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Analysis of differentially expressed genes in response to bacterial stimulation in hemocytes of the carpet-shell clam *Ruditapes decussatus*: Identification of new antimicrobial peptides

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Abstract

Suppression subtractive hybridization was used to identify differentially expressed genes in hemocytes from carpet-shell clam *Ruditapes decussatus* stimulated with a mixture of dead bacterial strains. Putative function could be assigned to 100 of the 253 sequenced cDNAs. Based on sequence homologies, 3.16% of the total identified genes were possibly related to immune functions. Clam myticin isoforms 1, 2 and 3, and clam mytilin, with similarity with myticins and mytilins previously reported on *Mytilus galloprovincialis* were identified and characterized for the first time in clams. The analysis of their expression levels by quantitative PCR showed that they were induced by bacterial challenge. The results obtained in this work could be the first step leading to the understanding of molecular mechanisms by which these economically important marine bivalves respond to pathogens.

Keywords: Antimicrobial peptides; Clam; Immune response; Myticin; Mytilin; *Ruditapes decussatus*; SSH

1. Introduction

In recent years bivalve culture has grown in importance worldwide but diseases, favored by high densities, cause significant economical loses. Diseases affecting these organisms have traditionally been studied using histological techniques, which have proved to be very useful to detect pathogens, but also to determine the lesions and the interaction of the pathogens with the host immune defense mechanisms (Figueras and Novoa, 2004). Today, most of the knowledge on clam (*Ruditapes decussatus*) innate immunity is based on functional assays (Ordás et al., 2000; Tafalla et al., 2003). However, almost nothing is known on the molecular basis of clam immune responses. Interest in bivalve genomics has emerged during the last decade, due to the importance of these organisms in aquaculture and fisheries and to their role in marine environmental science (Saavedra and Bachère, 2006). However, bivalve genomics is at its beginning, and the available data refers only to a very small number of genes (Venier et al., 2006). Thus, few studies have been conducted on the expressed immune genes in response to infections in bivalve molluscs (Tanguy et al., 2004; Gueguen et al., 2003; Kang et al., 2006).

Techniques such as mRNA differential display or suppression subtractive hybridization libraries may facilitate the identification of genes involved on bivalve immune response (Tanguy et al., 2004). The selection of genes induced by infection that could be related with resistance may be used as molecular markers of interest to assist in the selection of strains for aquaculture (Figueras and Novoa, 2004).

Bivalves lack a specific immune system and therefore do not possess immune memory, but they have developed an innate immune system involving cell-mediated and humoral components

Abbreviations: cDNA, DNA complementary to RNA; mRNA, messenger RNA; PAMPs, pathogen-associated molecular patterns; PRRs, host pattern recognition receptors, Cg-Def1, *Crassostrea gigas* defensin isoform 1; Cg-Def2, *Crassostrea gigas* defensin isoform 2; AOD, American oyster defensin; MGD1, *Mytilus galloprovincialis* defensin isoform 1; MGD2, *Mytilus galloprovincialis* defensin isoform 2; EST, expressed sequence tag; SSH, Suppression Subtractive Hybridization; rRNA, ribosomal RNA; qPCR, Quantitative PCR; AMP, Antimicrobial peptide; ORF, Open reading frame; RACE, Rapid amplification of cDNA ends; UTR, Untranslated region; NJ, Neighbour-Joining.

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used to recognize and eliminate pathogens. The hemocytes are the cells primarily involved in inflammation, wound repair, encapsulation and phagocytosis, and it is in the hemolymph where the most potent components of the bivalve immune response are localized (Pipe, 1990). They recognize unique and characteristic pathogen-associated molecular patterns (PAMPs) using host pattern recognition receptors (PRRs) (Medzhitov, and Janeway 2000; Kang et al., 2006). In addition, the cell free hemolymph contains specific soluble substances such as antimicrobial peptides secreted by the hemocytes, among others. These constitute important components of the immune system for all phyla from plants to animals including bacteria, being exceptionally diverse in sequence, structure and function (Cellura et al., 2007). In bivalve molluscs, antimicrobial peptides have only been identified in oysters *Crassostrea gigas* and *C. virginica* (Gonzalez et al., 2007; Seo et al., 2004) and mussels *Mytilus galloprovincialis* and *M. edulis* (Charlet et al., 1996; Hubert et al., 1996; Mitta et al., 1999a,b). Taking into account the features of their primary structure and their consensus cysteine array, these peptides were classified in four groups: i) mytilins, with five isoforms (A, B, C, D and G1) (Mitta et al., 2000a,b), ii) myticins, with three isoforms A, B (Mitta et al., 1999a,b), and C (Pallavicini et al., in press), iii) defensins, found in both mussel and oysters, with two isoforms (MGD1 and MGD2) in *M. galloprovincialis*; defensin A and B in *M. edulis*, Cg-Def1 and Cg-Def2 in *C. gigas*, and AOD (American oyster defensin) in *C. virginica* (Charlet et al., 1996; Mitta et al., 2000a, Gonzalez et al., 2007; Seo et al., 2004); and iv) mytimicin, partially characterized from *M. edulis* plasma (Charlet et al., 1996). However, until now no antimicrobial peptides have been described in carpet-shell clams, and only one expressed sequence tag (EST) obtained from a cDNA library from other clam species, *R. philippinarum* infected with *Perkinsus olseni*, had similarity with mussel defensin MGD-1 (Kang et al., 2006).

The main goal of this paper is the identification and characterization of genes involved in the immune response of the bivalve mollusc *R. decussatus*, one of the most economically important clam species cultured in Galicia (NW Spain). The use of the SSH technique will allow us to identify differentially expressed genes in response to a particular stimulus.

2. Materials and methods

2.1. Maintenance of clams

Carpet-shell clams, *R. decussatus*, were obtained from a commercial shellfish farm. Animals were maintained in open circuit filtered seawater tanks at 15 °C with aeration and they were fed daily with *Isochrysys galbana* (12×10^8 cells/animal), *Tetraselmis suecica* (10^7 cells/animal) and *Skeletonema costatum* (3×10^8 cells/animal). Prior to the experiments, bivalves were acclimatised for 1 week.

2.2. Immune stimulation, hemolymph withdrawal and RNA isolation

A total of 50 clams were notched in the shell in the area adjacent to the adductor muscles and injected into the adductor muscle with 100 µl (containing 10^7 cells/ml) of a mixture of dead bacteria (*Micrococcus lysodeikticus*, *Vibrio splendidus* and *Vibrio anguillarum*), kindly donated by Philippe Roch (UMR CNRS Ecolag, Université de Montpellier 2, France). Other group of 50 clams were injected with 100 µl of filtered sea water (FSW) and used as controls. After the stimulation, clams were returned to the tanks and maintained for 48 h at 15 °C until sampling.

Hemolymph (0.5–1 ml) was withdrawn from the adductor muscle of each animal with a disposable syringe. Hemolymph collected from fifty individual clams was pooled and centrifuged at 2500 ×g during 15 min at 4 °C. The pellet was re-suspended in 6 ml of Trizol (Invitrogen) and the RNA was extracted according to the manufacturer's protocol.

2.3. Suppression subtractive hybridization

The suppression subtractive hybridization technique (SSH) (Diatchenko et al., 1996) was used to characterize new genes involved in the carpet-shell clam's innate immune response against the mixture of dead bacteria 48 h after stimulation. Briefly, cDNA was synthesized from 1 µg of each hemocyte RNA sample (bacterial infected and control not infected) using the SMART PCR cDNA Synthesis Kit (Clontech). A SSH assay was then performed using the PCR-Select cDNA Subtraction Kit (Clontech) following manufacturer's instruction (Pallavicini et al., in press), and using the cDNA of infected tissues as tester, and the cDNA of non-infected tissues or control as driver. The PCR mixture of differentially expressed was cloned using the TOPO TA cloning kit (Invitrogen) and transformed in *E. coli* competent cells.

Selected colonies were amplified by PCR using Nested PCR primer 1 and 2R from PCR-Select cDNA Subtraction Kit (SSH technique). Agarose gel electrophoresis was performed to check and select by size the samples to be sequenced and arrayed. The PCR profile consisted of: initial denaturation for 5 min at 94 °C; 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 65 °C and 1.5 min elongation at 72 °C; final extension for 7 min at 72 °C. Excess primers and nucleotides were removed by enzymatic digestion using 10 U and 1 U of ExoI and SAP, respectively (Amersham Biosciences) at 37 °C for 1 h followed by inactivation of the enzymes at 80 °C for 15 min. DNA sequencing was performed using a BigDye terminator Cycle Sequencing Ready Reaction Kit and an automated DNA sequencer ABI 3730.

2.4. Sequencing analysis

Raw chromatograms were analysed with Chromas 231 software (Technelysium). Search for similarities with known genes was performed using BLAST (http://www.ncbi.nlm.nih.gov/blast/). Translation and protein analysis were carried out using the ExPaSy tools (http://us.expasy.org/tools). Multiple sequence alignments were generated with Clustal W (Thompson et al., 1997). Database search was performed using BlastX and the best annotated hit from the similarity search was retained. Novel ESTs were deposited in GeneBank and assigned accession numbers EL903716–EL903793.