

## Running title: Genes expressed in shrimp surviving Vibriosis

### Identification of genes that are differentially expressed in hemocytes of the Pacific blue shrimp (*Litopenaeus stylirostris*) surviving an infection with *Vibrio penaeicida*.

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**Abbreviations:** SSH, Suppression subtractive hybridization; EST, expressed sequence tag; EF-1 $\alpha$ , elongation factor-1alpha; PCR, polymerase chain reaction; rtPCR, real-time PCR; Ct, cycle threshold; TGase, transglutaminase; PEN, penaeidin.

#### Abstract:

Considerable progress has been made in the field of invertebrate immunity through the characterization of genes involved in the response to infection and/or stress. However, the mechanisms by which commercially important marine invertebrates can successfully survive an infection remain largely unknown. For the first time in an invertebrate model, we have searched to discover genes involved in the survival capacity of shrimp using the highly pathogenic bacteria, *Vibrio penaeicida*. In the present study, we applied the technique of

suppression subtractive hybridization (SSH) to hemocyte cDNAs from infected and uninfected shrimp, only using samples from individuals that had survived 96 hours post-infection. The resulting library contains 260 expressed sequence tagged (EST) cDNA clones potentially representing highly expressed genes in surviving shrimp. Sequence similarity comparisons were made and putative identities were assigned to clones that were at least 51 % identical to known genes. This analysis showed two functional categories that were highly represented: those of genes involved in immune reactions (10.7% of the ESTs) and those involved in proliferation-hematopoiesis (10.3%). Expression pattern profile analyses of selected ESTs at different times post-infection confirmed the differential expression of the genes and efficiency of the SSH method. Differences in gene transcript abundance, for select ESTs encoding antimicrobial effectors, were evidenced by real-time PCR between shrimp that survived acute *Vibrio* infection and those individuals that did not survive acute *Vibrio* infection. These results suggest there are basic differences at the level of transcript abundance for genes directly involved in immune and hematopoietic processes from shrimp that survive and do not survive infection.

**Keywords:** Crustacean, *Vibrio*, suppression subtractive hybridization, immune response, hematopoiesis.

## **Introduction**

Recent advances have been made in the study of immunity from commercially produced shrimp (order:Decapoda, family:Penaeidae) through the molecular characterization of immune effectors and the analysis of gene expression in response to microbial challenge or stress. In particular, much attention has been devoted to the penaeidins (PEN), a family of antimicrobial peptides whose expression appears to be specific to penaeid shrimps, and whose analyses of expression has advanced the understanding of the immune response of the penaeids to infection (4). Progress has also been made on a larger scale via genomic methods, namely expressed sequence tag (EST) projects, to implicate a more complete set of genes involved in mediating the immune response in penaeid shrimp. To date, several EST projects have been reported for immune cells and tissues (hemocytes and hepatopancreas) of individual non-immune challenged *Litopenaeus setiferus* and *L. vannamei* (13) or from a

variety of tissues from *Penaeus monodon* (24, 39). Results from these studies demonstrate a high level of conservation between immune effector cDNAs from different shrimp species, which include antimicrobial peptides (penaeidins and anti-LPS factor) and antimicrobial proteins (lysozyme and crustins) (13, 39). Certain EST programs have also been applied to identify genes implicated in the hemocyte response of *Penaeus japonicus* to the white-spot syndrome virus (WSSV), *e.g.* protease inhibitors, tumor-related proteins and apoptosis-related proteins (35). Analysis of differential gene expression appears to be a promising approach to identify and characterize genes involved in the host response against pathogens (5, 40, 16, 33).

Apart from viruses, bacterial infections, *e.g.* vibriosis, limit the production of shrimp in aquaculture systems, but these diseases tend to dominate the larval stages that are maintained in hatcheries (26, 37). In contrast, *Vibrio penaeicida* differs from other strains that cause vibriosis, as it predominantly affects juvenile and adult Pacific blue shrimp (*Litopenaeus stylirostris*) in New Caledonia rearing ponds (36) and kuruma prawn (*P. japonicus*) from Japanese aquaculture farms (20). The acquisition of susceptibility to *V. penaeicida* is correlated with the developmental stage of the shrimp, which most likely corresponds to immunological or physiological changes during the last post-larval molt (12). In previous work, we sought to examine the generalized immune response of *L. stylirostris* to *Vibrio* by analyzing sites of bacterial localization in the tissues and changes in the penaeidin (PEN) following experimental infection (29). Two phases of the response to *Vibrio* infection were evident, the first corresponding to a massive migration of granular hemocytes to the sites of infection where they lyse and discharge their granular content followed by a systemic proliferation of hemocytes, particularly granular hemocytes which produce PENs. Thus, these observations strongly suggested that the ability of shrimp to circumvent *Vibrio* infections is closely tied to the regulation of hematopoietic processes.

Considering the kinetic data relating to temporal variations in hemocytic reactions subsequent to infection (29), we have established an experimental protocol to facilitate the identification of genes differentially expressed in the shrimp that have survived infection, using a subtractive suppression hybridization approach. Herein, we describe two main categories of ESTs from circulating hemocytes; those directly related to immune function and those related to hematopoiesis and cell proliferation. Semi-quantitative analyses of selected ESTs confirmed the differential expression of the genes and efficiency of the SSH method. In addition, for select ESTs, we present evidence for differences in gene transcript abundance from pooled and individual samples, between shrimp that survived acute *Vibrio* infection and

those individuals that did not survive *Vibrio* infection. More information on every EST (BlastX results and functional classification) is available using the public and interactive *L. stylirostris* database available on the web site (<http://www.ifremer.fr/StyliBase/>).

## **Materials and methods**

### **Animals and Experimental Infections.**

Juvenile *L. stylirostris* (20-30 g) were obtained from the French Polynesia IFREMER laboratory (Taravao, Tahiti). Shrimp used for the subtracted library construction were individually identified using colored silicone injection under the 6<sup>th</sup> abdominal segment of the cuticle. The infections were carried out by immersing individual shrimp for 2 hours in seawater tanks containing  $1.3 \times 10^4$  colony forming unit (CFU) of *V. penaeicida* strain AM101 /ml, corresponding to LD50 conditions(36). Animals were then rinsed with clean seawater and transferred into 100 L tanks supplied with filtered (1 $\mu$ m) and aerated seawater. Non-infected animals were kept in a separate 100 L tank.

For the primary experimental infection, animals were divided into five groups, one of which was to remain uninfected for the duration of the experiment to assess mortality due to handling and intangibles not associated with the experimental infection. Hemolymph was collected from the first group of shrimp (T-12) 12h prior to the experimental infection (Figure 1). Shrimp were then infected, 12 hours following hemolymph withdrawal, with *V. penaeicida* and individually tagged animals were monitored for the next 96 hours. Shrimp that did not survive the 96 hour infection were noted and hemolymph samples were sorted according to whether or not individuals survived the 96 hour infection. The second, third and fourth group of shrimp (T+12, T+24 and T+48, respectively) were infected at the same time as the T-12 group, however, hemolymph was not withdrawn before the experiment. Rather hemolymph was withdrawn at 12, 24 or 48 hours post-infection for each group, respectively. Only live shrimp were sampled at the designated time period. Shrimp which died before the hemolymph extraction were not included in the sampling. Following hemolymph extraction, individuals from the three groups were immediately returned to their respective tanks for the duration of the 96 hour experimental infection. Individually tagged animals from T+12, T+24, and T+48 were monitored for the next 24, 72, and 48 hours, respectively. Shrimp from each group that did not survive the remaining infection period were noted and hemolymph samples were sorted as stated above. At the end of the coordinated experimental infection, hemolymph samples from surviving and non-surviving shrimp were stored RNAlater then categorized

according to sampling time and whether or not the individual shrimp was alive at the end of the experiment.

The 96 hour time point was selected based on the fact that mortalities due to *V. penaeicida* appear to peak from 20 hours to 24 hours after infection and are considerably lower at 96 hours post-infection (12). Approximately 50 shrimp were utilized per condition and infections were conducted in duplicate to ensure similar mortalities could be reproduced. Hemolymph was collected from the ventral sinus located at the base of the first abdominal segment as described previously (10).

For macroarray and Northern blot analyses of selected ESTs, new infections were performed using the same experimental conditions (50 shrimp per condition) and hemolymph sampling was conducted at two separate time points after infection for RNA extraction. Expression profiles were constructed for uninfected shrimp as a control (T-12), shrimp 12 hours post-infection (T+12, when mortalities appear) and shrimp at 96 hours post-infection (T+96, end of mortalities, surviving shrimp).

#### **RNA isolation and surviving shrimp subtractive cDNA library construction.**

Total RNA from hemocytes was isolated using Trizol reagent (Gibco BRL) (1 ml/10<sup>7</sup> cells). For subtractive library construction, poly(A)<sup>+</sup> RNA was purified using nucleoTrap mRNA purification kit (Clontech). For all expression analyses, total RNA was used.

To implicate genes differentially expressed in hemocytes from shrimp that survived the *Vibrio* infection, SSH libraries were constructed by subtracting a mixed pool of hemocyte mRNA from T+12, T+24, and T+48 (Tester) with a mixed pool of hemocyte mRNA from the T-12 samples (Driver). Hemocyte RNA samples utilized for the construction of the SSH library were taken only from shrimp surviving the 96h experimental infection (detailed in Figure 1), while samples from shrimp not surviving the 96 hours infection were omitted from the pools. SSH libraries were produced using the PCR-SELECT cDNA subtraction kit (Clontech). The tester cDNA was prepared using 2 µg of poly (A<sup>+</sup>) RNA and the driver cDNA was synthesized using 8 µg of poly (A<sup>+</sup>) RNA. Enzyme digestion, adapter ligation, hybridization, and PCR amplification were performed as according to protocols provided by the manufacturer (Clontech). PCR products were cloned into pCR®2.1-TOPO cloning vector using TOPO TA cloning kit (Invitrogen) and transformed into *Escherichia coli* TOP10 One Shot Chemically Competent cells (Invitrogen).

#### **Sequence analysis.**

Subtracted cDNA clones randomly selected were single-pass sequenced (MWG Biotch S.A.; France) and analyzed using BLASTX and BLASTN algorithms (1) available through NCBI (National Center for Biotechnology Information, USA). Vector sequences were removed and database searches were limited to ESTs > 100 bp in length. Remaining sequences were clustered using the CAP3 assembly program (19). EST sequences have been submitted to the dbEST and GenBank databases (GenBank accession nos. from CV699273 to CV699526 and from CV720543 to CV720548).

### **cDNA macroarray.**

#### *a)- Amplification and spotting of cDNA fragment*

Subtracted cDNAs were amplified using universal primers present on pCR®2.1-TOPO: M13 Forward (5'-GTAAAACGACGGCCAG-3') and M13 Reverse (5'-CAGGAAACAGCTATGAC-3'). PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and quantified. Each PCR product (1.8 µg) was precipitated by ethanol precipitation, resuspended in 1.8 ml of 0.4 M NaOH, 10 mM EDTA and denatured at 95°C for 10 minutes. Each sample was blotted in duplicate (150 ng per spot) on three identical positively charged Nylon membranes using Minifold I Spot Blot System (Schleicher & Schuell). The DNA dot blots were washed with 250 µl of 0.4 M NaOH per dot. Membranes were further washed in 2X SSC for 5 minutes, air dried and DNAs were fixed to the membrane by UV cross-linking. On each membrane, *L. stylirostris* elongation factor 1-alpha (EF-1α) cDNA fragment (382 bp) was spotted in duplicate for normalization (GenBank accession no. **AY117542**).

#### *b) cDNA probe labeling, hybridization, and post-hybridization processing*

The cDNA probes (uninfected shrimp, 12 h and 96 h post infection) were labeled with [ $\alpha$ -<sup>32</sup>P] dCTP from total RNA (7 µg) using the SuperScript II reverse transcription kit, according to the manufacturer's instruction (Invitrogen). Three cDNA macro-array membranes were prehybridized separately, at 42°C over-night in pre-hybridization solution (4X SSC, 10X Denhardt's solution, 50% formamide, 0.1% SDS, 50 mM Na<sub>2</sub>HPO<sub>4</sub> pH 7.2, 1mM EDTA and 100µg/ml salmon sperm DNA). Labeled cDNA probes from each experimental condition tested were added to the pre-hybridization solution and incubated 12 hours at 42°C. After hybridization, the membranes were washed twice at 65°C for 15 minutes in 2X SSC/0.1% SDS, once in 1X SSC/0.1% SDS and twice in 0.1X SSC/0.1% SDS. The hybridization signal of each spot was quantified using the STORM system technology from

Molecular Dynamics and corrected from background signals (PCR reaction without a cDNA template and a PCR reaction with the pCR®2.1-TOPO plasmid without insert). One of the arrays did not hybridize well, as determined by low signal and high background, and was omitted from the analysis. The remaining two membranes were treated as a single membrane and intensity values from each spot (4 spots total) were used to calculate a mean and standard deviation for the overall hybridization. Each spot intensity was first normalized to overall mean intensity for EF-1 $\alpha$  and the mean of four spots representing the same SSH product was determined. Relative expression levels were used to determine the expression profiles of genes that may be implicated in the surviving capacity of shrimp. Gene expression, which in the context of this study is the same as differential relative transcript abundance, was considered differential if relative pooled values varied greater than two-fold. Similar threshold criteria were considered for previously reported studies (23, 42).

#### **Northern blot analysis.**

Hemocyte total RNAs (10  $\mu$ g) from uninfected (T-12) and infected (T+12, 12 hours post-infection and T+96, 96 hours post-infection) shrimp were subjected to Northern blot analysis as previously described (14) using selected clones containing cDNA inserts from the SSH as probes. Probes were amplified by PCR using M13 Forward (5'-GTAAAACGACGGCCAG-3') and M13 Reverse (5'-CAGGAAACAGCTATGAC-3') primers, purified using QIAquick PCR Purification Kit (Qiagen) and radio-labelled with [ $\alpha$ -<sup>32</sup>P] dCTP by random priming using the Ready-to-go DNA labeling kit (Amersham Pharmacia Biotech). Hybridization signals were quantified using the STORM system technology (Molecular Dynamics) and each hybridization signal was normalized with the signal from EF-1 $\alpha$  to obtain relative expression levels.

#### **Real Time PCR analyses**

A preliminary real-time polymerase chain reaction (rtPCR) analysis was utilized to determine whether acute changes in selected RNA abundance could be detected from hemolymph sampled 12 and 24 hours post-infection. Pooled, hemocyte total RNA (15 individuals per pool) was utilized from: (i) uninfected shrimp (T-12); (ii) surviving infected shrimp collected at 12h (T+12s) and 24 hours (T+24s) post-infection; (iii) non-surviving infected shrimp collected at 12h (T+12ns) and 24 hours (T+24ns) post-infection. Total RNAs were treated with DNase (TURBO DNase, Ambion) to remove contaminating genomic DNA. The DNase was removed by phenol chloroform extraction. First-strand cDNA was

synthesized from 1 µg of total RNA, using SuperScript II reverse transcription kit, according to the manufacturer's instruction (Invitrogen), in 20µl of volume reaction. 1µl of each reverse transcription reaction served as template in 20µl of rtPCR reaction containing 1X SYBR Green master mix (Qiagen) and 0.5 µM of each primer. A list of oligonucleotide primers used to amplify specific gene products are shown in the Figure 3A. Each rtPCR reaction was done in triplicate with an initial denaturation step of 900s at 95°C followed with amplification of the target cDNA (35 cycles of denaturation at 95°C for 15 seconds, annealing between 54°C and 64°C for 15 seconds and extension time at 72°C for 15 seconds) and performed with the LightCycler (Roche Molecular Biomedicals). In addition, to determine the rtPCR efficiencies of each primer pair used, standard curves were generated using five serial dilutions of plasmid containing the insert of interest ( $10^3$  to  $10^7$  copies/µl). Results are presented here as changes in relative expression normalized to reference gene (EF-1α) using the method described by W. Pfaffl (34) and determined using the equation:

$$\text{Relative Expression} = [(E_{\text{target}})^{\Delta\text{CP}_{\text{target}}(\text{control-sample})}] / [(E_{\text{ref}})^{\Delta\text{CP}_{\text{ref}}(\text{control-sample})}] ;$$

where  $E_{\text{target}}$  = amplification efficiency of the target or gene of interest;  $E_{\text{ref}}$  = amplification efficiency of the reference (EF1-α), CP = crossing point of a designated threshold level. The corresponding rtPCR efficiency (E) of 1 cycle in the exponential phase was calculated according to the equation:  $E=10^{[-1/\text{slope}]}$  (34).

A second rtPCR experiment was conducted on randomly selected subsamples (5-6 individuals) belonging to T-12, T+24s and T+24ns. This analysis was conducted to verify differences suspected from the preliminary results by incorporating statistical methods. The quality of total RNA from each individual was analyzed by 1% agarose gel electrophoresis. Samples that appeared as a smear with a low abundance of ribosomal RNA were excluded from the sampling. Estimates of RNA abundance were made using rtPCR with conditions and analytical procedures identical for those listed for the preliminary rtPCR analysis. Statistical significance was determined using Students t-test between surviving and non-surviving shrimp sampled at 24 hours and differences were considered when  $P<0.05$ .

## Results

### SSH screening of mRNAs differentially expressed in surviving infected shrimp.

An SSH library was constructed using solely hemocyte mRNA samples from animals that survived an experimental infection (Figure 1). The RNA samples from shrimp that died were not used in the construction of the SSH library.

A total of 320 randomly selected clones were single-pass sequenced resulting in the characterization of 260 ESTs that were longer than 100 bp after eliminating vector sequences (Table 1). The average insert size was estimated to be 633 bp by PCR amplification of inserts from 50 randomly selected clones. We used the assembly program CAP3 to organize the redundant ESTs into overlapping contigs (19). These ESTs coalesced into 52 contigs and 132 singletons suggesting that the overall redundancy of the library was 49.2%. Comparison of EST sequences to non redundant SwissProt and GenBank databases revealed 87 distinct ESTs that shared high similarity to genes with known function and 9 other ESTs similar to genes with unknown function (E-values  $< 10^{-3}$ ), all of which were considered enriched in hemocytes from shrimp surviving the *Vibrio* infection. In addition, we constructed a database (<http://www.ifremer.fr/StyliBase/>), where all the ESTs and their corresponding functional classification as well as a complete list of BlastX matches can be found.

### **Functional SSH categories.**

All ESTs were assigned functions as predicted from sequence similarity and subsequently clustered into distinct functional categories (Table 1). ESTs that represented transcripts that encode ribosomal proteins (2% of the ESTs), proteins involved in cell structure (2.4%), DNA replication/repair/transcription/translation (5.5%), and cell signalling (6.3%) were all found in relatively low abundance. Numerous EST sequences that belonged to transcripts that encode proteins involved in metabolism (8.7%) were found in next greater proportion to those previously listed, with cytochrome-C oxidase being most represented (6 clones).

Two functional groups predominated the SSH library. First, 10.7% of the ESTs identified in the subtracted library belonged to a group designated as cell proliferation which included genes involved in the regulation of cell cycle and apoptosis or cell differentiation and hematopoiesis (Table 2). We have isolated two ESTs with sequence similarity to domino (6) and dMi-2, (22) required for cell viability during development and hemocyte proliferation in *Drosophila*, as well as many genes involved in the cell proliferation pathway. In particular, an apparent homolog of a Ras oncogene, described as a key regulator of cellular proliferation in vertebrates and invertebrates (27, 3), and genes with oncogenic potential belonging to the Rho GTPase activating protein family (43, 28) have been identified. Moreover, various transcripts of protein kinases potentially involved in the proliferation pathway were isolated; a serine/threonine kinase TAO2 component of stress-responsive MAP kinase cascades (8) and a

sequence homologue to the human ribosomal S6 kinase (p38MAPK) that has been shown to mediate cell proliferation (11, 7).

The second major group of genes preferentially isolated in the SSH library (10.3% of sequenced clones) are represented by ESTs that belong to transcripts which are known to encode proteins directly involved with immune function (Table 2). Briefly, several ESTs that belonged to transcripts that encode antimicrobial peptides were isolated [four clones of penaeidin 2 (*Litsty* PEN2) and two clones for penaeidin 3 (*Litsty* PEN3)] which were recently characterized in *L. stylirostris* (29), as well as one apparent sequence homologous to lysozyme identified from *L. vannamei* (38) and other shrimp species: *P. monodon* (39), *P. japonicus* (35), *L. setiferus* and *L. vannamei* (13). A transcript encoding a putative cysteine and proline-rich peptide was also identified. This transcript was similar to a mouse cryptdin-related mRNA (E-value: 0.24) (32) and a cryptdin-related protein 4C (E-value: 0.24; accession number AAA18210). Interestingly, transglutaminase (TGase) ESTs were not only the most redundant transcripts identified for immune function, but also the most redundant EST found throughout the whole subtracted cDNA library (10 clones). Ribosomal proteins and actin sequences were not very abundant (< 2%) revealing the efficiency of the SSH.

Compared to other shrimp SSH and EST studies, the surviving shrimp SSH library contains few proteases and inhibitors of proteases which are known to participate in various processes related to innate immunity and the anti-infectious response. Two clones for  $\alpha 2$ -macroglobulin, well known from studies of invertebrate immunity (2, 21), were present in the library, but no protease inhibitors or proteins involved in the prophenoloxidase cascade were represented.

### **Gene expression patterns from different functional categories during infection.**

Expression profiles of some selected genes assigned to different functional categories were analyzed by macroarray and Northern blot to examine the general pattern of expression during the course of infection. EF-1 $\alpha$  was used as the normalizing factor, but the same results of relative expression were obtained when actin was used for normalization (data not shown). A total of 19 SSH clones were chosen to represent the different functional categories identified (Figure 2A). Given the criteria that approximately two-fold changes in transcript abundance represent differential expression (23, 42), it appears that the abundance of RNA for all genes considered were relatively stable or differ very little at 12 hours and 96 hours compared with the uninfected samples regardless of functional category. Moreover, even if some modulation of gene expression can be inferred by two-fold changes in RNA abundance,

inter-array variation was too great to estimate whether changes in some RNA populations could be considered different. However, certain RNA populations appeared to be good candidates for further analysis of differential expression such as the immune genes, TGase and lysozyme, and a gene product related to cell proliferation and hematopoiesis such as domino (Figure 2A). Both TGase and domino transcripts appear to be elevated in circulating hemocytes at T+96 (relative increase of 2.19 and 2.64-fold, respectively versus uninfected shrimp). Different profiles were also observed for lysozyme that showed a decrease in relative transcript concentration at T+12 (by a factor of 0.45) followed by an elevation in abundance in surviving shrimp at T+96 (relative increase of 1.57-fold *versus* T-12 and 2.67-fold *versus* T+12; Figure 2A). Differences in select expression profiles were further verified by Northern blot (Figure 2B).

### **Comparison of hemocyte gene expression from shrimp surviving and not surviving a *Vibrio* infection by Real-time PCR.**

Real-time PCR efficiencies of selected ESTs varied between 1.89 and 1.98 (Figure 3A), however, as these efficiencies were not exactly 2.00 (representing 100% amplification efficiency at each cycle) we calculated relative abundance using an equation to correct for differences in efficiency as described by Pfaffl (34). Preliminary analysis of pooled samples for lysozyme and the cryptdin-like EST showed a paralleled fall in transcript abundance at 12 hours post-infection followed by a return to a level similar to that of the uninfected control (T-12) for the surviving shrimp at 24 hours (T+24s) (Figure 3B). However, hemocytes from non-surviving shrimp (T+24ns) did not show a return to T-12 levels after 24 hours. This expression profile was also observed to a lesser extent for *Litsty* PEN3 and seems to be common for immune effector transcripts identified from the SSH study. On the other hand, the expression profile of haematopoietic genes domino and TGase appears different in that there is a constant relative reduction in transcript abundance at both 12 and 24 hours post-infection for surviving and non-surviving shrimp. Based on the fact that variation in relative expression, between both non-surviving and surviving shrimp, was most dramatic at 24 hours for three immune effector or putative effector EST transcripts (lysozyme, PEN3, and cryptdin-like), we chose to investigate sample variation only at the 24 hours time point using individual samples (N = 5-6) to infer differences by statistical methods between surviving and non surviving groups for all five transcripts.

The second round of real-time PCR analysis revealed significant differences in transcript abundance for the three immune effector transcripts (results expressed as transcript

expression of surviving versus non-surviving shrimp collected at 24 hours post infection, relative to uninfected shrimp) : (i) *Litsty* PEN3 ( $0.81\pm 0.15$  versus  $0.32\pm 0.11$ , respectively;  $P<0.05$ ), (ii) lysozyme ( $3.74\pm 1.01$  versus  $0.10\pm 0.04$ , respectively,  $P<0.01$ ), and (iii) cryptdin-like ( $1.42\pm 0.17$  versus  $0.15\pm 0.05$ , respectively,  $P<0.05$ ) (Figure 3C). No differences in relative expression were detected between surviving and non-surviving shrimp at 24 hours for the transcripts TGase or domino ( $P>0.05$ ).

## Discussion

A clear understanding of the molecular mechanisms that control or contribute to the anti-infectious response, in economically important marine invertebrates, is a major prerequisite leading to the effective management and future progress of the aquaculture industry. In particular, understanding immunity in these animals is of prime interest for developing strategies to limit the impact of disease (4).

The aim of this study was to identify genes which may be involved in a successful immune response of a penaeid shrimp to *V. penaeicida* infection using suppression subtractive hybridization. Knowledge of *Vibrio* infection kinetics allowed us to design an appropriate experimental infection protocol to investigate such an immune response using the subtraction the hemocyte mRNAs from non-infected animals and infected animals which were able to survive an experimental infection. Three different groups of shrimp representing three infection times were sampled to identify changes in early and late RNA abundance that likely represent differentially expressed genes involved in the response to *Vibrio* infection and may be included in the generalized immune response of the shrimp.

About 260 ESTs, representing differentially abundant RNAs from hemocytes of surviving infected shrimp, were analysed. The efficacy of the subtraction was controlled in particular by the weak proportion of housekeeping or constitutively expressed genes we obtained, compared to results usually seen with other EST projects. This is also shown by the low number of PEN transcripts found in our SSH library (2 clones), whereas this gene is considered to be constitutively expressed in shrimp granular hemocytes (10) and appears to represent a relatively high fraction of the immune related sequences in hemocyte EST libraries from *L. setiferus* and *L. vannamei* (82% and 73%, respectively; (13)).

As expected from hemocytes, the dominant functional class of transcripts isolated from the subtracted cDNA library belongs to immune-related function (10.7%). Among the various components of the immune system, a number of antimicrobial molecules were identified in our SSH library which include members of the PEN family (*Litsty* PEN2 and *Litsty* PEN3) and lysozyme, but did not include anti-LPS factor or crustins - two antimicrobial molecules that are typically found in shrimp hemocyte EST collections (13). One hypothesis to explain the absence of these antimicrobial effector transcripts from our SSH library is that their abundance does not vary to any great extent between our experimental driver and tester population of shrimp hemocytes. Interestingly, a new transcript encoding a putative cysteine and proline-rich peptide was identified that present similarity with a mouse cryptdin-related mRNA (32). In our experiments, this cryptdin-like transcript is differentially abundant between surviving and non-surviving shrimp, possibly evidence of differential expression in response to *Vibrio penaeicida*. Whereas the nature and properties of this putative molecule remain to be elucidated, one can assume it plays a role in the immune response of shrimp surviving the infection. With regard to PENs, apart from the isolation of *Litsty* PEN3 sequences whose expression is considered to be up-regulated in granular hemocytes during the systemic and proliferative immune response occurring about 48 to 96 hours after microbial challenge (30), sequences of the *Litsty* PEN2 class were also identified. In *L. vannamei*, this peptide presents a similar range of antimicrobial activity compared to PEN3 (9), but nothing is yet known about its expression profile and function during the immune response. The identification of *Litsty* PEN2 in the SSH library would suggest this class of PEN may also be differentially expressed during *Vibrio* infection in surviving shrimp.

The lysozyme sequence we identified from *L. stylirostris* hemocytes may be differentially expressed in the hemocytes of surviving shrimp. Expression analyses performed either by macroarray, Northern blot or rtPCR, reveal a clear difference in lysozyme transcript abundance during the course of *Vibrio* infection. A decrease in lysozyme transcript abundance was seen early after infection (*c.* 12 hours) and was elevated in surviving shrimp at 96 hours. This profile is very similar to that of PENs, further supporting the idea that the expression profile is linked to modifications of circulating hemocyte population during infection (30). If this is the case, then lysozyme would likely be expressed in granular hemocytes, which have been shown to leave the blood circulation and infiltrate different tissues in response to an infection (30). These provoking observations shed light on the difficulties encountered when working with a complex and dynamic cell assemblage such as hemocytes. Further work will be necessary to determine whether lysozyme expression is regulated independently within

each hemocyte or whether the expression profile is due to variations in hemocyte population composition. Whatever the mechanism underlying the increase in lysozyme transcript, the striking difference in lysozyme transcript abundance shown by rtPCR at 24 hours post infection between hemocytes from non-surviving and surviving shrimp (Figure 3B) reveals a potential for involvement of this effector in the successful immune response of these shrimp to *Vibrio* infection. This result is even more intriguing when one considers that recombinant lysozyme from *P. japonicus* has been shown to be effective against different *Vibrio* strains (17), thus making this effector highly relevant in the *L. stylirostris* bacterial defense response.

Transglutaminase (TGase) was found to be most redundant in the differential library and appeared to be elevated after 96 hours post-infection, suggesting that these transcripts were not immediately elevated after infection (Table 2, Figure 2). For the various vertebrate lineages, TGases are implicated in numerous processes related to wound healing, inflammation, cell proliferation and migration, apoptosis, and a variety of processes which contribute to tissue and cellular homeostasis (41). However, in crustaceans and chelicerates TGases are primarily known for participating in blood coagulation, which is considered a powerful immune defense reaction (15, 31). Similar to the response shown for microbially challenged oysters (14), TGase transcripts were significantly over-represented in hemocytes from *L. stylirostris* that had survived a *Vibrio* infection, perhaps signifying a greater level of importance not previously attributed to this molecule (Figure 2). The fact that TGase transcripts appear to be elevated in surviving shrimp at 96 hours post infection is notable and (Figure 2) may reflect the appearance of newly released or synthesized TGase-expressing hemocytes into the blood, as was previously suggested to describe changes in abundance of PEN transcripts (30, 29). This phenomenon appears to occur during the late phase of the shrimp immune response corresponding to the proliferative and systemic reaction (reviewed in (4)). Interestingly, TGase expression has been detected in hematopoietic tissues of the shrimp *P. monodon*, suggesting a relationship between hemocyte proliferation and TGase synthesis (18), but it cannot be excluded that TGase gene transcription is up-regulated for individual circulating hemocytes of surviving shrimp. Due to the high prevalence of TGase in the SSH library (11%) and the increase of transcripts after 96 hours post-infection, it is possible that this molecule plays a large role in the proliferation phase of the immune response.

The fact that stimulation of hematopoiesis is a major element of the shrimp immune response (4) is supported by the high representativeness of genes involved in cell proliferation pathway in the SSH library (Table 2). Expression pattern of sequences homologous to domino

gene from *Drosophila* has been considered in our study. As shown for TGase, the abundance of domino transcripts appear elevated in hemocytes of surviving shrimp observed at 96 hours post-infection, but clear differences in level of expression were not seen at 12 and 24 hours relative to the survival capacity of the shrimp. Moreover, domino expression appears to be elevated later in the immune response as evidenced by an increase in transcript abundance (Figure 2). A correlation in late expression genes such as TGase and domino, both of which are known to play roles in hemocyte proliferation, may indicate an up-regulation of gene transcription through a common regulatory pathway. Finally, the SSH library from surviving shrimp contains a number of genes involved in apoptosis, which have already been shown to play a role in the shrimp anti-viral response (35) and cell cycle regulation.

From the analyses of expression, it appears that transcripts identified by SSH are moderately expressed in non-infected animals and thus are present prior to infection. Further, gene transcript abundance appears weakly modulated upon infection for a majority of EST transcripts analyzed. This may be a feature intrinsic to the regulation of these gene products, but may also reflect the method used to analyze RNA abundance since macroarrays are less sensitive compared with lower throughput RNA analysis techniques (25). The use of pooled RNA samples to examine differences in transcript abundance may also account for some reduction in gene specific transcript abundance between the different populations of shrimp tested, since pooling does not allow an estimate of population variation to be determined. However, given the small amount of RNA contained in circulating hemocytes, pooling was successfully utilized as a preliminary screen to identify candidate transcripts so that a more sensitive technical approach such as rtPCR could be utilized for a more rigorous examination. Although somewhat limited, we found different expression profiles for several interesting transcripts which have been confirmed by Northern blot and rtPCR. The expression profile of genes encoding immune effectors such as *Litsty* PEN3, lysozyme or a cryptdin-like molecule is characterized by a decrease in transcripts during the first phase of the immune response and a restoration or increase during the proliferative stage of the immune response. This expression profile was consistently reduced for certain transcripts (e.g. lysozyme, *Litsty* PEN3 and cryptdin-like at 24 hours) in animals which will succumb to infection. Thus, our study reveals that the differential RNA transcript abundance profiles of these genes could be good markers for monitoring the capacity of shrimp to further survive a pathogenic infection or for other health monitoring purposes.

These data and the isolation of ESTs in shrimp that have been able to survive an experimental *V. penaeicida* infection may greatly contribute to the progress in understanding

the host-pathogen immune response in shrimp tolerant of infection. Further analyses of expression of all the SSH products obtained may also contribute to the identification of genes related to *Vibrio* resistance in other shrimp species and could serve as markers for selection purposes or prophylactic surveys in shrimp aquaculture.

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**Figure 1. Original experimental protocol for the identification of genes differentially expressed in shrimp surviving *Vibrio* infection by subtractive suppression hybridization.**

A: Details on the time table sampling of shrimp hemocytes for the SSH library construction. Four groups of shrimp were set up (T-12, T+12, T+24 and T+48) and each shrimp of these groups was individually identified by silicone tagging. In the first group (T-12) or driver, hemocytes collected 12 hours before the infection represent the samples of healthy shrimps. Then, the shrimp of the four groups were infected by *Vibrio penaeicida* in DL50 conditions. For the three groups (T+12, T+24 and T+48) or tester, hemocytes were collected at 12, 24 and 48 hours post-infection (period of mortality). For the four experimental groups, hemolymph was individually sampled. Mortalities were monitored until 96 hours (end of period of acute mortalities) and hemocytes samples from the individual shrimp which had survived the infection have been selected to make the subtracted library. B: Experimental infection, record of mortalities and effective of shrimp used for the construction of the hemocyte SSH library.

**Figure 2. Expression patterns during *Vibrio penaeicida* infection of some genes as representative of the different functional groups identified in the SSH library.**

Shrimp hemocytes were collected from uninfected shrimps (T-12: □), 12 hours post-infection (T+12: ■) and 96 hours post-infection (T+96: ■). Fifty shrimps were used for each point. Graphics represents relative expressions normalizing with the elongation factor-1alpha from the Molecular Dynamics Storm system quantifications. A: Macro-array analysis of 19 genes from SSH, involved in different functional categories: metabolism (A, uracil phosphoribosyl transferase; B, NADH deshydrogenase; C, UMP synthase); immunity (D, TGase; E, PEN3; F, lysozyme; G, cyclophilin); replication, repair, transcription, translation (H, splicing factor; I, noisette); cell signalling (J, sterol regulatory element binding protein 1; K, lung seven transmembrane receptor 1); ribosomal proteins (L, ribosomal protein L12); cell division and proliferation (M, activator of S phase kinase; N, domino; O, dMi-2); cell structure (P, profilin; Q, alpha-spectrin; R, actin) and other (S, annexin). Among tested genes, only the expression profiles (surrounded) appeared modulated during the infection (E, G and O). B: Northern blot hybridization of six SSH sequences (TGase, domino, lysozyme, PEN3, annexin and profilin) during the infection, on ten micrograms of hemocyte of total RNA per lane. Corresponding graphs present relative expression levels during the infection.

**Figure 3. Real-time PCR analyses of transcripts identified by SSH from hemocytes of surviving and non surviving shrimp post infection with *V. penaeicida*.**

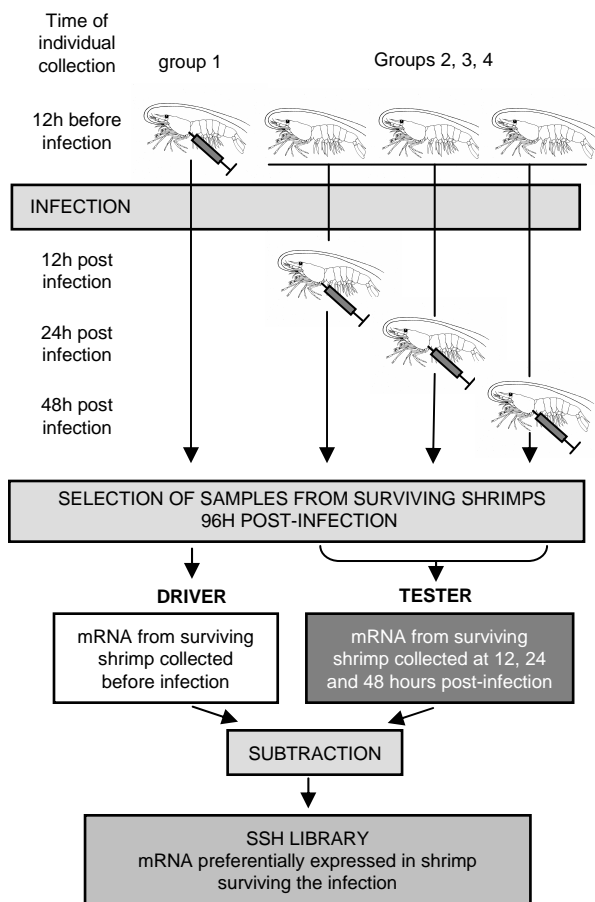
A: Real time PCR primers forward and reverse primers used to determine transcript abundance of lysozyme, PEN3, cryptdin-like, domino, TGase and EF-1 $\alpha$ . Specific annealing temperature of each primer pair is noted as well as the PCR efficiency; calculated by the equation:  $E=10[-1/\text{slope}]$  (Rasmussen 2001).

B: Relative expression of lysozyme, PEN3, cryptdin-like, domino and TGase from pooled samples (15 shrimp per condition) of uninfected shrimp (T-12:  $\square$ ) and at two time points post-infection (12 and 24 hours post-infection) from shrimp surviving infection (T+12s, T+24s:  $\blacksquare$ ) and not surviving infection (T+12ns, T+24ns:  $\blacksquare$ ). Relative expression levels were normalized with EF-1 $\alpha$  and the values during the infection were calculated in reference to uninfected shrimp (relative expression = 1) according the  $2^{-\Delta\Delta C_t}$  method corrected for efficiency (Pfaffl 2001). C: Expression analysis of lysozyme, PEN3, cryptdin-like, domino and TGase at the individual level. Results are expressed as mean values  $\pm$  SEM from five or six shrimp collected 24 hours post-infection, which have survived infection (T+24s:  $\blacksquare$ ) or did not survive infection (T+24ns:  $\blacksquare$ ). \* denotes statistical difference between the two infected groups,  $p < 0.05$  (Student's  $t$  test).

**Table 1.** General characteristics of the *Litopenaeus stylirostris* hemocyte SSH EST library from surviving shrimp.

**Table 2.** ESTs similar to genes potentially involved in immune mechanisms and in cell proliferation, hematopoiesis and apoptosis.

A



B

	Infected				Non infected
	Driver (group 1)	Tester (groups 2, 3, 4)			Control (group 5)
Time of collection	12h before infection	12h post infection	24h post infection	48h post infection	12h post infection
Starting number of shrimp	112	93	88	48	30
Surviving shrimp to 96h post infection	55	48	38	33	27

Figure 1

Table 1

General characteristics of *Litopenaeus stylirostris* hemocyte ESTs from SSH.

Total number of cDNAs sequenced	320
Total number of cDNA analyzed <sup>a</sup>	260
Average insert size (range)	633bp (200-1200)
Average EST length	440 bp
EST clusters <sup>b</sup>	52
Singletons <sup>c</sup>	158
ESTs assembled in clusters	128
Redundancy <sup>d</sup>	49.2%
<b>Functional classification</b>	
No hits	49.0%
Immunity	10.7%
Proliferation	10.3%
Metabolism	8.7%
Cell signalling	6.3%
Replication, repair, transcription, translation	5.5%
Unknown	3.6%
Cell structure	2.4%
Ribosomal proteins	2.0%
Others	1.6%

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

<sup>b</sup> ESTs with 75% or greater identity over a 40 bp region were clustered together forming 52 EST clusters.

<sup>c</sup> 158 sequences did not sufficiently match any sequence in the data set to allow assembly.

<sup>d</sup> Redundancy = number of ESTs assembled in clusters / total ESTs.

Table 1

Table 2

ESTs similar to genes potentially involved in immune response and hemocyte proliferation period.

<b>Putative function</b>	<b>GenBank accession no.</b>	<b>Closest species (GenBank accession no.)</b>	<b>E-value</b>	<b>Redundancy</b>
<b>Immunity</b>				
Lysozyme	CV699332	<i>Litopenaeus vannamei</i> (AF425673)	1.E-07	1
Penaeidin2	CV699319	<i>Litopenaeus stylirostris</i> (AAQ62565)	2.E-12	4
Penaeidin3i	CV699309	<i>Litopenaeus stylirostris</i> (AAQ62566)	1.E-25	2
Transglutaminase	CV699330	<i>Penaeus monodon</i> (AAL78166)	1.E-74	10
Alpha-2-macroglobulin	CV699313	<i>Limulus sp.</i> (BAA19844)	4.E-12	2
Monocyte/neutrophil elastase inhibitor	CV699318	<i>Homo sapiens</i> (AAC31394)	6.E-31	1
Amyloid precursor protein	CV699314	<i>Sus scrofa</i> (BAA84580)	1.E-10	1
Ninjurin1	CV699312	<i>Rattus norvegicus</i> (AAB17559)	2.E-09	2
t-complex protein 11	CV699278	<i>Mus musculus</i> (AAH16610)	2.E-06	1
Heparan sulfate	CV699317	<i>Mus musculus</i> (AAB84387)	8.E-31	1
Cyclophilin isoform 5	CV699305	<i>Caenorhabditis elegans</i> (AAC47126)	2.E-55	1
Dipeptidyl peptidase III	CV699350	<i>Rattus norvegicus</i> (BAA24608)	9.E-05	1
Cryptdin-related protein 4C	CV699287	<i>Mus musculus</i> (AAA18210)	0.24*	1
<b>Hematopoiesis-differentiation</b>				
Ral-A exchange factor RalGPS2	CV699276	<i>Mus musculus</i> (BAB31312)	8.E-05	1
RhoGap protein	CV699298	<i>Gallus gallus</i> (AAB07998)	2.E-15	1
RAS oncogene family member RAP1B	CV699291	<i>Homo sapiens</i> (CAA90983)	1.E-38	1
Lsc protein	CV699295	<i>Rattus norvegicus</i> (CAA15426)	8.E-30	1
Serine/threonine protein kinase TAO2	CV699275	<i>Rattus norvegicus</i> (AAD39480)	5.E-40	1
Ribosome S6 protein kinase	CV699287	<i>Homo sapiens</i> (AAQ24165)	1.E-54	2
Mi protein	CV699281	<i>Mus musculus</i> (CAA80600)	1.E-04	3
dMi protein	CV699293	<i>Drosophila melanogaster</i> (AAD17276)	1.E-28	3
Helicase DOMINO A	CV699301	<i>Drosophila melanogaster</i> (AAF82185)	1.E-59	1
ATP binding protein associated with cell differentiation	CV699289	<i>Homo sapiens</i> (AAH05968)	4.E-57	1
<b>Cell cycle regulators-apoptosis</b>				
Anaphase-promoting complex subunit 4	CV699286	<i>Homo sapiens</i> (AAF05752)	9.E-05	1
Activator of S phase kinase	CV699290	<i>Homo sapiens</i> (BAA78326)	2.E-06	1
Fizzy-related protein	CV699294	<i>Drosophila melanogaster</i> (CAA74575)	1.E-30	1
Peanut gene product	CV699284	<i>Drosophila melanogaster</i> (AAA19603)	9.E-19	2
Apoptosis 1 inhibitor	CV699279	<i>Danio rerio</i> (AAL33679)	3.E-08	1
Programmed cell death 6 interacting protein	CV699302	<i>Mus musculus</i> (AAH30340)	7.E-52	2
Radixin	CV699296	<i>Homo sapiens</i> (AAA36541)	2.E-20	2
Na <sup>+</sup> /K <sup>+</sup> ATPase alpha subunit	CV699283	<i>Callinectes sapidus</i> (AAG47843)	2.E-25	1

\* E-value > 10<sup>-3</sup>

Table 2

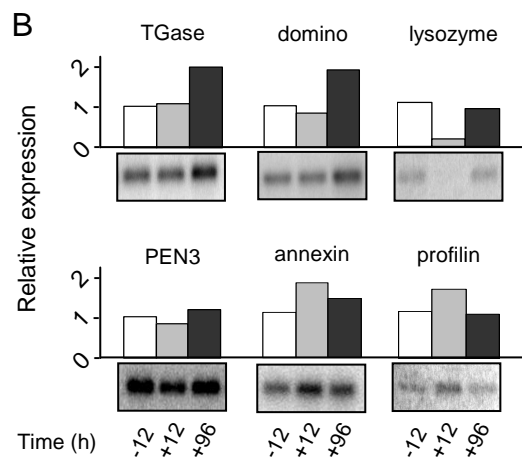
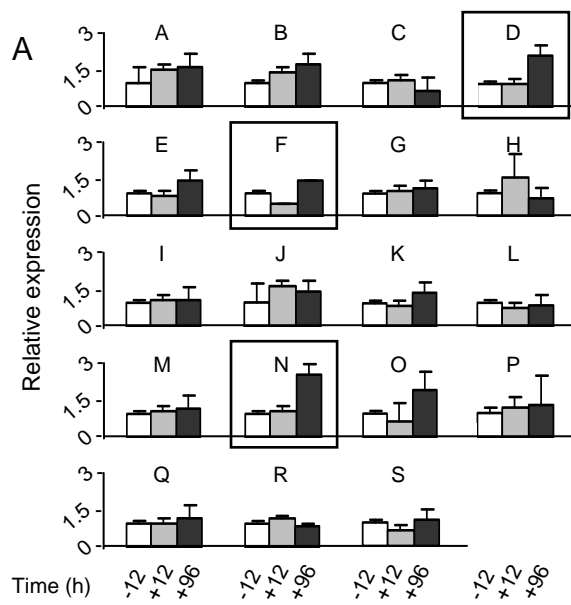
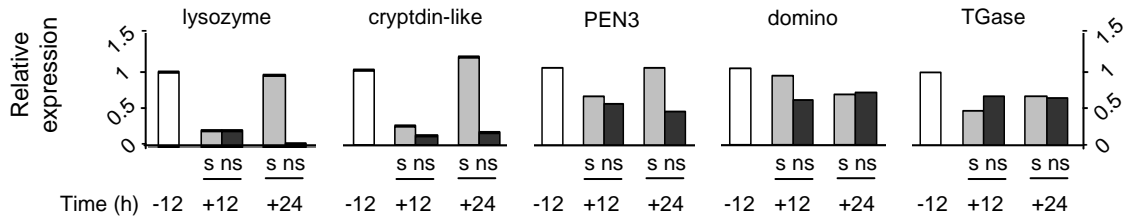


Figure 2

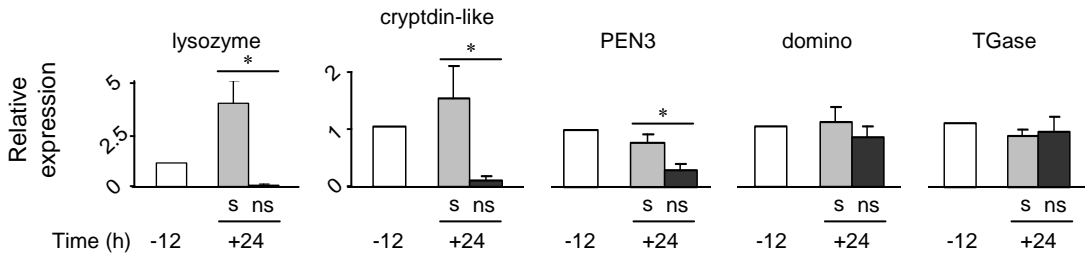
**A**

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing (°C)	PCR efficiency
lysozyme	GGCTTGGCACCAGGGTTACC	CGTCTGCACGTCAGCTGTG	59	1.93
PEN3	CCATGCGCCTCGTGGTCTG	GAACGCGCTTGTAAGGTGGTAA	64	1.98
cryptdin-like	GCCCTAAGTGCCCATATGA	GCCGCATTACATCCTATA	54	1.89
domino	CGATGAGATGGGTCTTGTTAA	CGGAGGTAGGGACAACAATC	55	1.94
TGase	ATCAAGAGCATTGTCATCTGC	TATCAGGCTTGTAACCTCCA	58	1.93
EF-1 $\alpha$	GGTGCTGGACAAGCTGAAGGC	CGTTCGGTGATCATGTTCTTGATG	60	1.96

**B**



**C**



**Figure 3**