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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.

Key words: antimicrobial peptides, clam, immune-response, Myticin, Mytilin, *Ruditapes decussatus*, SSH.

1. Introduction

In recent years bivalve culture has grown in importance worldwide but diseases, favoured 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 & 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 & 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 & 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 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, & 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; Mitta et al., 1999b). 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; Mitta et al., 2000b), ii) myticins, with three isoforms A, B (Mita et al., 1999), 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 *Ruditapes 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. Material 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 15ºC with aeration and they were fed daily with *Isochrysys galbana* ($12x10^8$ cells/animal), *Tetraselmis suecica* (10^7 cells/animal) and *Skeletonema costatum* ($3x10^8$ 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 hours at 15ºC 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 2500xg during 15 minutes at 4ºC. 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 1µg 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 94ºC; 35 cycles of 30 s denaturation at 94ºC, 30 s annealing at 65ºC and 1.5 min elongation at 72ºC; final extension for 7 min at 72ºC. Excess primers and nucleotides were removed by enzymatic digestion using 10 U and 1 U of ExoI and SAP, respectively (Amersham Biosciences) at 37ºC for 1 h followed by inactivation of the enzymes at 80ºC 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.

2.5. Identification and characterization of new AMP. Isolation of full-length transcripts

In order to get the full length ORF cDNA of selected sequences, those ESTs with high similarity with the known AMPs mytilin and myticin sequences were used to design primers for 5' cDNA. RACE reactions were performed using SMART RACE cDNA Amplification Kit from Clontech according to the manufacturer's instructions. A 5'RACE primer (MYTL-CC.1: 5'- GGAAAGGTCTATTAACACAGGTCG-3') for mytilin, and a 5' RACE (MYT-CC.2: 5'-GTGCCAATGGAGACTCAGCTCTGCGAC-3') for myticin were designed by Primer 3 software (Rozen and Skaletsky, 2000). After cloning in pGem-T Vector System (Promega) and transformation in Top 10F' competent bacteria (Invitrogen), several clones were sequenced from both ends with M13 forward and reverse primers using BigDye terminator Cycle Sequencing Ready Reaction Kit and an automated DNA sequencer ABI 3730.

A retro transcription was performed to obtain cDNAs using the SuperScriptII RNAase H- Reverse Transcriptase (Invitrogen) from 5 μg of the original RNA. The resulting cDNA was subjected to a PCR reaction using a primer pairs (MYTL-CC F3: 5'-GTTCAATATCTACATCTTTAAG-3' and MYTL-CC R2: 5' -CTATTAACACAGGTCGAATC-3') for mytilin, and (MYT-CC F: 5'-AGTACGCGGGGGCTTCTAC-3' and MYT-CC R: 5'-AGCAGAAAACGTCGAAAGCG-3') for myticin, that were designed in order to check the full length AMP sequences obtained by RACE. The cleavage site for the signal peptide was determined by analysis with SignalP (Bendtsen et al., 2004).

2.6. Phylogenetic analysis

In addition to the sequences obtained in this study, sequences of the complete ORF of mytilins and myticins obtained from the GenBank were also included in the analysis: myticin A (AAD47638); myticin B (AAD47639); mytilin B (AAD52661); mytilin C (AAU44785). rRNA sequences were aligned with ClustalW (Thompson et al., 1997) including in MEGA 3 (Kumar et al., 2004). A phylogenetic tree based on deduced amino acid ORF sequences was performed using the Neighbour-Joining (NJ) algorithm

with the MEGA3 software programme. Statistical confidence on the inferred phylogenetic relationships was assessed by bootstrap of 1000 replicates.

2.7. Expression analysis of the identified AMP genes

In order to confirm the differentially expression of the antimicrobial peptides identified as up-regulated in the SSH library at 48 h post injection, and to evaluate and quantify their relative expression, a real time SYBR Green PCR assay was carried out using the same hemocytes cDNA previously used for the SSH library. Results were obtained from three different qPCRs.

An additional experiment was carried out to determine the expression of the clam AMPs after infection with dead bacteria (the same bacterial mixture previously used) or live *V. anguillarum*. For each treatment, three pools of 4-5 clams were injected into the adductor muscle with 100 µl (containing 10^7 cells/ml) of dead or live bacteria. The same protocol was used for controls injecting FSW. Hemolymph and RNA samples were obtained as previously described.

Quantitative PCR assays and data analysis were performed using 7300 Real Time PCR System (Applied Biosystems). The 25 µl PCR mixture include of 12.5 µl of SYBR Green PCR master mix (Applied Biosystems) with 0.5 µl of primers pairs 10 µM (MYT-CCQ F: 5'-ACAAACTTCATCCCGGCAAA-3'; MYT-CCQ R: 5'-GGTCCAAATGTTCCATCTCATTC-3' for Myticins, and MYTL-CCQ F: 5'-AAGGTGAAACTGGAATGTAAAGAGAAG-3' and MYTL-CCQ R: 5'-AAGGTCTATTAACACAGGTCGAATCA-3' for mytilins), and 1 µl of a 1:5 dilution of the cDNA. Amplification was carried out at the standard cycling conditions of 95 º for 10 min, followed by 40 cycles of 95 º 15 s and 60 º for 1 min. The comparative CT method (2-ΔΔCT method) was used to determine the expression level of analyzed genes (Livak and Schmittgen, 2001). The expression of the candidate genes was normalized using 18S fragment as a housekeeping gene by the specific primers 18S-F: 5'-GGGAAGAGCGCTTTTGTTAG-3' and 18S-R: 5'- GCATAGCACGTACCATCGAC-3'. Fold units were calculated dividing the normalized expression values of infected tissues by the normalized expression values of the controls. Results are given as the mean and standard deviation of three replicates and pools.

3. Results

3.1. ESTs sequencing and identification

A total of 253 clones obtained from hemocyte SSH libraries were isolated, amplified by PCR and sequenced. BLASTx analysis and comparison of ESTs against GenBank databases was performed to find the sequence similarity. A total of 119 sequences grouped in 78 contigs were potential protein-coding genes. Of them, 100 sequences, grouped in 64 contigs showed a significant match to known proteins (Table 1). A total of 75 transcripts were redundant (representing 63% sequences). An overview of the obtained results is displayed in Table 2. Only matches with e-values smaller than $10e^{-3}$ were retained as significant. The genes identified were clustered and graphically represented into eight functional categories (Fig. 1): (1) Some of the identified transcripts (6 clone clusters, including 8 ESTs, representing 3.16 %) were probably involved in immune defense; (2) a total of 7 clone clusters (including 10 ESTs, 3.95 %) showed similarity with genes involved in cell signaling and adhesion; (3) a total of 14 clone clusters, (including 26 ESTs, 10.28 %) were potentially related to cytoskeleton structure; (4) 17 clusters (32 ESTs, 12.65 %) showed similarity with genes involved in cell cycle; (5) a total of 14 clone clusters (including 18 ESTs, 7.11%) showed similarity with genes involved in cell metabolism and respiratory chain; (6) and 6 clone clusters (6 ESTs, 2.37 %) were ribosomal proteins. The remaining 19 obtained transcripts (7.5% of the total sequences) were not classified, and considered to have unknown functions (Table. 1). A total of 134 analyzed transcripts showed no significant result or no hit.

Among the ESTs with direct or indirect immune functions: two clusters showed similarity with proteins involved in stress (HSP 70 and ferritin), one cluster had similarity with proteins of apoptosis inhibition (IAP), one with the polypeptide “Naegleriapore” and two with antimicrobial peptides.

3.2. Analysis of antimicrobial peptides

The sequence clustering and BLASTx similarity searching of the SSH obtained ESTs showed two independent EST clusters which can be putatively identified as precursors of the antimicrobial peptides myticin and mytilin respectively. After amplification of the complete ORF by 5’ RACE, cloning and sequencing, a total of 3 sequences coding for different amino acid sequences showed evident similarity with the described myticins. Moreover, one sequence presented similarity with the described mytilins. The full length ORF nucleotide and aminoacid sequences of the three new isolated myticin and mytilin forms from *R. decussatus* are presented in Fig. 2 and 3 respectively.

The new myticin forms that we have named “clam myticin” presented a 300 bp ORF encoding 100 amino acid residues. The 5’ untranslated region (UTR) was only partially sequenced. However, the 3’ UTR was sequenced until the Poly A, and contains 163 base pairs. The analysis of the aminoacid sequence of the three forms showed a shared common structure. The consensus cystein array characteristic of Myticin family was conserved. A signal peptide of 20 amino acids was localized in the N-terminal region. The cleavage site for the signal peptidase was located after the A preceding the Q, at position 20 for the form 1, and after the S preceding the V, at position 22 for the forms 2 and 3 (Fig. 2). A 40-residue sequence corresponding to the mature peptide, as well as a highly conserved C-terminal extension of also 40 residues were observed. Aminoacidic diversity was found in 13 positions between the three different forms (9 between form 1 and form 2; 10 between form 1 and form 3; and 7 between form 2 and form 3), and in 55 positions compared with the previously described myticins A and B (Fig. 4A). The new mytilin form that we have named “clam mytilin” presented a partial sequence of 508 bp long, with a 297 bp complete ORF that code for 99 amino acid residues. The partial 5’ untranslated region (UTR) contained 39 nucleotides, and the complete 3’ UTR region contains 173 nucleotides until the Poly A. The consensus cystein array characteristic of Mytilin family was detected. A signal peptide of 24 amino acids was localized in the N-terminal region (Fig. 3). This mytilin peptide differs only in 2 amino acidic residues from the mytilin C previously described in mussels (Fig. 4B). A 34-residue sequence corresponding to the mature peptide was observed.

The phylogenetic tree constructed with the amino acid sequences of the herein described clam AMPs, together with all the described groups of Myticins and Mytilins identified in mussels, showed two main clusters. Neighbor-Joining phylogenetic analysis supported clam myticin forms as a separated sister taxon to the mussel myticins, with a bootstrap of 100%, and clam mytilin as a sister taxon of mussel mytilins. The high bootstrap (98%) strongly favors the position of clam mytilin close to mytilin C. (Fig. 5).

3.3. *Expression patterns of AMP transcripts*

Quantitative PCR analysis were performed to determine the relative expression pattern of the two different antimicrobial peptides identified as up-regulated in the hemocyte SSH library at 48 h post-infection, and also to validate this technique. A significant induction of the expression levels of both AMPs was detected in bacterial

stimulated clams (1109.6± 636.9 for clam myticin, and 886.1± 459.4 for clam mytilin), in contrast with non-infected controls (0.139±0.238; and 0.562± 0.605 respectively). Both AMPs were only slightly expressed in controls. However, bacterial infections induced an increase of 7982.7 and 1576.7 fold for clam myticin and clam mytilin respectively (Fig. 6A).

In addition, Q-PCRs performed on hemocytes from clams injected with dead bacteria or *V. anguillarum* showed a similar expression pattern after 24 and 48 h post-injection, although the increment with respect to controls was lower if we compare it with that obtained using the cDNA of the SSH library. Both myticin and mytilin expression levels were increased after bacterial challenge (Fig. 6B). The highest expression levels were observed 48 h post infection in both challenges.

4. Discussion

The understanding of the molecular mechanisms by which clams, bivalves, and invertebrates in general, are able to mount an immune response to pathogens remains largely unknown. To our knowledge there are few studies on immune-related genes identification and their expression carried out in bivalve molluscs (Gueguen et al., 2003; Kang et al., 2006 and Venier et al. 2006). The study of differential gene expression by SSH is a promising approach to identify and characterize genes involved in the host response against pathogens not only in bivalves, but in different aquatic invertebrate and vertebrate groups (Ursic-Bedoya et al., 2007; Lorgeril et al., 2005; Bayne et al., 2001 Tanguy et al., 2004; He et al. 2004; Pan et al., 2005, Jenny et al., 2006, Dios et al, 2007). Recently, sea urchin immune genes have been widely investigated (Hibino et al., 2006; Rast et at., 2006; Lapraz et al., 2006; Nair et al., 2005; Courtney Smith et al., 2001). Despite some specific findings (Gueguen et al., 2003; Tanguy et al., 2004; Kang et al., 2006), mostly on oysters, very little is known concerning the molecular mechanisms involved in the recognition, activation and effector molecules of bivalves immune response to pathogens. The only work related to the molecular basis of clam immune response infected by pathogens was by Kang et al. (2006), that prepared a Manila clam cDNA library and also studied the expression of two lectins in *Perkinsus olseni* or by *Vibrio tapetis* infected clams.

The subtracted cDNA library enriched with gene transcripts differentially expressed in bacterial stimulated clams, showed that several transcripts obtained from hemocytes, the main cellular effectors in invertebrate immune defense, could be

classified as stress and immune response related. Other ESTs had several developmental and metabolic cell functions. Most of the sequences characterized in the present work are new ESTs, and some of them have unknown functions in clams up. Further studies are needed to characterize those genes, which will be the goal of future researches.

Among the various components that putatively could have an immune role associated, two stress proteins, HSP70 and ferritin, were identified as being up-regulated in the bacterial infected clams. Heat-shock proteins are molecular chaperones that protect the cell and maintain homeostasis under stressful conditions. The HSP70 is a well known stress related protein already described in several bivalve species, including the oyster (*Crassostrea gigas*), mediterranean mussel (*Mytilus galloprovinciallis*), the manila clam (*Ruditappes philipinarum*) (Tanguy et al., 2004; Cellura et al., 2007 and Kang et al., 2006), and also in the pacific abalone (*Haliotis discus hannai*) (Cheng et al., 2007), a marine gasteropod with growing economic importance. This protein is inducible by a variety of stressful conditions, not only heat shock and contaminants, but also by pathogens and pathophysiological conditions related to inflammation (Cheng et al., 2007). The ferritin was the other stress protein found as being up-regulated in our experimentally infected clams. This protein had been classified as stress protein due to their similarity with proteins involved in detoxification processes triggered by stress (Gueguen et al., 2003). Beck et al. (2002), indicated that ferritin can be considered an acute phase protein that regulate iron and free cations as a defense mechanism against the alteration of the arrangement of plasma proteins and serum concentrations of the cations copper, zinc and iron during inflammation process.

Proteins of apoptosis inhibition (IAP), which could be related to the inhibition of the apoptosis induced by the stress and the infection, and Naegleriapore, a pore forming polypeptide of the pathogenic protozoan *Naegleria fowleri* (Herbst et al., 2002), were also identified as being up-regulated in our experiments.

Additionally, two AMPs which presented similarity with already known mussel antimicrobial peptides were also identified. In this work we report for the first time the full length of three different clam myticin isoforms and a clam mytilin. These peptides showed similarity with known mussel mytilin and myticin genes, sharing their polypeptide structure and consensus cystein array characteristic of each group. However, aminoacidic diversity of the clam myticin forms identified was found in 13 positions among the three different forms (9 between form 1 and form 2; 10 between form 1 and form 3; and 7 between form 2 and form 3), and in 55 positions compared

with the previously described mussel myticins A and B. Aminoacidic diversity was found with the newly described myticin C (Pallavicini et al., in press). Clam mytilin differs highly from mussel mytilins A, B, G1 and D, but only in 2 amino acidic residues from Mytilin C.

It is accepted that myticins are essentially active against gram-positive bacteria, and less active against gram-negative bacteria and fungi. However, mytilins display a wider spectrum of activity according to isoforms (Mitta et al., 2000c). Mussel hemocytes are a site of storage and production of AMPs. They are sensible to different microbial substances and after stimulation, hemocytes degranulate and release into the extracellular fluid a series of substances involved in immune defense, including "specific" antimicrobial peptides (Mitta et al., 1999a). The variation in aminoacidic residues of the different AMP types or forms can give as a result the variation in the protein produced, which could generate a specific differentiation for recognition of different types of pathogens.

Only one AMP (an EST with similarity to mussel defensin MGD-1, Kang et al. 2006) has previously been identified in *Perkinsus olseni* infected Manila clams. Defensins are involved in the anti-infectious process, but no striking discrepancies have been observed in haemocyte expression between naïve and bacteria challenged oysters and mussels at different infection times (González et al., 2007). The expression of this AMP was not induced after bacterial challenge (Mitta et al., 1999b; Gueguen et al., 2006). The constitutive expression of these AMPs, and their unaltered or even decreased transcription level after bacterial challenge, together with the limited sequencing effort, could explain their absence from our SSH library.

Clam myticin and mytilin showed increased expression levels by Q-PCR, 24 and 48 h post injection in clams injected with the mixture of dead bacteria and on those injected with live *V. anguillarum*. Q-PCR results obtained at 48 h post injection confirmed those obtained in SSH library cDNA. However clam myticin and mytilin expression levels were significantly higher when Q-PCR was performed using SSH cDNA. This difference could be due to several reasons such as differences in clam stocks or to seasonal variations in the immune response as it has been shown in mussels (Santarem et al. 1994)

The present work constitutes one of the first studies on *Ruditapes decussatus* gene identification and their modulation by bacterial infection using the suppression subtractive hybridization technique. Although we have focused on the two AMPs

characterized by the first time on clam hemocytes, most of the analyzed genes were not previously identified in molluscs. The relatively high diversity of AMP types and their isoforms and their possible specificity in the immune response constitutes them as an interesting research topic. Moreover, further study of the described genes is needed since the identification of molecular markers related to bacterial resistance could be very useful for selection purposes in bivalve aquaculture.

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Figure Legends:

Fig. 1. Functional classification of *R. decussatus* hemocyte ESTs. The 253 ESTs were clustered into 6 categories according with their putative biological function.

Fig 2. Complete ORF nucleotide and deduced amino acid sequences of the tree different Clam myticin forms. Signal peptides are in grey. (▼) Indicate the cleavage sites. (*) Indicate the stop codons. A. Clam myticin form 1; B. Clam myticin form 2; C. Clam myticin form 3.

Fig 3. Complete ORF nucleotide and deduced amino acid sequence of the Clam mytilin sequence. Signal peptide is in grey. (▼) Indicate the cleavage site. (*) Indicate the stop codon.

Fig. 4. A. Multiple alignment of clam myticin forms compared with mussel myticin A and B. B Multiple alignment of amino acidic sequences of the clam mytilin compared with related mytilins. (*) identical residues, (:) conserved substitutions and (.) semiconserved substitutions. The most similar sequences are highlighted in grey, and the amino acidic differences between these two forms are in bold. The cystein consensus array, in grey, is also shown.

Fig. 5. Neighbour-Joining (NJ) tree of the ORF aminoacid sequence data shows the phylogenetic relationships among the three forms of the Clam myticin AMP (form 1, 2 and 3), the Clam mytilin, and the complete ORF of myticins and mytilins previously reported in mussels. Bootstrap: 100 repetitions.

Fig. 6. Quantitative expression of clam myticin and clam mytilin in hemocyte samples of *R. decussatus*. A. AMPs expression in hemocyte cDNA obtained by SSH library at 48 h post-infection with a mixture of dead bacteria. B. AMPs expression in clams injected with a mixture of dead bacteria or *V. anguillarum* after 24 and 48 h post-infection. Results are mean ± SD. Bars represent the relative expression transcript levels of infected clams referred to controls, previously normalized to 18S transcript levels.

Figure 1

Fig. 1.

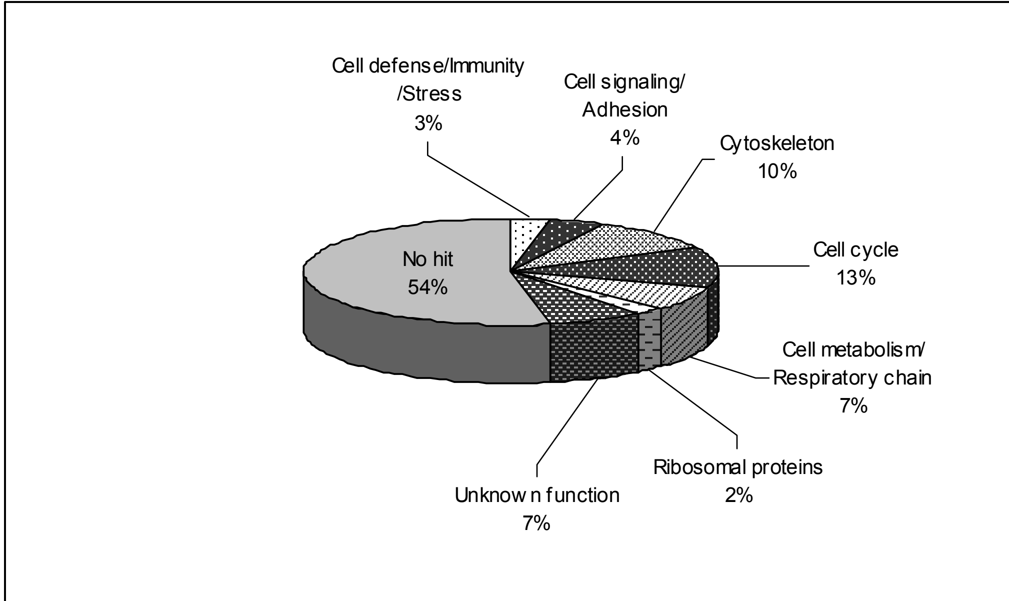


Figure 2

Fig 2.

A

```
1     cggcttagtacgcggggggcttctacaagattgtaaaaaaggtttctaattaagtattcc  60
                                  M  K  A  T  I  L  L  A  V  V  V  A  V
61    tcaaaactcaaaacattcaacatgaaggcaacgatcttgttagctgttgtagtggcagtc  120
      I  V  G  V  Q  E  A ▼Q  S  V  A  C  T  S  Y  Y  C  S  K  F
121   attgttggagttcaggaagcccaatcagtagcttgtacatcatactactgtagtaagttc  180
      C  G  S  A  G  C  S  L  Y  G  C  Y  K  L  H  P  G  K  I  C
181   tgtgggtctgctggttgctcattatatggatgttacaaacttcatcctggcaaaatttgc  240
      Y  C  L  H  C  R  R  A  E  S  P  L  A  L  S  G  S  A  R  N
241   tactgccttcattgtcgcagagctgagtctccattggcactttctggaagcgctaggaat  300
      V  N  D  K  N  N  E  M  D  N  S  P  V  M  N  E  V  E  N  L
301   gtgaacgacaagaacaacgagatggacaactctccagtgatgaatgaggtggaaaatttg  360
      D  Q  E  M  D  M  F  *
361   gaccaagaaatggatatgttctagacagatatttgatcaagagctaacttagaaaatcag  420
421   ctatacttctttccttgattgtgaactctttgtgcaaagtctgttataaacttgaaaatt  480
481   tattaaatcttctgtcatacgctttcgacgttttctgcttgaagtaacattaaatattta  540
541   ataaggaaaaaaaaaaaaaaaaaa  565
```

B

```
1     cggcttagtacgcggggggcttctacaagattgtaaaaaaggtttctaattaagtattcc  60
                                  M  K  A  T  I  L  L  A  V  V  V  A  V
61    tcaaaactcaaagcattcatcatgaaggcaacgatcttgttagctgttgtagtggcagtc  120
      I  V  G  V  Q  E  V  Q  S ▼V  P  C  A  S  T  Y  C  A  R  F
121   attgttggagttcaagaagtccaatcagtaccttgtgcatcaacctactgtgctaggttc  180
      C  G  S  A  G  C  L  K  Y  G  C  Y  R  L  H  P  G  K  I  C
181   tgtgggtctgctggttgcttaaaatatggatgttacagacttcatcccggcaaaatttgc  240
      Y  C  L  H  C  R  R  A  E  S  P  L  A  L  S  G  S  A  R  N
241   tactgccttcattgtagaagagctgagtctccattggcactttctggaagcgctaggaat  300
      V  N  D  K  N  N  E  M  D  N  S  P  V  M  N  E  M  E  N  L
301   gtgaacgacaagaacaacgagatggacaactctccagtgatgaatgagatggaaaatttg  360
      D  Q  E  M  D  M  F  *
361   gaccaagaaatggatatgttctagacagatatttgatcaagagctaacttcaaaaatcag  420
421   ctatacttcttccttgatggtgaacactttgcgcaaagtctgttataaacttgacaatta  480
481   ttaaatcttctgtcatacgctttcgacgttttctgcttgaagtaacattaaatatttaat  540
541   aaggaaaaaaaaaaaaaaaaaa  563
```

C

```
            M  K  A  T  I  L  L  A  V  V  V  A  V  I  V  G  V  Q
1     ttgaacatgaaggcaacgatcttgttagctgttgtagtggcagtcattgttggagttcag  60
      E  V  Q  S ▼V  P  C  A  S  T  L  C  S  R  F  C  G  S  A  G
61    gaagtccaatcagtaccttgtgcatcaaccttgtgtagtaggttctgtgggtctgctggt  120
      C  R  L  Y  G  C  Y  R  L  H  P  G  K  I  C  Y  C  L  H  C
121   tgcagattatatggatgttacagacttcatcccggcaaaatttgctactgccttcattgt  180
      R  R  A  E  S  P  L  A  L  S  G  S  A  R  N  V  N  D  Q  N
181   cgcagagctgagtctccattggcactttctggaagcgctaggaatgtgaacgatcagaac  240
      K  E  M  D  N  S  P  V  M  N  E  M  E  H  L  D  Q  E  M  D
241   aaagagatggacaactctccagtgatgaatgagatggaacatttggaccaagaaatggat  300
      M  F  *
301   atgttctagacagatatttgatcaagagctaacttagaaaatcagctatacttctttcct  360
361   tgatggtgaacaatttgttcaaagtctgttataaacttgacaatttattaaatcttctgt  420
421   catacgctttcgacgttttctgataagccacattaaatatttaataaggaaaaaaaaaaa  480
481   aaaaaaaa  488
```

Figure 3

Fig 3.

```
                                           M  K  L  A  V  I  L
1    aattcggcttgttcaatatctacatctttaagatccaatatgaagttagcagttatccta  60
     A  I  A  L  A  V  L  L  I  V  Q  D  A  D  A ▼S  C  A  S  R
61   gccatcgcccttgcagtacttcttatagtgcaagacgcagatgcaagctgtgcttccaga  120
     C  K  S  R  C  R  A  R  R  C  R  Y  Y  V  S  V  R  Y  G  W
121  tgtaaatctcgttgtagagccagacgctgtagatattacgtgtcagtcagatatggatgg  180
     F  C  Y  C  R  C  L  R  C  S  S  E  H  T  M  K  F  S  P  E
181  ttttgctattgcagatgtctccgttgttccagcgagcataccatgaaattctcacctgaa  240
     S  E  G  P  A  E  M  P  A  Q  M  N  D  H  E  Q  F  Q  D  M
241  agtgaaggaccagctgagatgccagcacagatgaatgaccatgagcaattccaggacatg  300
     Q  K  G  E  T  E  Q  G  E  T  G  M  *
301  cagaaaggagaaaccgaacaaggtgaaactggaatgtaaagagaaggccttataaagtga  360
361  cgttgatgcacattctgtttcagagtgatattctgattcgacctgtgttaatagaagccg  420
421  ccctttcgtttcacttgtataaaatgtcttagatttctgtaattgttatctaatatcata  480
481  gattctaaataaaattgtgtttcaactcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa  540
```

Figure 4

Fig. 4.

A

```
Clam Myticin form 3     MKATILLAVVVAVIVGVQEVQSVPCASTLCSRFCGSAGCRLYGCYRLHPGKICYCLHCRRAESPLALSGSARNVNDQNKEMDNSPVMNEMEHLDQEMDMF
Clam Myticin form 2     MKATILLAVVVAVIVGVQEVQSVPCASTYCARFCGSAGCLKYGCYRLHPGKICYCLHCRRAESPLALSGSARNVNDKNNEMDNSPVMNEMENLDQEMDMF
Clam Myticin form 1     MKATILLAVVVAVIVGVQEAQSVACTSYYCSKFCGSAGCSLYGCYKLHPGKICYCLHCRRAESPLALSGSARNVNDKNNEMDNSPVMNEVENLDQEMDMF
Myticin A               MKATILLAVLVAVFVAGTEAHSHACTSYWCGKFCGTASCTHYLCRVLHPGKMCACVHCSRVNNPFRVNQVAKSIN----DLDYTPIMKSMENLDNGMDML
Myticin B               MKATMLLAVVVAVFVAGTEAHPHVCTSYYCSKFCGTAGCTRYGCRNLHRGKLCFCLHCSRVKFPFGATQDAKSMN----ELEYTPIMKSMENLDNGMDML
                        ****.****.***:*.  *::.: *:*::*::***:*.*  *.*: **.**:*:*.**:*:::*:::::*:::*     ::.::*:*:::*.**: ***:
Consensus cystein array                         CxxxxCxxxCxxxxCxxxxCxxxxxxxxCxCxxC
```

B

```
Mytilin_C               MKLAVILAIALAVLLIVQDADASCASRCKSRCRSRRCRYYVSVRYGWFCYCKCLRCSSEHTMKFSPESEGP----AEMPAQMNDHEQFQDMQKGETEQGETGM

Clam Mytilin            MKLAVILAIALAVLLIVQDADASCASRCKSRCRARRCRYYVSVRYGWFCYCRCLRCSSEHTMKFSPESEGP----AEMPAQMNDHEQFQDMQKGETEQGETGM
Mytilin B               MKAAVILAIALVAILAVHEAEASCASRCKGHCRARRCGYYVSVLYRGRCYCKCLRCSSEHSMKFPENEGSSPSDMMPQMNENENTEFGQDMPTGETEQGETGI
Mytilin G1              --------------------VVTCGSLCKAHCTFRKCGYFMSVLYHGRCYCRCLLC-----------------------------------------------
Mytilin A               ----------------------GCASRCKAKCAGRRCKGWASASFRGRCYCKCFRC-----------------------------------------------
Mytilin D               ----------------------GCASTCKAKCAGRRCKGWASASFRRRCYCKCFRC-----------------------------------------------
                                               *.* **.:*  *:*  : *. :   ***:*: *
Consensus cystein array                        CxxxCxxxCxxxxCxxxxxxxxxxxCxCxCxxC
```

Figure 5

Fig. 5.

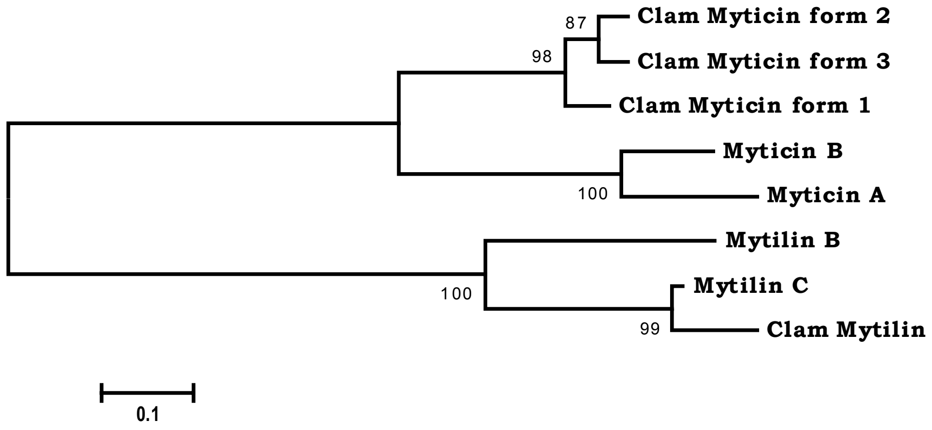
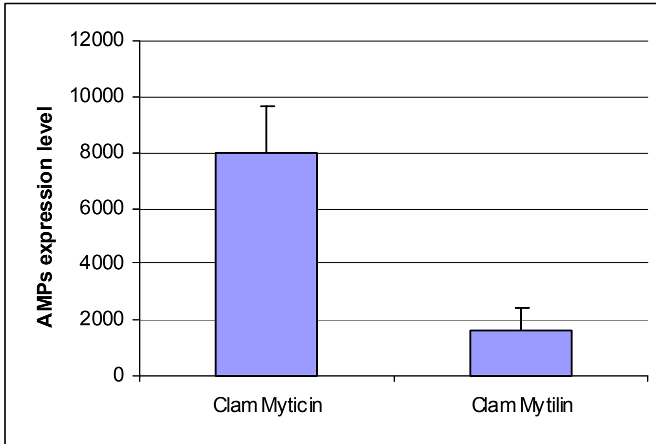


Figure 6

Fig. 6.

A.



B.

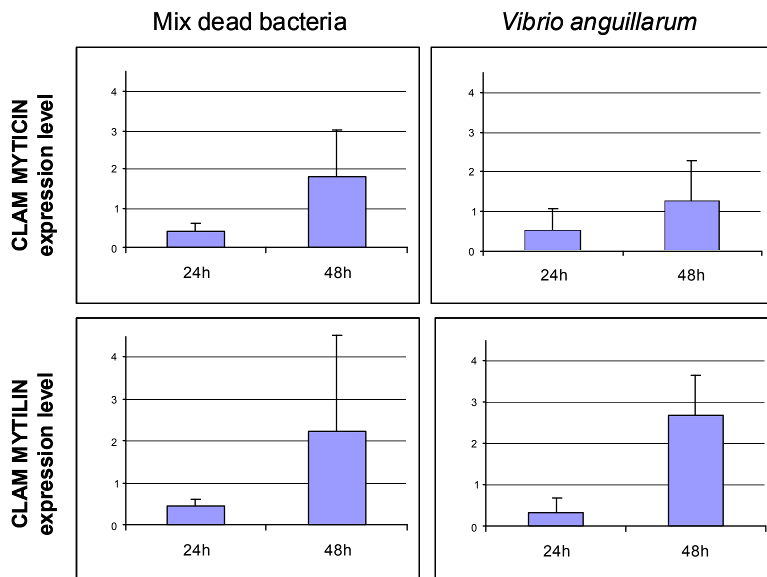


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Table 1

Table 1. Identified SSH up-regulated clones in dead bacterial challenged *R. decussatus* with significant database match.

Category and gene identity. Blast-X	Genbank Accession nº	Insert size (bp)	Homolog species	e-value	nº clones./nºcontigs
Cell defense/ immunity/ stress					
Myticin-B precursor	EL903763	386	*Mytilus galloprovincialis*	4e-14	1
Mytilin C precursor	EL903793	280	*Mytilus trossulus*	3e-21	1
Inhibitor of apoptosis protein 2	EL903725	308	*Danio rerio*	1e-12	1
Heat shock cognate 70	EL903717	761	*M. galloprovincialis*	1e-113	2/1
Ferritin	EL903718	900	*Crassostrea gigas*	1e-20	2/1
Naegleriapore A pore-forming peptide precursor	EL903762	610	*Naegleria fowler*	3e-11	1
Cell signaling/ Adhesion					
CDH1-D (E- Cadherin)	EL903726	720	*Gallus gallus*	2e-37	4 /1
G-Cadherin	EL903749	514	*Strongylocentrotus purpuratus*	7e-5	1
EFHC2 (EF-hand domain. Calcium ion binding)	EL903729	337	*S .purpuratus*	1e-49	1
Signal sequence receptor beta-like protein	EL903767	483	*C. gigas*	2e-44	1
Proline-rich transmembrane protein 1	EL903760	387	*Homo sapiens*	5e-7	1
CG14991-PA, isoform A	EL903779	402	*Tribolium castaneum*	3e-45	1
Longin-like	EL903778	711	*Medicago truncatula*	3e-8	1
Cytoskeleton					
Actin	EL903720	462	*Mus musculus*	3e-75	5 /3
	EL903721	384			
	EL903722	585			
Actin-binding/filamin-like protein	EL903719	235	*Schistosoma mansoni*	5e-23	3/1
ENSANGP00000003616 (filamin family)	EL903761	564	*Anopheles gambiae*	2e-33	5/1
Ppp2r1a-provprotein	EL903748	418	*Xenopus laevis*	2e-63	1
Actophorin	EL903747	616	*Entamoeba histolytica*	1e-15	2/1
Collagen, type VIII	EL903784	220	*G. gallus*	3e-5	1
High-glycine tyrosine keratin type II.4	EL903783	649	*M. musculus*	2e-19	1
Inter-alpha-trypsin inhibitor heavy chain H3	EL903781	653	*H. sapiens*	3e-44	1
Paramyosin protein	EL903739	563	*C. gigas*	6e-22	1
Radixin	EL903738	721	*M. musculus*	1e-100	1
CG33253-PA	EL903768	458	*T. castaneum*	1e-15	2/1
TYB12_ONCMY Thymosin beta-12	EL903750	460	*Oncorhynchus mykiss*	2e-12	3/1
Cell cycle					
Deoxyhypusine synthase	EL903737	445	*M. musculus*	2e-67	1
Mantle gene 6	EL903751	451	*Pinctada fucata*	2e-6	8 /3
	EL903752	682			
	EL903753	474			
Heavy metal-binding protein HIP	EL903785	305	*M. edulis*	3e-3	1
Metal-dependent RNase	EL903770	274	*Lactococcus lactis*	1e-4	1
Polyubiquitin	EL903769	212	*S. purpuratus*	5e-32	1
Ubiquitin A-52 residue ribosomal protein fusion product 1	EL903780	175	*S. purpuratus*	3e-20	1
60kD Ro/SSA autoantigen isoform 2 or Sjogren	EL903728	772	*Pan troglodytes*	2e-33	1

syndrome antigen A2.					
Elongation factor-1 alpha	EL903744	343	*Calyptogena soyoae*	3e-57	9/1
Elongation factor 1 gamma	EL903745	567	*Atypus snetsingeri*	6e-37	1
AF240834_1 Elongation factor-2	EL903746	393	*Nereis virens*	8e-55	1
CHK1	EL903758	134	*X. tropicalis*	2e-5	2/1
Splicing factor 3a, subunit 1	EL903740	310	*P. troglodytes*	8e-4	1
Transcription factor IIB	EL903764	96	*Aedes aegypti*	1e-6	2/1
Transcriptional intermediary factor 1 gamma TIF1gamma or TRIM33)	EL903759	452	*D. rerio*	6e-25	1
Delta isoform of regulatory subunit B56	EL903777	385	*T. castaneum*	1e-17	1
<u>Cell Metabolism/ Respiratory chain</u>					
Cytochrome oxidase subunit I	EL903756	460	*Ruditapes decussates*	3e-76	1
Cytochrome c subunit II	EL903741	295	*Ruditapes philippinarum*	7e-27	1
Hypothetical proteinXP_780193	EL903788	637	*S. purpuratus*	9e-3	1
Proliferated association protein 1 isoform	EL903731	697	*S. purpuratus*	4e-88	1
MGC80785 protein	EL903757	322	*X. laevis*	3e-24	1
Sarcoplasmic calcium binding protein	EL903754	557	*Pseudocardium sybillae*	2e-4	3/1
Acetyl-coenzyme A acyltransferase 2	EL903743	374	*X. laevis*	2e-36	1
GTP-binding protein	EL903782	698	*P. fucata*	2e-135	1
Similar to placental protein 11	EL903716	702	*S. purpuratus*	5e-37	2/1
Carbonic anhydrase	EL903732	735	*Anthopleura elegantísima*	1e-26	1
Rab GTPase	EL903742	358	*Dictyostelium discoideum*	4e-15	2/1
Receptor of Activated Kinase C 1	EL903730	307	*Mya arenaria*	5e-44	1
Purine-rich binding protein-alpha	EL903791	382	*S. purpuratus*	3e-8	1
Hypothetical protein TTHERM_02141640	EL903735	450	*Tetrahymena thermophila*	1E-22	1
<u>Ribosomal proteins</u>					
Ribosomal protein L13	EL903773	306	*D. rerio*	6e-23	1
Ribosomal protein L28	EL903775	209	*Haliotis asinina*	3e-12	1
Ribosomal protein P2-like	EL903774	400	*Culicoides sonorensis*	3e-18	1
Ribosomal protein L19	EL903771	240	*C. gigas*	3e-34	1
40S ribosomal protein Sa	EL903727	221	*Ictalurus punctatus*	5e-33	1
Ribosomal protein L7	EL903772	244	*C. gigas*	3 e-15	1
<u>Unknown function</u>					
HRPII (histidine-rich protein)	EL903733	758	*Plasmodium falciparum*	1e-3	1
Vitelline membrane outer layer protein I	EL903723	765	*G. gallus*	8e-33	2/1
Y38H6C.19 (contains Vitellin domain)	EL903724	393	*Caenorhabditis elegans*	1e-9	2/1
Z29075 myophilin antigen	EL903734	343	*Schistosoma japonicum*	9e-13	1
GM2 activator protein (ganglioside M2)	EL903736	350	*M. musculus*	1e-8	1
Hypothetical protein TTHERM_02641280	EL903765	376	*T. thermophila*	2e-16	3/1
Conserved hypothetical protein	EL903787	373	*A. aegypti*	1 e-18	2/1
CG7997-PA	EL903755	345	*T. castaneum*	9e-40	1
CG3612-PA isoform 2	EL903776	438	*T.castaneum*	4e-75	1
GA14397-PA .	EL903789	367	*Drosophila pseudoobscura*	4e-8	1
Hypothetical protein LOC556586	EL903790	545	*D. rerio*	5e-14	1
LSM14 homolog B	EL903792	873	*M.musculus*	1e-7	1

Hypothetical protein	EL903766	316	*Rattus norvegicus*	5e-8	1
Hypothetical protein M04C7.2	EL903786	382	*Caenorhabditis elegans*	3e-3	1

Table 2

Table 2. General characteristic of ESTS obtained from hemocytes of *R. decussatus* challenged with a mixture of dead bacteria.

Category	**Total ESTs**
Total number of EST sequenced	283
Total number of EST analyzed	253
Significant matches to known proteins	119
EST clusters	78
Singletons	44
No significant match to database	134
Redundancy	63%