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Sequence analysis of a normalized cDNA library of *Mytilus edulis* hemocytes exposed to *Vibrio splendidus* LGP32 strain☆

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ABSTRACT

In the past decades, reports on bivalves' pathogens and associated mortalities have steadily increased. To face pathogenic micro-organisms, bivalves rely on innate defenses established in hemocytes which are essentially based on phagocytosis and cytotoxic reactions. As a step towards a better understanding of the molecular mechanisms involved in the mussel *Mytilus edulis* innate immune system, we constructed and sequenced a normalized cDNA library specific to *M. edulis* hemocytes unchallenged (control) and challenged with *Vibrio splendidus* LGP32 strain for 2, 4 and 6 h. A total of 1,024,708 nucleotide reads have been generated using 454 pyrosequencing. These reads have been assembled and annotated into 19,622 sequences which we believe cover most of the *M. edulis* hemocytes transcriptome. These sequences were successfully assigned to biological process, cellular component, and molecular function Gene Ontology (GO) categories. Several transcripts related to immunity and stress such as some fibrinogen related proteins and Toll-like receptors, the complement C1qDC, some antioxidant enzymes and antimicrobial peptides have already been identified. In addition, Toll-like receptors signaling pathways and the lysosome and apoptosis mechanisms were compared to KEGG reference pathways. As an attempt for large scale RNA sequencing, this study focuses on identifying and annotating transcripts from *M. edulis* hemocytes regulated during an *in vitro* experimental challenge with *V. splendidus*. The bioinformatic analysis provided a reference transcriptome, which could be used in studies aiming to quantify the level of transcripts using high-throughput analysis such as RNA-Seq.

1. Introduction

Bivalve mollusk culture is an important and rapidly expanding area of the world aquaculture production [1]. The blue mussel *Mytilus edulis*, tolerant to a wide range of environmental changes, combines a significant economic importance and a key role in bio-surveillance programs as a sentinel species in many areas of the world [2]. In the past decades, reports on bivalves' pathogens and associated mortalities have steadily increased [3–5]. Among the opportunistic bacteria, members of the Gram-negative genus *Vibrio* are the most frequently isolated bacteria from mollusks. Several species belonging to this genus have been related with mortalities and diseases in larvae and juvenile individuals [6–11]. For example, the strain LGP32 has been associated with massive mortality events in the production of *Crassostrea gigas* oysters in France [12]. Recent studies have explored the route of infection and pathogenic processes of this strain [13]. A metalloprotease has been linked to toxicity [14,15] and the outer membrane protein (OMP) OmpU has recently been shown to be pivotal to LGP32 virulence [13,16].

To face pathogenic micro-organisms, bivalves rely on innate defenses triggered by hemocytes which are essentially based on phagocytosis and cytotoxic reactions. Innate defenses are able to recognize unique and characteristic molecules present at the surface of microorganisms, such as lipopolysaccharides (Gram negative bacteria) or peptidoglycans (Gram positive bacteria), known as pathogen-associated molecular patterns (PAMP). Indeed, hemocytes recognize PAMP through lectins, and membrane bound receptors, like Toll-like

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receptors, which are referred to as pathogen recognition receptors (PRR) [17]. Different types of lectins (C-type lectin, sialic acid binding lectin, fucolectin and galectin) have been characterized in *M. galloprovincialis* [18]. The diversity of C-type lectin sequences may answer to the variety of pathogens. Therefore, C-type lectins are considered as PRR by some authors [19–21]. In addition, Toll-like receptors have already been identified in various bivalves [22–25] included in *Mytilus* species [20,26]. Activation of Toll-like receptor pathways is essential for inducing immune related-gene expression in the defense against bacterial infections in invertebrates [27].

For an efficient defense mechanism, both cellular and humoral processes are involved in a coordinated way. In contact with bacteria, hemocytes first phagocyte the invaders and then degrade them by stimulating their phagolysosomal activities. Associated with the phagocytic activity, the NADPH oxidase as well as nitric oxide (NO) synthase are activated, thus leading to the production of reactive oxygen species (ROS), such as hydroxyl radical (OH^-) or singlet oxygen ($^1O_2$) and the production of NO enabling the oxidation of the foreign invaders [28–32]. In parallel, the antimicrobial peptides (AMPs) involved in innate immunity are synthetized and released in the hemolymph. These humoral molecules, known as cysteine-rich peptides, can destroy bacteria by destabilizing their membrane permeability [33]. In *Drosophilia* and shrimps, the AMP-encoding genes are regulated by the Toll and *Immune deficiency* (Imd) pathways [34,35]. In mussels, four groups of AMPs (defensins, mytilins, myticins and mytimycins), which play a key role in immune defense, have been identified and characterized [36]. These AMPs have specific and complementary antimicrobial activities. Defensins and myticins are more active against Gram-positive bacteria than against the Gram-negative ones. Mytimicins are exclusively antifungal [37]. On the other hand, mytilins act both on Gram-negative and Gram-positive bacteria, including vibrios [36,38].

In the last years, host-pathogen interaction models in aquatic species have gained more popularity since they constitute useful tools for understanding the pathogenicity of diseases in cultured and wild populations [20,39–43].

Several studies have focused on the molecular mechanisms involved in the response of hemocytes to *V. splendidus* LGP32 strain. Differential gene expression levels associated with immune responses such as AMPs, lysozyme and antioxidant enzymes genes were found in *M. galloprovincialis* and *M. edulis* hemocytes exposed to *V. splendidus* LGP32 [20,40,41,43]. Also, differentially expressed immune genes such as ficolin, killer cell lectin-like receptor, TLR-2, mitogen-activated protein kinases (MAPK), ferritin, heat shock proteins 90 (HSP90) and cathepsin have been observed in *Mya arenaria* hemocytes exposed to *V. splendidus* LGP32 [22,39]. These studies demonstrated that *V. splendidus* LGP32 has the ability to regulate the expression of the genes involved in innate immunity of bivalve mollusks during the first hours of bacterial challenge.

Knowledge of the transcriptome has been developed in the last decade: the sequence data available for bivalve species have been steadily growing, especially through EST collections [18,44] and pyrosequencing [23,26,45–48]. To date, approximately 369,093 ESTs, 55,541 proteins and 921 genes from the class Bivalvia have been inventoried in public databases. However, only a few thousand EST sequences are related to *M. edulis* [26,49,50] and *M. galloprovincialis* [18,20,47,51].

Unraveling molecular mechanisms involved in innate immune system of marine bivalves is essential for both scientific research and aquaculture. These processes will be more readily addressed when the hemocyte transcriptome of *Mytilus* species is available. The main focus of this study was to generate a cDNA sequences library specific to *M. edulis* hemocytes challenged with *V. splendidus* LGP32 strain with a short term exposure (0–6 h). These sequences will be available as a reference transcriptome for further high-throughput analysis such as RNA-Seq or microarrays.

2. Materials and methods

2.1. Mussels and hemolymph collection

Blue mussels, *M. edulis* (3–5 cm in shell length) were sampled from wild population in Prince Edward Island (Gulf of Saint Lawrence, Canada). Mussels were kept and maintained in a 300 L tank with re-circulating artificial seawater (Instant Ocean®) at a temperature of 16–17 °C and a salinity of 30 ppt. Animals were fed daily with Spat Formula (Innovative Aquaculture Products Ltd., Canada).

Hemolymph was withdrawn from the adductor muscle using 3 mL syringes fitted with 25 gauge needles. The hemolymph quality of individual mussels was checked using an inverted microscope (ZEISS, Germany). Hemolymph was pooled into a sterilized 30 mL tube and cell concentration in hemolymph was determined using a hemocytometer. Only hemocytes with prolonged pseudopodia were kept for further analysis.

2.2. In vitro hemocyte challenge

One million hemocytes were added to each well of 24 well plates previously filled with 1 mL of L-15 growth media (Leibovitz L-15 medium (Sigma, ON, Canada)). The L-15 was slightly modified by the addition of 20.2 g/L NaCl, 0.54 g/L KCl, 0.6 g/L $CaCl_2$, 1 g/L $MgSO_4$, 83 g/L $MgCl_2$–$6H_2O$, 10% of glucose and 10% of heat inactivated Fetal Bovine Serum (FBS). Primary cell culture was incubated for 1 h at 16 °C so that hemocytes were able to adhere to the bottom of the wells. Cell viability was assessed using the Trypan blue exclusion method.

Bacteria were cultured overnight to reach exponential growth phase in Trypticase Soy Broth (TSB, BD-BactoTM) supplemented with 2% NaCl at 16 °C in 250 mL Erlenmeyer flasks and shaken at 100 rpm. The bacterial concentration was determined using a spectrophotometer (UNICO Spectrophotometer, Biotech, Inc., Québec, Canada) at 540 nm. Bacteria were added to hemocytes at a (1:3) hemocyte:bacteria ratio. Controls represent hemocytes incubated in L-15 media without bacteria. Hemocytes were exposed to bacteria for 2, 4 and 6 h. For each exposure time, 6 replicates were performed and cell viability was assayed by using the Trypan blue exclusion technique. More than 90% of the hemocytes were viable before RNA extraction (data not shown).

2.3. RNA extraction, cDNA library and 454 pyrosequencing

2.3.1. RNA extraction

For each exposure time, total RNA from hemocytes was extracted using a Qiagen RNeasy Mini Kit according to the manufacturer's protocol (Invitrogen, ON, Canada). RNA was quantified using a NanoDrop spectrophotometer (Thermo-Fisher Scientific, DE, US) and RNA quality was assessed using the Experion RNA StdSens Analysis Kit (Bio-Rad Ltd. ON, Canada). Then, RNA from the control and challenged hemocytes (2, 4 and 6 h) was pooled to achieve 5 μg of total RNA necessary for cDNA library synthesis.

2.3.2. cDNA synthesis and PCR amplification

cDNA synthesis was carried out by Clontech Inc. (CA, US) and performed using SMART PCR kit (Clontech Inc. CA, US). Briefly, first strand cDNA synthesis was performed using primer annealing mixture (5 μL) containing 0.3 μg of total RNA; 10 pmol SMART-Sfi1A oligonucleotide (5′-aagcagtggtatcaacgcagagtggccattacggccrgrgrg-3′); 10 pmol CDS-Sfi1B primer (5′-aagcagtggtatcaacgcagagtggccgaggcggccd(T)20-3′). The reaction mixture was heated at 72 °C for 2 min and cooled on ice for 2 min. First-strand cDNA synthesis was then initiated by mixing the annealed primer-RNA with Reverse Transcriptase in a final volume of 10 μL, containing 1 × First-Strand Buffer (50 mM Tris–HCl (pH 8.3); 75 mM KCl; 6 mM $MgCl_2$); 2 mM DTT; 1 mM of each dNTP.

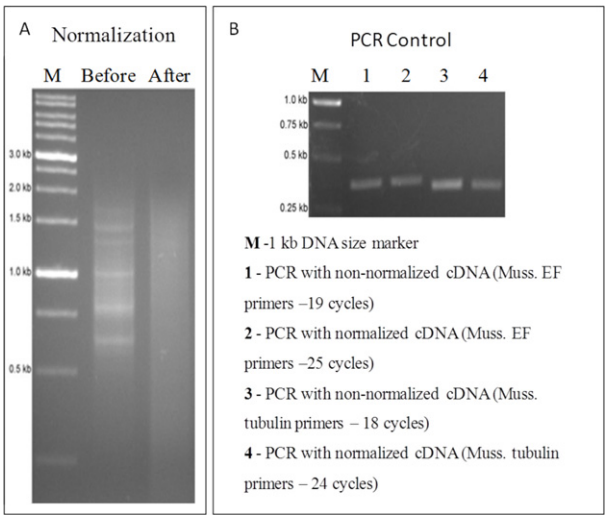

Fig. 1. (A) cDNA profile before and after normalization; (B) PCR Control after normalization. Following extraction and quality control, RNA from the control and challenged hemocytes (2, 4 and 6 h) were pooled before conducting cDNA first strand synthesis. cDNA normalization was perform by Clontech Inc. CA, US using duplex-Specific Nuclease (DSN) treatment. Electrophoresis profiles were compared before (A, before) and after cDNA normalization (A, after). Following normalization, all major bands disappeared. To further control normalization, cDNAs to Elongation Factor alpha (EF) and tubulin were PCR-amplified with different cycle numbers and amplification products were compared. M: molecular size markers.

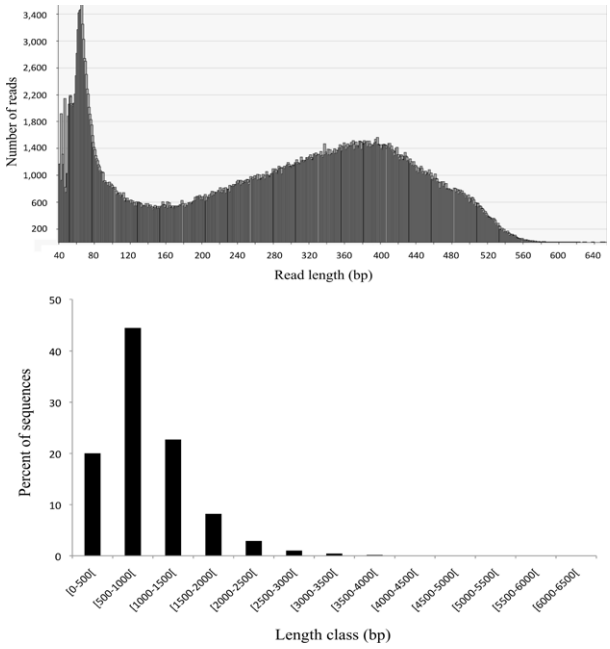

Fig. 2. (A) lengths distribution of all reads generated and (B) lengths distribution of the assembled sequences. A total of 1,024,708 reads were generated by 454 sequencing with a mean sequence length of 256 pb (histogram A plotting number of reads per read length). Following Newbler assembly, 19,622 sequences with an average length of 925 bp were generated, most of them (44%) ranged between 500 and 1000 bp in length (histogram B plotting percent of sequence in function of transcripts size ranges).

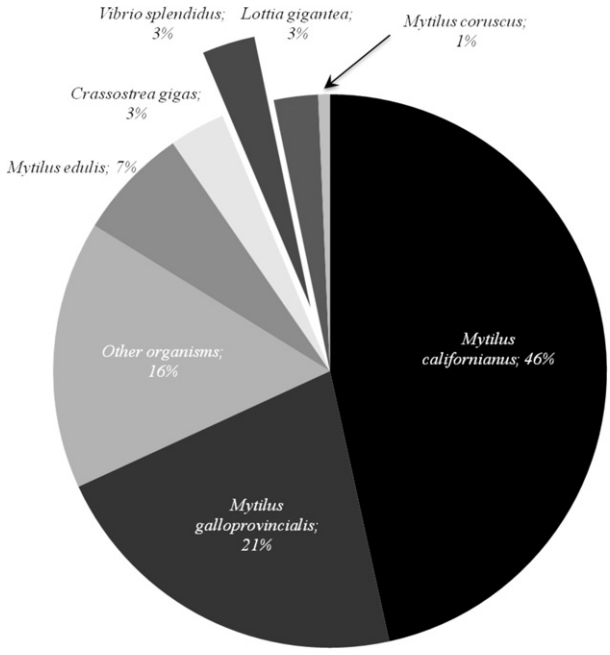

Fig. 3. Organisms that match to the assembled sequences of *Mytilus edulis* hemocytes exposed to *Vibrio splendidus* LGP32. Percentage of transcripts finding similarity with various species in the non-redundant sequence databases (Megablast program against nucleic acids).

The first-strand cDNA synthesis reaction was incubated at 42 °C for 2 h in an air incubator and then cooled on ice.

Then, first-strand cDNA was diluted 5 times with TE buffer, heated at 70 °C for 7 min and used for amplification by Long-Distance PCR. PCR reaction (50 μL) was performed using 1 μL diluted first-strand cDNA; 1 × Advantage 2 reaction buffer; 200 μM dNTPs; 0.3 μM SMART PCR primer (5-aagcagtggtatcaacgcagagt-3′); 1 × Advantage 2 Polymerize mix. The PCR conditions were: initiation at 95 °C for 7 s; annealing at 66 °C for 20 s and extension at 72 °C for 3 min, 19 cycles. PCR products were purified using QIAquick PCR Purification Kit (Qiagen Inc., CA, US) and concentrated using ethanol precipitation. DNA pellet was diluted in ultrapure water to a final cDNA concentration of 50 ng/μL.

2.3.3. cDNA normalization and duplex-specific nuclease (DSN) treatment

Hybridization reaction was performed using 3 μL (about 150 ng) purified ds cDNA and 1 μL of 4 × Hybridization Buffer (200 mM HEPES-HCl, pH 8.0; 2 M NaCl). The reaction mixture was overlaid with one drop of mineral oil and incubated as follows: 98 °C for 3 min and 68 °C for 5 h.

The following preheated reagents were added to the hybridization reaction at 68 °C: 3.5 μL ultrapure water; 1 μL of 5 × DNAse buffer (500 mM Tris–HCl, pH 8.0; 50 mM $MgCl_2$, 10 mM DTT); 0.5 μL DSN enzyme. Then, incubation was extended at 67 °C for 20 min. On completion of DSN treatment, the DSN enzyme was inactivated by heating at 97 °C for 5 min.

2.3.4. Amplification of normalized cDNA and 454 pyrosequencing

cDNA sample was diluted by adding 30 μL ultrapure water and used for PCR amplification. PCR reaction (50 μL) contained: 1 μL diluted cDNA; 1 × Advantage 2 reaction buffer; 200 μM dNTPs; 0.3 μM SMART PCR primer; 1 × Advantage 2 Polymerize mix. PCR was carried out on MJ Research PTC-200 DNA Thermal Cycler (GMI Inc., MI, US). Eighteen PCR reaction cycles were performed as follow: 95 °C for 7 s; 65 °C for 20 s; 72 °C for 3 min. Roche GS-FLX 454 pyrosequencing was conducted by Genome Quebec at McGill University (Montreal, Canada).

2.4. Data analysis

Raw data generated from 454 sequencing were assembled by Genome Quebec. Adaptors and primer sequences were trimmed

and low quality sequences were discarded. *De novo* sequence assembly was performed using Newbler program. The generated contigs and isotigs were blasted against non-redundant (nr) sequence databases (National Centre for Biotechnology Information (Bethesda, USA), NCBI) using the Megablast program at Genomequest (USA). Sequences were also blasted against protein databases (Genpept, Translated Genbank, Protein Data Bank, RefSeq, Swiss-Prot and Translated EMBL), by using BlastX program. The 5 best hits were kept and *e*-value cut-off of 10^{-3} was applied by default. Gene ontology searches were performed on the slim GO terms using Amigo program. Sequences were submitted to BLAST comparison against the KEGG GENES database to obtain KO (KEGG Orthology) assignments and to generate KEGG pathways. The threshold >60 is applied to BLAST bit scores.

3. Results and discussion

3.1. Normalized cDNA library

During the last decade, numerous studies have been undertaken to better understand the molecular mechanisms of responses of hemocytes challenged with *V. splendidus*. In this context, the new generation of sequencing technologies represents an unprecedented opportunity to identify a wide range of molecular actors, as well as to unravel the molecular mechanisms involved in this process. A normalized cDNA library is a very relevant tool for mussel transcriptomics and can be further exploited as an effective source of novel mRNAs [18].

In this study, RNA was extracted from hemocytes of *M. edulis* unchallenged (control) and challenged with *V. splendidus* LGP32 strain for 2, 4 and 6 h. Then, the total RNA samples were pooled and a normalized cDNA library was produced by equilibrating the final representation of abundant and rare transcripts. This strategy was adopted in order to enhance the probability of regulated transcripts during short-term infection by *Vibrio* to be represented. Quality control of the normalization step was conducted by agarose gel electrophoresis and by PCR amplification of elongation factor 1 alpha (EF1-α) and tubulin transcripts (Fig. 1). As expected, before normalization, some transcripts were highly expressed, whereas, after normalization, only a smear was observed on the gel indicating an equalization of transcript levels (Fig. 1A). Consequently, the probability to sequence several times the same sequence is decreased and the probability to sequence rare transcripts is increased. PCR controls were then carried out to analyze the quality of the normalization process. Abundant transcripts such as EF1-α and tubulin were used as a control. Bands of the targeted transcripts EF1-α and tubulin were observed before and after normalization. After normalization, amplification of the targeted cDNA needed more cycles (25 and 24 cycles for EF1-α and tubulin respectively) in comparison to non-normalized library (19 and 18 cycles for EF1-α and tubulin respectively) showing a decrease of their abundance (Fig. 1B).

3.2. Pyrosequencing and assembly

The normalized cDNA library from hemocytes of *M. edulis* exposed to *V. splendidus* LGP32 was pyrosequenced on a Roche GS-FLX 454 sequencing apparatus by using the Titanium chemistry. A total of 1,024,708 nucleotide reads was generated with an average length of 265 bp. The longest and shortest reads were 660 bp and less than 50 bp long, respectively (Fig. 2A). Most of the reads ranged between 360 and 420 bp.

The different steps for the sequences analysis all was performed using the Genomequest bioinformatic platform or at genome Quebec (http://gqinnovationcenter.com/). A trimming step was conducted to remove adaptors, primers and low quality sequences. Then, reads were submitted for assembly using Newbler program, generating 19,622 sequences which included 148 contigs and 19,474 isotigs. The average length was 925 bp. The length of the sequences varied from 3 to 6,371 bp. Most sequences (44%) were ranging between 500 and 1000 bp. Length distribution of the sequences is shown in Fig. 2B.

To examine the coverage of the generated contigs and isotigs, a similarity research on non-redundant (nr) nucleotide sequence databases (National Centre for Biotechnology Information (Bethesda, USA), NCBI) was performed using Megablast. A total of 65,832 hits was obtained and covered 748 different organisms. Among the best hits, 49,471 (75%) matched with *Mytilus* species (46% *M. californianus*; 21% *M. galloprovinciallis,* 7% *M. edulis and* 1% *M. coruscus*) (Fig. 3). As anticipated, several sequences likely corresponded to *Vibrio* sp or to *Vibrio splendidus* (3.1%). Interestingly, some sequences had best blast hits with *Ciona intestinalis* cDNAs. The aquaculture industry in Prince Edward Island, where the mussels were collected, faces an invasion issue with this species. In some areas of the island, production problems, including handling difficulties and resource competition with the cultured blue mussel *Mytilus edulis* has been observed [52]. Mussel could filter and accumulate *C. intestinalis* larvae which could explain the identification of sequences with high homology with *C. intestinalis.*

For a number of sequences, the match between our assembled transcripts and sequences listed in Genbank was perfect. This was in particular the case for *Mytilus* species (262 perfect matches) and for *Vibrio splendidus* LGP32 strain (255 perfect matches), suggesting a good accuracy and quality of sequencing and assembly.

3.3. Annotation

For annotation, nucleotide sequences of contigs and isotigs were also blasted against protein databases using the Megasearch program in the 6 frames. A score cut-off of 30 was applied and results were limited to 5 per query. A total of 19,793 hits were obtained, but in the end, only the very best hit for each query sequence was kept. For these 4198 selected best hits, the resulting E values ranged from 3.2 to 0. A total of 4146 of these best hits had an *e*-value lower than 10^{-5}. The very best similarity was obtained with UniProtKB/TREMBL accession number C0Z203 which corresponds to *Mytilus galloprovincialis* hsp90. The corresponding alignment was 664 aminoacids in length, the Megasearch score was 3383 and residue identity equaled 98.8%. Again, some of the best hits did correspond to *Vibrio splendidus* protein sequences, which is consistent with megasearches conducted at the nucleotide level.

The genome size of *Mytilus* is estimated to be 1.56×10^9 bp (http://www.genomesize.com/). Therefore, a simplistic guess on the number of genes would be about 15,000 assuming that the genome does not contain a particularly high proportion of sequence repeats [47]. Expressed sequence tag collections from *M. galloprovincialis* tissues represent around 7000 non-redundant sequences [47]. On this basis, we assume that the set of 19,622 sequences we isolated is likely to cover the major part of *M. edulis* hemocytes transcriptome, even though some sequences are very likely to be related to *Vibrio* and to a lesser extent to *Ciona*.

3.4. Gene ontology analysis

Gene ontology (GO) has been widely used to perform gene classification and functional annotation using controlled vocabulary and hierarchy including molecular function, biological process and cellular components [53]. Gene ontology (GO) analysis was conducted using Amigo (http://amigo.geneontology.org/). Go slim is a cut down version of the GO containing a subset of the terms of the whole Gene ontology. These slim annotations give a broad overview of the ontology content without the details of the specific fine grained terms. The sequences were successfully assigned to biological process, cellular

component, and molecular function GO categories. Distributions of the GO term for the three categories are shown in Table 1.

For biological process category, the most abundant terms were "metabolic process" (GO:0008152, 77.9%) and "cellular process" (GO:0009987, 68.7%), followed by "small molecule metabolic process" (GO:0044281, 19.9%), "transport" (GO:0006810, 7%), "cellular macromolecule biosynthetic process" (GO:0034645, 19.5%) and "gene expression" (GO:0010467, 17.8%).

Furthermore, for the cellular component GO the most evident matches were within the "cell part" (GO:0044464, 91.6%) and "intracellular" (GO:0005622, 54%) terms, followed by "membrane" (GO:0016020, 48.9%) and "intracellular part" (GO:0044424, 45.7%). There were also "cytoplasm" (GO:0005737, 37.5%), "cytoplasmic part" (GO:0044444, 21.5%) and "intracellular membrane-bounded organelle" (GO:0043231, 20.2%).

Finally, the matches of molecular function terms were most prevalent within the "catalytic activity" (GO:0003824, 66.8%), the "binding" (GO:0005488, 60.3%), the "nucleic acid binding" (GO:0003676, 21.7%), the "hydrolase activity" (GO:0016787, 21.1%) and "transferase activity" (GO:0016740, 20.2%). In other studies with various bivalve species, from different tissues ESTs, authors globally found these same dominant Go slim terms [18,45,48].

3.5. Identification of immune related sequences in M. edulis hemocytes

The cellular immune system is linked to competent cells, referred to hemocytes which are the circulatory cells of molluscs. They have various known functions including digestion, transport of nutrients, formation and mending of the shell, repair of wounds, excretion and internal defense [54,55]. Several transcripts related to immunity and stress were identified in our cDNA library.

3.5.1. Pathogen recognition

The initial step of the immune responses is the detection and recognition of foreign invaders by hemocytes. Different proteins and receptors, such as lectins, Toll-like receptors (TLRs) and peptidoglycan recognition receptors (PGRPs), have been reported to be involved in pathogens recognition on the cellular surface [20,23,26,56].

Lectins are a large group of carbohydrate-recognition proteins with a high structural diversity. They have the ability to recognize carbohydrates endogenous to the animal or presented by microbial invaders and can be found in soluble and membrane associated forms. In this way, they play crucial roles in multi-process of host immune responses, such as pathogen recognition, immune signaling transduction, cellular adhesion and inflammation [57].

Different types of lectins (C-type lectin, sialic acid binding lectin, fucolectin and galectin) have been characterized in *M. galloprovincialis* [18,20]. In *M. edulis* transcriptome, after applying BlastX, we found only 4 transcripts homologous to galectins, and 34 homologous to Fibrinogen-related protein (FREPs-1, -2, -4, -5, -6 and undefined). Galectins are characterized by a conserved sequence motif in their carbohydrate recognition domain and a specific affinity for β-galactosides. Fibrinogen-related proteins (FREPs) contain in the C-terminal portion fibrinogen-like domains but differ in the N-terminal region. Many members of this family play important roles as pattern recognition receptors in innate immune responses [58]. In *M. edulis*, there is a very diverse set of FREP sequences among and within individuals suggesting the capacity to recognize and eliminate different kinds of pathogens [56].

The Toll-like receptor (TLR) signaling pathway is an ancient pathway. It depends on specific families of pattern recognition receptors responsible for detecting microbial pathogens on the cellular surface and generating innate immune responses [59]. A total of 22 KO (KEGG Orthology) were associated with this pathway. TLRs are characterized by N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain [59]. A single TLR was identified in previous studies in *Chlamys farreri* (CfToll-1), *Mya arenaria* (TLR-2) and *Crassostrea gigas* (CgToll-1) [22,24,25] and 2 transcripts in *Mytilus galloprovincialis* [20]. More recently, TLR-2, 6 and 13 were detected by pyrosequencing of *Ruditapes philippinarum* hemocytes [23] and 27 putative TLR were identified in *Mytilus edulis* transcriptome [26]. Some transcripts represented in the normalized cDNA library had high similarities with TLR-1, 2, 6, 4 and 3. TLR-2 forms a heterodimer with either TLR-1 or TLR-6 which allows various pathogens such as bacteria, mycoplasma, fungi and viruses to be identified [59]. TLR-4 recognizes lipopolysaccharide (LPS) together with myeloid differentiation factor 2 (MD2) on the cell surface. However, no MD2 in the *M. edulis* transcriptome was detected. TLR3 detects viral double-stranded (ds) RNA in the endolysosome.

Fig. 4 shows the TLR signaling pathway with the corresponding molecules found in the *M. edulis* transcriptome compared to the KEGG reference pathway. In vertebrates, TLR signaling pathways are separated into two groups: a MyD88-dependent pathway that leads to the production of pro-inflammatory cytokines with quick activation of NF-κB and MAPK, and a MyD88-independent pathway associated with the induction of IFN-beta and IFN-inducible genes, and a slow activation of NF-κB and MAPK. Many components of the MyD88 dependent TLR pathway are found such as MyD88, IRAK-4, TRAF-6 and more (Fig. 4). Fewer components are found in a MyD88-independent pathway, which is in accordance with Philipp et al. [26].

Peptidoglycan recognition proteins (PGRPs) are conserved from insects to mammals and recognize bacteria and their major cell wall component, peptidoglycan [60]. In bivalves, PGRPs have been identified in the scallops *A. irradians* and *C. farreri*, the clams *R. philippinarum* and *S. grandis* and the oyster *C. gigas* [23,61–64]. To our knowledge, PGRPs have not been identified yet in *Mytilus* species. Following BlastX, a transcript was identified as homologous to PGRP S1S from *C. gigas* (57% identity with *E* value = $6.26e^{-83}$).

Also, 99 transcripts with homologies with the complement C1q were present in our library. The C1qdomain-containing (C1qDC) proteins constitute a family of proteins characterized by a globular C1q (gC1q) domain in their C-terminus. In vertebrates, they are involved in various cellular processes and are considered as major effector arms in immune responses as a key bridge between innate and adaptative immunity [65,66]. Some complement-like factors have also been identified in various bivalves [67,68] including in *Mytilus* species [20,69,70] and are involved in the recognition of invading microorganisms probably as pattern recognition molecules. In *M. galloprovincialis* hemocytes, both Gram-positive (*Micrococcus lysodeikticus*) and Gram-negative (*V. anguillarum*) bacteria lead to an increase in C1qDC transcript levels [69,70].

No cytokines-like sequence were identified. This would indicate that the corresponding transcripts are present at relatively low levels in *M. edulis*, or alternately these sequences have reduced similarities with orthologs listed in bioinformatics data banks. However, TNF-alpha receptor and IL-1 receptor associated kinase 4 were represented.

3.5.2. Phagocytosis and mechanisms involved

After recognition and chemotactic migration of hemocytes towards invading pathogens and following attachment and endocytosis of pathogens [71], hemocytes phagocyte and kill these invaders by producing lysozymes, antimicrobial peptides (AMPs) and toxic radicals.

During the phagocytosis process, phagosomes formed progressively acquire digestive characteristics. This maturation of phagosomes involves a regulated interaction with the other membrane organelles, including recycling endosomes, late endosomes and lysosomes [72]. In the cDNA library, 38 KO were associated with the phagosome (data not shown). After the fusion of phagosomes and lysosomes, toxic products were released which permit to kill most bacteria and degrade them into fragments. Phagolysosome contains

Table 1
Distribution of the Gene ontology terms (Go slim) of *Mytilus edulis* hemocytes exposed to *Vibrio splendidus* annotated sequences. The sequences have been classified in Biological Process (I), Cellular component (II) and Molecular function (III). Because one sequence can be assigned to more than one GO term, the percentage of all terms is larger than 100%.

Description	Frequency (%)	Description	Frequency (%)
I. Biological process			
Metabolic process	77.89	Establishment of localization in cell	1.32
Cellular process	68.62	Locomotion	1.31
Cellular biosynthetic process	29.66	Multicellular organismal process	1.25
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	28.64	Anatomical structure development	1.21
Small molecule metabolic process	19.81	Cellular component assembly at cellular level	1.14
Transport	19.70	Sulfur compound metabolic process	1.08
Cellular macromolecule biosynthetic process	19.50	Protein folding	0.96
Gene expression	17.85	Homeostatic process	0.93
Regulation of biological process	14.45	Cell death	0.87
RNA metabolic process	14.18	Organelle organization	0.79
Regulation of cellular process	13.89	Intracellular transport	0.77
Response to stimulus	10.63	Ribosome biogenesis	0.77
Cellular protein metabolic process	9.95	Cellular component morphogenesis	0.75
Generation of precursor metabolites and energy	7.61	Multicellular organismal development	0.74
Cellular response to stimulus	7.17	Cell morphogenesis	0.74
Cellular ketone metabolic process	7.07	Cellular macromolecular complex assembly	0.70
DNA metabolic process	6.94	Immune system process	0.65
Carboxylic acid metabolic process	6.84	mRNA processing	0.61
Carbohydrate metabolic process	6.77	Cellular protein localization	0.60
Cellular catabolic process	5.74	Protein complex assembly	0.57
Macromolecule modification	5.48	Intracellular protein transport	0.53
Cellular amino acid metabolic process	5.39	Cellular component movement	0.50
Signaling	5.10	Cell motility	0.47
Signal transduction	5.04	Neurological system process	0.42
Translation	4.88	Chromosome organization	0.41
Response to stress	4.44	Cell differentiation	0.36
Nucleobase, nucleoside, nucleotide and nucleic acid catabolic process	3.93	Vesicle-mediated transport	0.30
Protein modification process	3.89	Protein targeting	0.27
Interspecies interaction between organisms	3.72	Nucleocytoplasmic transport	0.21
Cellular component organization	3.52	Cellular membrane organization	0.20
Cofactor metabolic process	3.43	Cytoskeleton organization	0.18
Reproduction	2.75	Growth	0.17
Lipid metabolic process	2.69	Anatomical structure formation involved in morphogenesis	0.16
ncRNA metabolic process	2.62	Cell proliferation	0.13
RNA processing	2.52	Cell cycle phase	0.13
Macromolecule localization	2.17	Mitotic cell cycle	0.11
tRNA metabolic process	2.03	M phase	0.10
Cellular component organization at cellular level	1.90	Cell–cell signaling	0.09
Regulation of biological quality	1.82	Mitochondrion organization	0.04
Protein transport	1.64	Ribonucleoprotein complex assembly	0.04
Symbiosis, encompassing mutualism through parasitism	1.55	Circulatory system process	0.03
Cellular localization	1.40	Cell junction organization	0.01
II. Cellular component			
Cell part	91.55	Cell projection	1.59
Intracellular	54.00	Chromosome	1.37
Membrane	48.90	Cytoskeleton	1.02
Intracellular part	45.66	Nuclear part	0.87
Cytoplasm	37.50	Endomembrane system	0.81
Cytoplasmic part	21.51	Cytoskeletal part	0.77
Intracellular membrane-bounded organelle	20.19	Cytosol	0.75
macromolecular complex	13.14	Endoplasmic reticulum	0.70
Iintracellular organelle part	12.09	Microtubule cytoskeleton	0.55
Mitochondrion	10.62	Golgi apparatus	0.37
Plasma membrane	10.26	Nucleoplasm	0.35
Intracellular non-membrane-bounded organelle	8.16	Cytoplasmic membrane-bounded vesicle	0.23
Protein complex	7.75	Nucleolus	0.20
Organelle envelope	7.50	Nuclear chromosome	0.15
Extracellular region	7.22	Endosome	0.08
Ribonucleoprotein complex	5.09	Microtubule organizing center	0.08
Nucleus	4.77	Cilium	0.03
III. Molecular functions			
Catalytic activity	66.79	Ligase activity	3.47
Binding	60.27	Protein binding	2.81
Nucleic acid binding	21.68	Isomerase activity	2.59
Hydrolase activity	21.09	Methyltransferase activity	2.40
Transferase activity	20.18	Transferase activity, transferring acyl groups	2.29
Ion binding	17.90	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	1.51
Oxidoreductase activity	16.62	Helicase activity	1.27
DNA binding	11.89	Translation factor activity, nucleic acid binding	0.95
Transferase activity, transferring phosphorus-containing groups	9.93	GTPase activity	0.88
RNA binding	7.60	Phosphatase activity	0.83
Structural molecule activity	5.02	Enzyme regulator activity	0.68
Peptidase activity	4.66	Protein transporter activity	0.60
Nucleic acid binding transcription factor activity	4.64	Protein binding transcription factor activity	0.50
Nucleotidyltransferase activity	4.58	Transcription factor binding	0.24
Kinase activity	4.57	Cytoskeletal protein binding	0.18
ATPase activity	4.39	Enzyme binding	0.15

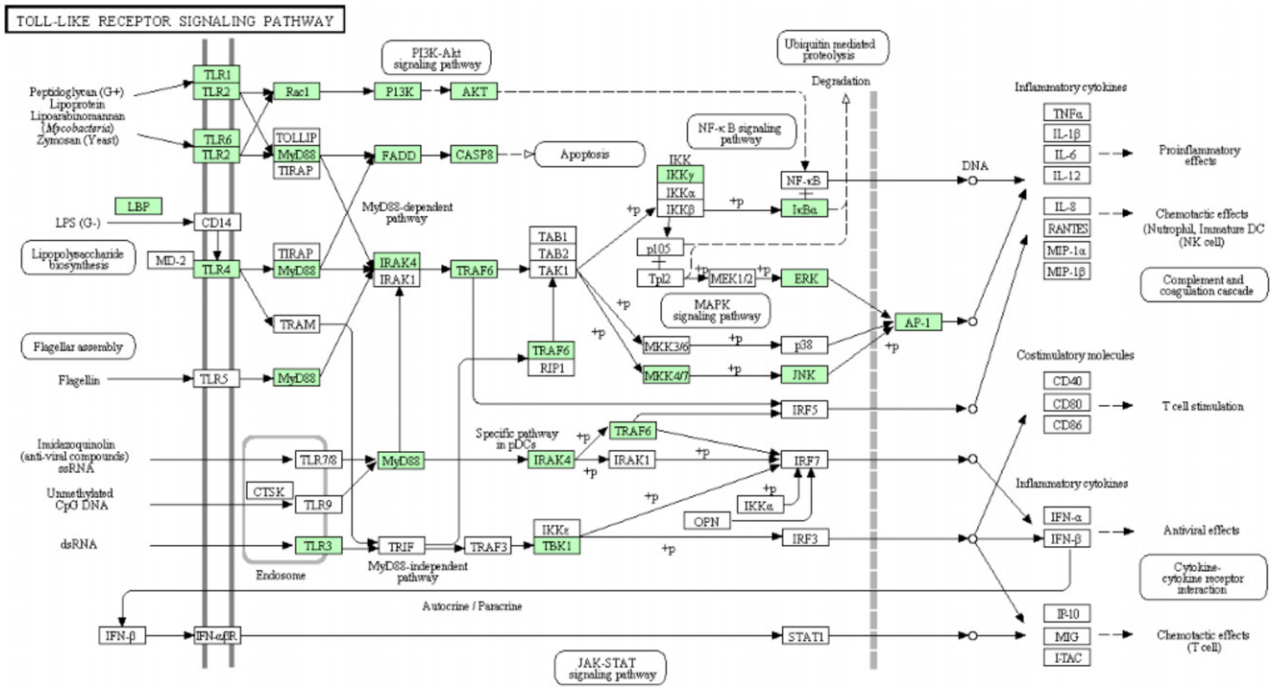

Fig. 4. Schematic comparison of *Mytilus* and KEGG reference TLR pathway members. Shaded boxes indicate proteins identified in our 454 results and white boxes the absent ones.

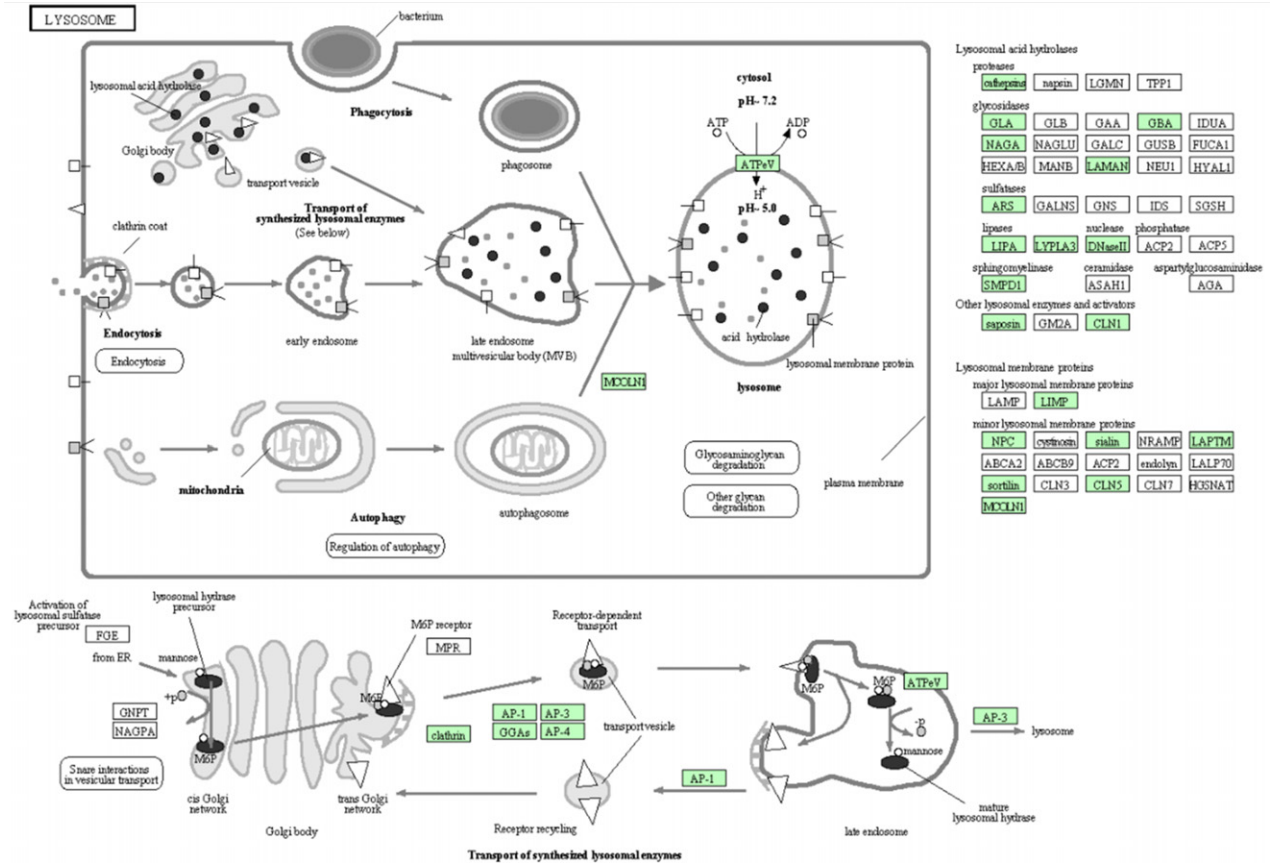

Fig. 5. Schematic comparison of *Mytilus* and KEGG reference lysosome mechanisms. Shaded boxes indicate proteins identified in our 454 results and white boxes the absent ones.

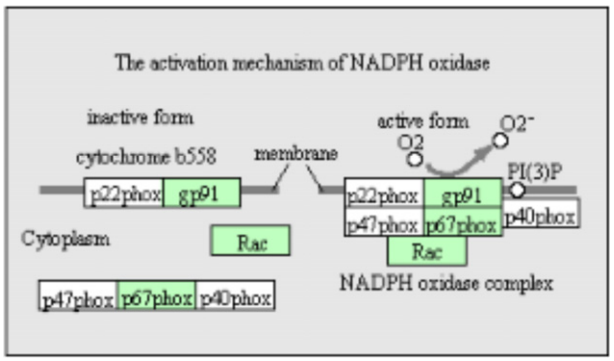

Fig. 6. Schematic comparison of *Mytilus* and KEGG reference activation mechanism of NADPH oxidase. Shaded boxes indicate proteins identified in our 454 results and white boxes the absent ones.

all elements which allow the degradation of bacteria: an acidic environment that impedes microbial growth, reactive oxygen and nitrogen species toxic for bacteria, antimicrobial peptides and proteins such as defensins and some endopeptidases and exopeptidases, hydrolases and proteases [72].

In the cDNA library, 39 KO were associated with the lysosome (Fig. 5). Among the lysosomal acid hydrolase, some transcripts showed high similarity with cathepsins, some glycosidases (GLA, GBA, NAGA and LAMAN), sulfatases (ARS) and lipases (LIPA, LYPLA3) (Fig. 5), which enables bacteria to be degraded.

AMPs and lysozymes can destroy bacteria by destabilizing their membrane permeability [33]. They are stored in granules as active forms and, after stimulation, they are involved in the destruction of bacteria inside phagocytes, before being released by exocytosis into hemolymph to participate in systemic responses [41,71]. Recognition of an infection by the Toll and *Immune deficiency* (Imd) pathway leads to a signaling cascade that typically results in the activation of AMPs genes. The Imd pathway is activated mainly by Gram-negative bacteria, such as *Vibrio* species [34,35,67]. Imd receptors were not identify in the cDNA library, but lysozyme (7 sequences related) and AMPs active against Gram-negative bacteria such as defensin, mytilin B, C and D (respectively 2, 6, 1 and 2 transcripts related) were highlighted. They probably activated by the Toll pathways. Also, 34 sequences with homology with myticin were found (32 for myticin C and 2 for myticin B). In *M. galloprovincialis*, myticin C was found to have a high polymorphic variability as well as chemotactic and immunoregulatory roles [73,74].

Associated with the phagocytic activity, the NADPH oxidase as well as nitric oxide (NO) synthase are activated to produce toxic radicals [28–32]. Fig. 6 shows the activation mechanisms of NADPH oxidase of phagocytic cells with the corresponding molecule found in the *M. edulis* transcriptome compared to the KEGG reference pathway. The activation of the NADPH oxidase enzyme proceeds through a multistep assembly at the plasma membrane of several components including the membrane-bound ($p22^{phox}$ and $gp91^{phox}$) and cytoplasmic subunits ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) and the small GTP-binding proteins (Rac) (Heyworth et al., 1993). In mussel hemocytes, transcripts having similarities with Rac, $gp91^{phox}$ and $p67^{phox}$ were present (Fig. 6).

In addition, our investigation led to the identification of a transcript homologous to putative cyclooxygenase. ROS can also be generated in the cytosol by other enzymes, including cyclooxygenase. Cyclooxygenase is involved in the first step of arachidonic acid oxidation leading to the production of prostaglandins, which are readily induced during inflammatory reactions in many tissues of the mussel [75]. This enzyme is also involved in the signaling pathways leading to hemocyte bactericidal activity [76].

To protect themselves from damage caused by toxic radicals, organisms use antioxidants, such as superoxide dismutases, catalase, glutathione peroxidase, thioredoxin reductase and gluthatione S-transferases to eliminate these free radicals by converting them to less toxic compounds [77]. Some of these antioxidant enzymes, the superoxide dismutase (7 sequences related) and the glutathione peroxidase (2 sequences related) were identified after BlastX.

In addition, 2 transcripts homologous to ferritin were present in the cDNA library. Ferritin is an iron chelating protein which has been classified as a stress protein due to its similarity with proteins involved in detoxification processes triggered by various stresses [78]. It is a critical component of iron homeostasis in various organisms. Iron is involved in respiratory burst activity by catalyzing the fenton reaction, which leads to the production of reactive oxygen species [79]. Hence, ferritin can regulate iron concentration to destroy microbial agents and at the same time protect cells from oxidative stress [80].

Furthermore, many endogenous substances are eliminated after being oxidized and conjugated to an anionic group (glutathione, glucuronate or sulfate) and then transported across the plasma membrane to the extracellular space. The latter step is mediated by integral membrane glycoprotein belonging to the superfamily of ATP-Binding Cassette (ABC) transporters. A subfamily includes the multidrug resistance-associated proteins (MRPs) [81]. MRPs are of vital importance in detoxification and cellular homeostasis [82]. MRPs have been identified in mussels [83,84] as well as in our cDNA library (1 sequence related).

3.5.3. *Other important molecules and pathways*

Heat shock proteins (HSPs) are rapidly synthesized in response to stress. They are essential for several important processes such as protein folding, protection of proteins from denaturation or aggregation, and facilitation of protein transport through membrane channels. Besides molecular chaperones, HSPs also have a number of significant functions in the innate immune response [85] and they are well studied in bivalves [40,86–90]. For *M. edulis* hemocyte transcriptome, 39 transcripts with homologies with different HSPs were found (HSP70, HSP90, HSP40, HSP60).

Apoptosis plays a key role in immune system homeostasis and function, both in vertebrates and invertebrates [91,92]. A key characteristic of the majority of apoptotic pathways is the involvement of a family of proteases called caspases that cleave target proteins at specific sites typically containing aspartic acid residues followed by a caspase-specific three amino acid sequence [93]. Fig. 7 shows the apoptosis pathways with the corresponding molecule found in the *M. edulis* transcriptome compared to the KEGG reference pathway. 20 KO were associated with these pathways. Phillip et al. [26] found various transcripts for apoptosis related genes in the *M. edulis* transcriptome: a high number of TNF receptor like transcripts were identified, as well as various members of the Bcl-2 family and the apoptosis-inducing factor family (AIFs) and caspase-like transcripts. Also, caspases were characterized in the mussel *Mytilus galloprovincialis* and caspase-specific responses were observed to pathogens [94]. Authors suggest that the apoptotic process in *Mytilus* species has a similar complexity to that of vertebrates [26,94]. In the present analysis, some transcripts had similarities with caspases (CASP8, CASP3, CASP7 and CASP6) and other components such as the Fas-associated death domain (FADD) (Fig. 7).

4. Conclusion

In the present work, a cDNA library was constructed and sequenced, which probably covers the major part of the transcriptome of the *M. edulis* hemocytes challenged with *V. splendidus* LGP32 strain. A total of 19,622 sequences were assembled and annotated. As revealed by homologies at nucleic and protein levels and with KEGG

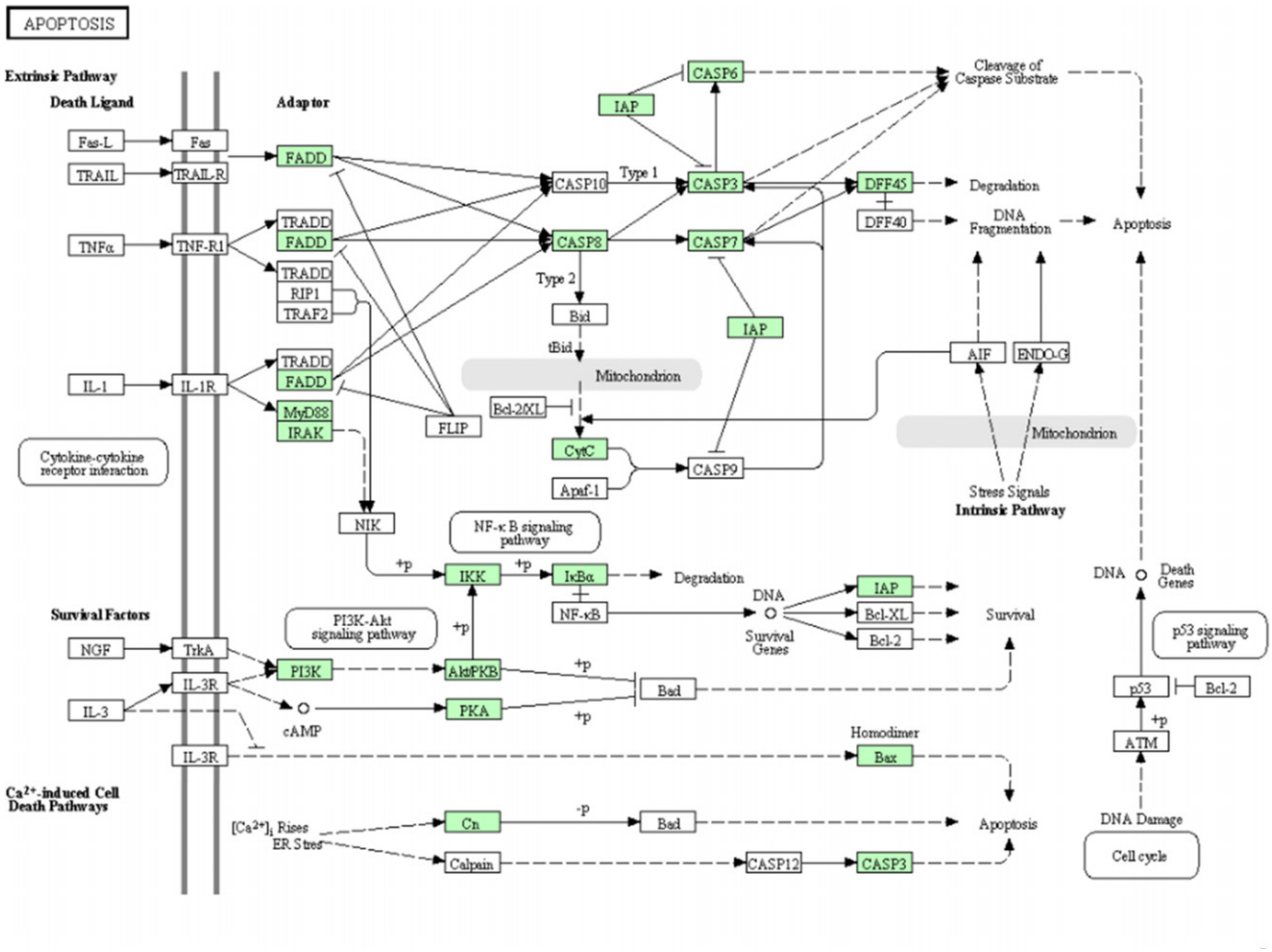

Fig. 7. Schematic comparison of *Mytilus* and KEGG reference apoptosis pathways. Shaded boxes indicate proteins identified in our 454 results and white boxes the absent ones.

annotations, some of the annotated sequences encoded to stress responses proteins, immune recognition receptors, immune effectors, Toll-receptor transduction pathway. Further studies will be focused on the quantification of gene expression levels of hemocytes at different exposure times. In this context, the sequences reported in this study could be used as a reference transcriptome for further high-throughput analysis such as RNA sequencing or microarrays.

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