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

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Submitted 29 November 2004; accepted in final form 18 February 2005

Identification of genes that are differentially expressed in hemocytes of the Pacific blue shrimp (Litopenaeus stylirostris) surviving an infection with Vibrio paraeica. Physiol Genomics 21: 174–183, 2005. First published February 22, 2005; doi:10.1152/physiolgenomics.00281.2004.—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 paraeica. 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 h postinfection. 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 postinfection 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.

Apartment 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 paraeica differs from other strains that cause vibriosis, as it predominantly affects juvenile and adult Pacific blue shrimp (L. stylirostris) in New Caledonia rearing ponds (36) and kuruma prawn (P. japonicus) from Japanese aquaculture farms (20). The acquisition of susceptibility to V. paraeica is correlated with the developmental stage of the shrimp, which most likely corresponds to immunological or physiological changes during the last postlarval 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 PEN following experimental infection (29). Two phases of the response to Vibrio infection were evidenced, 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.

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 antimicrobially 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 nonimmune challenged Litopenaeus setiferus and L. vannamei (13) or from a variety of tissues from Peneaus 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-lipopolysaccharide (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 P. 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, 16, 33, 40).

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 paraeica differs from other strains that cause vibriosis, as it predominantly affects juvenile and adult Pacific blue shrimp (L. stylirostris) in New Caledonia rearing ponds (36) and kuruma prawn (P. japonicus) from Japanese aquaculture farms (20). The acquisition of susceptibility to V. paraeica is correlated with the developmental stage of the shrimp, which most likely corresponds to immunological or physiological changes during the last postlarval 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 PEN following experimental infection (29). Two phases of the response to Vibrio infection were evidenced, 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 suppression subtractive hybridization (SSH) 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. Semiquantitative 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 StyliBase web site (http://www.ifremer.fr/StyliBase/).

**MATERIALS AND METHODS**

**Animals and Experimental Infections**

Juvenil *L. stylirostris* (20–30 g) were obtained from the French Polynesia Institut Français de Recherche pour l’Exploitation de la Mer (IFREMER) laboratory (Taravao, Tahiti). Shrimp used for the subtracted library construction were individually identified using colored silicone injection under the 6th abdominal segment of the cuticle. The infections were carried out by immersing individual shrimp for 2 h in seawater tanks containing 1.3 × 10^10^ colony forming units (CFU) of *V. penaeicida* strain AM101 per milliliter, corresponding to LD50 conditions (36). Animals were then rinsed with clean seawater and transferred into 100-liter tanks supplied with filtered (1 μm) and aerated seawater. Noninfected animals were kept in a separate 100-liter 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/H11001) 12 h prior to the experimental infection (Fig. 1). Shrimp were then infected, 12 h following hemolymph withdrawal, with *V. penaeicida*, and individually tagged animals were monitored for the next 96 h. Shrimp that did not survive the 96-h infection were noted, and hemolymph samples were sorted according to whether individuals survived the 96-h infection. The second, third, and fourth group of shrimp (T/H11001, T/H11002, and T/H11003, respectively) were infected at the same time as the T/H11001 group; however, hemolymph was not withdrawn before the experiment. Rather, hemolymph was withdrawn at 12, 24, or 48 h postinfection for each group, respectively. Only live shrimp were sampled at the designated time period. Shrimp that 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-h experimental infection. Individually tagged animals from T/H11001, T/H11002, and T/H11003 groups were monitored for the next 24, 72, and 48 h, 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 nonsurviving shrimp were stored with RNAlater, then categorized according to sampling time and whether the individual shrimp was alive at the end of the experiment.

The 96-h time point was selected on the basis of the fact that mortalities due to *V. penaeicida* appear to peak from 20 to 24 h after infection and are considerably lower at 96 h postinfection (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 at 12 h postinfection (T+12, when mortalities appear), and shrimp at 96 h postinfection (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/103 cells). For subtractive library construction, poly(A)+ 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 96-h experimental infection (detailed in Fig. 1), whereas samples from shrimp not surviving the 96-h 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)+ RNA, and the driver cDNA was synthesized using 8 μg of poly(A)+ 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 pCR2.1-TOPO cloning vector using TOPO TA cloning kit (Invitrogen) and transformed into One Shot TOP10 chemically competent Escherichia coli cells (Invitrogen).

Sequence Analysis

Subtracted cDNA clones randomly selected were single-pass sequenced (MWG Biotech; France) and analyzed using BlastX and BlastN algorithms (1) available through the National Center for Biotechnology Information (NCBI). 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

Amplification and spotting of cDNA fragment. Subtracted cDNAs were amplified using universal primers present on pCR2.1-TOPO: M13 forward (5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′) primers. 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 and 10 mM EDTA, and denatured at 95°C for 10 min. Each sample was blotted in duplicate (150 ng/spot) on three identical positively charged nylon membranes using MiniFold I Spot-Blot System (Schleicher and Schuell). The DNA dot blots were washed with 250 μl of 0.4 M NaOH per dot. Membranes were further washed in 2× SSC for 5 min, and air dried, and DNAs were fixed to the membrane by UV cross-linking. On each membrane, L. stylirostris elongation factor-1α (EF-1α) cDNA fragment (382 bp) was spotted in duplicate for normalization (GenBank accession no. AY117542).

cDNA probe labeling, hybridization, and posthybridization processing. The cDNA probes (uninfected shrimp, 12 h and 96 h postinfection) were labeled with [α-32P]dCTP from total RNA (7 μg) using the SuperScript II reverse transcription kit, according to the manufacturer’s instruction (Invitrogen). Three cDNA macroarray membranes were prehybridized separately, at 42°C overnight in prehybridization solution (4× SSC, 10× Denhardt’s solution, 50% formamide, 0.1% SDS, 50 mM Na2HPO4, pH 7.2, 1 mM EDTA, and 100 μg/ml salmon sperm DNA). Labeled cDNA probes from each experimental condition tested were added to the prehybridization solution and incubated 2 h at 42°C. After hybridization, the membranes were washed twice at 65°C for 15 min in 2× SSC/0.1% SDS, once in 1× SSC/0.1% SDS and twice in 0.1× 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 pCR2.1-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-1α, 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 twofold. Similar threshold criteria were considered for previously reported studies (23, 42).

Northern Blot analysis

Hemocyte total RNAs (10 μg) from uninfected (T−12) and infected (T+12, 12 h postinfection; and T+96, 96 h postinfection) 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 radiolabeled with [α-32P]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-1α 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 h postinfection. Pooled, hemocyte total RNA (15 individuals per pool) was used from T+12, 24 h postinfection shrimp collected at 12 h (T+12s) and 24 h (T+24s) postinfection, and T+96, 96 h postinfection shrimp collected at 12 h (T+12ns) and 24 h (T+24ns) postinfection. 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 instructions (Invitrogen), in 20 μl of volume reaction. One microliter of each reverse transcription reaction served as template in 20 μl of rtPCR reaction containing 1× 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 Fig. 3a. Each rtPCR reaction was done in triplicate with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. 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 h postinfection. Pooled, hemocyte total RNA (15 individuals per pool) was used from T+12, 24 h postinfection shrimp collected at 12 h (T+12s) and 24 h (T+24s) postinfection, and T+96, 96 h postinfection shrimp collected at 12 h (T+12ns) and 24 h (T+24ns) postinfection. 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 instructions (Invitrogen), in 20 μl of volume reaction. One microliter of each reverse transcription reaction served as template in 20 μl of rtPCR reaction containing 1× 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 Fig. 3a. Each rtPCR reaction was done in triplicate with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.
of 900 s at 95°C followed with amplification of the target cDNA (35 cycles of denaturation at 95°C for 15 s, annealing between 54°C and 64°C for 15 s, and extension time at 72°C for 15 s) 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 Pfaffl (34) and determined using the equation:

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\text{Relative Expression} = \left[\frac{(E_{\text{target}})\Delta C_{\text{Target (control-sample)}}}{(E_{\text{ref}})\Delta C_{\text{Ref (control-sample)}}}\right]^{1/slope}
\]

where \(E_{\text{target}}\) = amplification efficiency of the target or gene of interest; \(E_{\text{ref}}\) = amplification efficiency of the reference (EF-1α); and 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: \(E = 10^{-1/slope}\) (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 to those listed for the preliminary rtPCR analysis. Statistical significance was determined using Student’s t-test between surviving and nonsurviving shrimp sampled at 24 h, 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 (Fig. 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 nonredundant 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^-7), 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/translation (5.5%), and cell signaling (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 (3, 27), and genes with oncogenic potential belonging to the Rho GTPase activating protein family (28, 43) 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 homolog to the human ribosomal S6 kinase (p38MAPK) that has been shown to mediate cell proliferation (7, 11).

The second major group of genes preferentially isolated in the SSH library (10.3% of sequenced clones) is 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), and 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 ESTs found throughout the whole subtracted cDNA library (10 clones). Ribosomal proteins and actin sequences were not very abundant (10 clones). Differences in select expression profiles were further verified by Northern blot (Fig. 2A).
Comparison of Hemocyte Gene Expression from Shrimp Surviving and Not Surviving a Vibrio Infection by rtPCR

The rtPCR efficiencies of selected ESTs varied between 1.89 and 1.98 (Fig. 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 parallel fall in transcript abundance at 12 h postinfection followed by a return to a level similar to that of the uninfected control (T−12) for the surviving shrimp at 24 h (T+24s) (Fig. 3B). However, hemocytes from nonsurviving shrimp (T+24ns) did not show a return to T−12 levels after 24 h. 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 hematopoietic genes domino and TGase appears different in that there is a constant relative reduction in transcript abundance at both 12 and 24 h postinfection for surviving and nonsurviving shrimp. Based on the fact that variation in relative expression, between both nonsurviving and surviving shrimp, was most dramatic at 24 h for three immune effector or putative effector EST transcripts (lysozyme, PEN3, and cryptdin-like), we chose to investigate sample variation only at the 24 h time point using individual samples (n = 5–6) to infer differences by statistical methods between surviving and nonsurviving groups for all five transcripts.

The second round of rtPCR analysis revealed significant differences in transcript abundance for the three immune effector transcripts (results expressed as transcript expression of surviving vs. nonsurviving shrimp collected at 24 h postinfection, relative to uninfected shrimp): 1) Litsty PEN3 (0.81 ± 0.15 vs. 0.32 ± 0.11, respectively; P < 0.05), 2) lysozyme (3.74 ± 1.01 vs. 1.01 ± 0.40, respectively, P < 0.01), and 3) cryptdin-like (1.42 ± 0.17 vs. 1.15 ± 0.05, respectively, P < 0.05) (Fig. 3C). No differences in relative expression were detected between surviving and nonsurviving shrimp at 24 h 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 that may be involved in a successful immune response of a penaeid shrimp to *V. penaeicida* infection using SSH. Knowledge of *Vibrio* infection kinetics allowed us to design an appropriate experimental infection protocol to investigate such an immune response using the subtraction of the hemocyte mRNAs from noninfected animals and infected animals that 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 analyzed. The efficacy of the subtraction was controlled in particular by...
the weak proportion of housekeeping or constitutively expressed genes we obtained, compared with 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; Ref. 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 presented similarity with a mouse cryptdin-related mRNA (32). In our experiments, this cryptdin-like transcript is differentially abundant between surviving and nonsurviving 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 upregulated in granular hemocytes during the systemic and proliferative immune response occurring about 48–96 h 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 with 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.

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**Fig. 3.** Real-time PCR (rPCR) analyses of transcripts identified by SSH from hemocytes of surviving and nonsurviving shrimp postinfection with *V. penaeicida*. A: rPCR forward and reverse primers used to determine transcript abundance of lysozyme, PEN3, cryptdin-like, domino, TGase, and EF-1α. Specific annealing temperature of each primer pair is noted, as well as the PCR efficiency, calculated by the equation $E = 10^{-1/\text{slope}}$ (34). B: relative expression of lysozyme, PEN3, cryptdin-like, domino, and TGase from pooled samples (15 shrimp per condition) of uninfected shrimp (T=12, open bars) and at two time points postinfection (12 and 24 h postinfection) from shrimp surviving infection (T=12s and T=24s, gray bars) and not surviving infection (T=12ns and T=24ns, solid black bars). Relative expression levels were normalized with EF-1α, and the values during the infection were calculated in reference to uninfected shrimp (relative expression = 1) according the $2^{-\Delta\Delta Ct}$ method corrected for efficiency (34). C: expression analysis of lysozyme, PEN3, cryptdin-like, domino, and TGase at the individual level. Results are means ± SE from five or six shrimp collected 24 h postinfection, which have survived infection (T=24s, solid black bars) or did not survive infection (T=24ns, gray bars). *Statistical difference between the two infected groups, $P < 0.05$ (Student’s t-test).
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 (~12 h) and was elevated in surviving shrimp at 96 h. 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 h postinfection between hemocytes from nonsurviving and surviving shrimp (Fig. 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.

TGase was found to be most redundant in the differential library and appeared to be elevated after 96 h postinfection, suggesting that these transcripts were not immediately elevated after infection (Table 2, Fig. 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 that 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 overrepresented in hemocytes from _L. stylirostris_ that had survived a _Vibrio_ infection, perhaps signifying a greater level of importance not previously attributed to this molecule (Fig. 2). The fact that TGase transcripts appear to be elevated in surviving shrimp at 96 h postinfection is notable and (Fig. 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 Ref. 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 upregulated 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 h postinfection, 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 appears elevated in hemocytes of surviving shrimp observed at 96 h postinfection, but clear differences in level of expression were not seen at 12 and 24 h 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 (Fig. 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 upregulation 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 antiviral response (35) and cell cycle regulation.

From the analyses of expression, it appears that transcripts identified by SSH are moderately expressed in noninfected animals and thus are present prior to infection. Furthermore, 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 that have been confirmed by Northern blot and rtPCR. The expression profile of genes encoding immune effectors such as _Litsy_ 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, _Litsy_ PEN3, and cryptdin-like at 24 h) in animals that 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. parahaemolyticus_ 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.

**ACKNOWLEDGMENTS**

We thank P. Cotty and O. Gontier for StyliBase project support.

**GRANTS**

This study was part of a collaborative project supported by the European Commission, DG XII, in the program International Cooperation with Developing Countries (INCO-DC), contract ICA4-CT-2001-10023 (IMUNAQUA). J. de Lorderil was supported by doctoral funding from the New Caledonian Territories and the IFREMER. M. G. Janech is supported by the National Science Foundation-International Research Fellowship Program. The work was also funded by the Centre National de la Recherche Scientifique, the IFREMER, and the University of Montpellier II.

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