present in the hemolymph and on the plasma membrane of hemocytes in oysters and considered as important defense molecules in the hemolymph of invertebrates (Olafsen, 1995).

In *C. virginica* libraries, we also identified a gene that shows a strong homology with lanthionine synthase in rats and humans, and which is suggested to act as a peptide-modifying enzyme synthesizing anti-microbial peptides (Mayer et al., 2001). This result suggests that antimicrobial peptides could also be present in oysters as they are in the mussel, *Mytilus edulis* (Mitta et al., 1999). Surprisingly, no lectin-coding gene was identified in *C. gigas* libraries but one gene, beta-1,3-glucan binding protein, was identified. This protein is thought to play an important role in the innate immune response of crustaceans and insects because binding of lipopolysaccharide and beta-1,3-glucan binding protein activates the prophenoloxidase (proPO) cascade (Jimenez-Vega et al., 2002). This beta-1,3-glucan binding protein gene is also known to be up-regulated by bacterial and fungal infections in shrimp and the protein produced could be an inducible acute-phase protein that may play a critical role in host-parasite interactions (Roux et al., 2002). This gene was detected in both gill and hemocyte *C. gigas* Day-10 SSH libraries but not in the Day-45 libraries. The absence of the beta-1,3-glucan binding protein gene in *C. virginica* libraries could suggest a differential regulation of this gene or a differential efficiency in the proPO cascade activation in the two species. As previously proposed, *C. gigas* may degrade parasites more effectively than *C. virginica* and the proPO cascade, or a similar pathway, may play a role in parasite destruction in oysters, as it does in other invertebrates.

We also identified a gene coding for a protein called pernin or cavortin among *C. virginica* hemocyte up-regulated genes. This protein was first discovered in the mussel *Perna viridis* as large, aggregate structures of several hundred units that comprise almost all of the protein in cell-free hemolymph (Scotti et al., 2001). Pernin is a non-pigmented, glycosylated protein with a serine protease inhibitor activity, and is associated with iron, indicating that it may function as an iron chelator and inhibit *P. marinus* growth (Gauthier and Vasta, 1994). A 3-fold increase of pernin mRNA expression was observed in *C. virginica* after 45 days of *P. marinus* exposure suggesting the possible involvement of pernin in the response to parasite infection.

The genes coding for 3 proteins involved in lysosomal activity were detected in the *C. virginica* SSH libraries and

are up-regulated in response to *P. marinus*: deoxyribonuclease II, the Beige protein and the adaptor–related complex. Two such genes were identified in the *C. gigas* library: the proteasome iota chain and the lysosomal associated transmembrane protein. The Beige protein is a cytosolic protein responsible for the human autosomal recessive disorder, Chediak-Higashi syndrome, which results in hypopigmentation, bleeding and immune cell dysfunction (Perou et al., 1997). Perou et al. (1997) showed that cultured mouse fibroblasts, in which the Beige protein was over-expressed, had smaller than normal lysosomes that were more peripherally distributed than in control cells, suggesting that the Beige protein could regulate lysosomal fission. The over-expression of the Beige protein in *C. virginica* hemocytes could reflect lysosomal malfunction so the Beige protein could be envisaged as an indicator of physiological disturbance by intra-hemocytic *P. marinus.*

In both species, but especially in *C. virginica*, several genes corresponding to membrane receptors were identified that seem to be induced in response to *P. marinus* exposure. Among them, the Toll-like receptor 4 is known to have a key role in the recognition of microbial components (Akira et al., 2003). Its activation has been clearly demonstrated to be the first step for activation of mitogen activated protein kinase (MAPK) and the transcription factors, nitric oxide (NO) and Tumor Necrosis Factor (TNF), during invasion of vertebrate immune-system cells by parasites (Royle et al., 2003). The MAPK signal transduction cascade (MAPKKK–MAPKK–MAPK) is evolutionarily conserved from yeast to humans and plays pivotal roles in many cellular processes including cell growth, differentiation, and apoptosis. Interestingly, genes for MAPK 8 interacting protein 3 also called JIP-3 and a Tumor Necrosis Factor receptor associated protein (called TRAP-1) were also present in the same *C. virginica* library suggesting the involvement of the MAPK cascade in the oyster response to parasite infection. More, it was demonstrated that JNK-interacting protein 3 could be associated with Toll-like receptor 4 and was involved in lipopolysaccharides-mediated JNK activation in mouse cell (Matsuguchi et al., 2003). By RT-PCR, we showed that all these genes had a higher level of expression after 10 days than after 45 days post challenge, despite, or perhaps because of, a higher level of infection after 45 days. TRAP-1 has been identified as an interacting partner for several proteins involved in different cellular functions including cell cycle progression, cell differentiation and immune response (Felts et al., 2000) and was initially identified as a protein that binds to the intracellular domain of the type 1 receptor for tumor necrosis factor (Song et al., 1995). The presence of TRAP-1 highlight the question about the potential presence of TNF in oyster immune system. The over-expression of TRAP-1 gene observed in *P. marinus* inoculated *C. virginica*, but not in *C. gigas*, may well reflect greater cellular disorder caused by parasite in the former species.

Several other proteins involved in the signaling pathway and cellular structure were also identified in *C. virginica*. Among them, the p21-activated kinases (PAK) interacting exchange factor has been reported to mediate the recruitment of PAK into focal adhesions in rats (Wang et al., 2001). Moreover, the PAK interacting exchange factor is required for Rho family GTPase activation that is itself involved in many signaling pathways and cellular functions, including the organization of the actin cytoskeleton, regulation of transcription, cell motility and cell division (Ku et al., 2001). In our study, we found upregulation of RhoA, which is a member of the Ras super-family of small GTP-binding proteins (Ridley, 1997), as well as actin and tubulin, both being involved in cytoskeleton structure. In previous studies, the effect of parasite on host cytoskeleton has been clearly demonstrated (Gruenheid and Finlay, 2003). We also identified proteins of the β-thymosin family to be up-regulated in challenged *C. virginica*. Beta-thymosin are small (5kda) peptides involved in the vertebrate host immune response and cell differentiation and also found in echinoderms and molluscs (Safer and Chowrashi, 1997).

In both species, genes involved in metabolism and particularly in gluconeogenesis have been identified as being differentially expressed. Genes for phosphoenolpyruvate carboxykinase in *C. virginica* and glycogen phosphorylase in *C. gigas* were over-expressed in challenged oysters 45 days after inoculation, indicating a higher energetic requirement by host cells. Interestingly, the expression of the genes involved in respiratory chain and ATP production such as NADH oxidoreductase show a pattern that differs between the two species. We observed an over-expression of this gene in *C. gigas* after both 10 and 45 days of exposure but in *C. virginica*, this gene seems to be inhibited after 45 days. Differences in the way the two species produce and use energy after exposure to *P. marinus* could be a fruitful avenue of research, which could lead to a better understanding of the interactions of the parasite with a susceptible and a resistant oyster.

GenBank searches also yielded matches to unexpected genes, whose function in oysters may differ from other organisms. Some of the matches may involve homology at non-active domain of the proteins involved. For example, we observed increases in expression of a gene identified as coding for a T-cell receptor chain alpha c6.1 fusion protein that is known to be implicated in pathogenesis of chronic/pro-lymphocytic leukaemia of the T-cell lineage. In vertebrate genome, C6.1 A gene is linked to c6.1B gene that may be important in the initial clonal proliferation of T lymphocytes (Thick et al., 1994). It is unlikely that oysters have T-cell receptors or related functions.

This work initiates a complementary approach to EST mass sequencing by identification of highly up-regulated genes in response to parasite challenge. Most of the genes identified in this work have not yet been characterized in molluscs. Our data will complement those already obtained on the same species and will provide more material to

further develop cDNA microarrays. This comparison between *C. gigas* and *C. virginica* genes expressed in response to *P. marinus* infection does not explain the difference in susceptibility between the two species, even though some of the identified genes have immune-system functions in vertebrates. On the other hand, it did identify several genes, especially in *C. virginica* (in which defense was largely unsuccessful) whose over-expression reflects cellular disorder that is probably caused by heavy infections and may well be over-expressed as a consequence of disease rather than a specific defense-related activity. Nevertheless, characterization of over-expressed genes, especially those found in *C. gigas*, may eventually lead to the identification of genes for resistance to *P. marinus*. The genes and ESTs identified in this study can also be mapped onto genetic linkage maps and potentially serve as candidates for mapped disease-resistant genes/Quantitative Traits of Loci (QTLs).

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