CMLS, Cell. Mol. Life Sci. 61 (2004) 961–972
1420-682X/04/080961-12
DOI 10.1007/s00018-003-3441-9
© Birkhäuser Verlag, Basel, 2004

CMLS Cellular and Molecular Life Sciences

Research Article

Involvement of penaeidins in defense reactions of the shrimp *Litopenaeus stylirostris* to a pathogenic vibrio

M. Muñoz[a], F. Vandenbulcke[b], J. Garnier[a], Y. Gueguen[a], P. Bulet[c,‡], D. Saulnier[d] and E. Bachère[a,*]

[a] Ifremer/CNRS/Université de Montpellier 2, 'Défense et Résistance chez les Invertébrés Marins', CC 80, 2 Place Eugène Bataillon, 34095 Montpellier (France), Fax: +33 4 67 14 46 22, e-mail: ebachere@ifremer.fr
[b] Laboratoire de Neuroimmunologie des Annélides, CNRS 8017, Université des Sciences et Technologies de Lille (France)
[c] Institut de Biologie Moléculaire et Cellulaire, UPR 9022, CNRS, 'Réponse Immunitaire et Développement chez les Insectes', 15, rue René Descartes, 67084 Strasbourg Cedex (France)
[d] Ifremer, Ronce les Bains, BP 133, 17390 La Tremblade (France)

Received 25 November 2003; received after revision 8 January 2004; accepted 21 January 2004

Abstract. The present study reports for the first time the involvement of an antimicrobial peptide in the defense reactions of a shrimp infected by a pathogenic *Vibrio*, *Vibrio penaeicida*. New members of the penaeidin family were characterized in the shrimp *Litopenaeus stylirostris* by RT-PCR and RACE-PCR from hemocyte total RNAs, and by mass spectrometry detection and immunolocalization of mature peptides in shrimp hemocytes. In infected shrimps, bacteria and penaeidin distribution colocalized in the gills and the lymphoid organ that represented the main infected sites. Moreover, the shrimp immune response to infection involved massive hemocyte recruitment to infection sites where released penaeidin may participate in the isolation and elimination of the bacteria, We show that the ability of the shrimps to circumvent shrimp infections is closely related to a recovery phase based on the hematopoietic process.

Key words. Crustacea; antimicrobial peptide; immune response; *Vibrio* infection; hemocyte; agglutination; chemotaxis; hematopoiesis.

Infectious diseases represent one of the main limitations for sustainable development of shrimp aquaculture in all producing countries. Bacteria are etiological agents responsible for diseases, and Vibrionaceae represent the most important group of opportunistic pathogens of both larvae and juvenile shrimps [1–4]. In this context, when prevention of infectious diseases appears to be a priority for the shrimp culture industry, immunology can be considered as a target to establish strategies dealing with the management and control of disease in aquaculture [5].

Defense reactions in crustaceans rely on both cellular and humoral mechanisms. These include phagocytosis, encapsulation of foreign material [6], activation of the prophenoloxidase system (proPO system) and the clot-

* Corresponding author.
‡ Present address: Atheris Laboratories, CP 128, 1233 Bern, Geneva (Switzerland)

Access numbers from Swiss-Prot Data Base for penaeidins from *Litopenaeus vannamei* discussed in this study are: *Litvan* PEN2 (Pen-2): P81057, *Litvan* PEN3-1 (Pen-3a); P81058, *Litvan* PEN3-2 (Pen-3b): P81059 and *Litvan* PEN3-3 (Pen-3c): P81060. The nucleotide sequences reported in this paper have been submitted to GenBank with accession numbers *Litsty* PEN2: AY351655 and *Litsty* PEN3-1: AY351656.

ting process [7, 8], which have been already well studied. More recently, progress has been made in understanding the immune response in penaeid shrimps, with the characterization of antimicrobial peptides [9]. Among these, antifungal peptides derived from the C terminus of hemocyanin have been identified in the plasma of both *Litopenaeus vannamei* and *L. stylirostris* as the result of microbial stimulation [10]. Using the expressed sequence tag (EST) approach and a sequence homology search, various antimicrobial peptides have been identified in several shrimp species, namely peptides homologous to antilipopolysaccharide factor from horseshoe crab, 11.5-kDa antibacterial polypeptides identified in crab, and numerous sequences homologous to penaeidins [11]. To date, the penaeidins have been the most extensively studied peptides. This original family of antimicrobial peptides was initially characterized from the hemocytes of *L. vannamei* [12]. The penaeidins have been shown to be active, in vitro, mainly against Gram-positive bacteria and filamentous fungi [13]. They are synthesized and stored in the shrimp granular hemocytes, and partly released into the blood circulation in response to stimulation by injection of heat-killed microorganisms. Moreover, these peptides were shown to bind to the shrimp cuticle through their chitin-binding domain [14]. More recently, in vivo studies demonstrated that injection of heat-killed microorganisms induced important hemocyte reactions such as a massive migration to the site of injection and hemocyte lysis, which are followed by a proliferative recovery phase [15].

In the present study, we considered the defense reactions of the shrimp *L. stylirostris* to the pathogenic *Vibrio* strain, *Vibrio penaeicida* AM101 [16], which was associated in New Caledonia to a seasonal outbreak disease affecting predominantly juvenile shrimps [17, 18]. Surprisingly, early post-larvae developmental stages are less affected by these bacteria [19]. For further understanding of this bacterial disease in *L. stylirostris*, we proposed to analyze penaeidin gene expression and peptide distribution as tools to approach the shrimp immune response to *Vibrio* infections. First, two new members of the penaeidin family were characterized in the hemocytes of *L. stylirostris*. Then, we considered analyses of the relative localization of *Vibrio* and penaeidins in shrimps following experimental infections. This study highlighted that the ability of shrimps to circumvent *V. penaeicida* infection may rely on the isolation of the *Vibrio* by hemocytes for further elimination, but also on the activation of the hematopoietic process.

Materials and methods

Animals and immune challenge

Juvenile shrimp *L. stylirostris* (8–10 g) were obtained from the French Polynesia Ifremer laboratory. Experimental infection was performed as previously described [16] using *V. penaeicida* AM101, a highly virulent strain. Shrimps (20 individuals) were infected by immersion for 2 h in 10-l seawater tanks containing 1.5×10^5 colony-forming units (CFU) of bacteria/ml. Animals were carefully rinsed with clear seawater and transferred into 100-l tanks supplied with filtered (1 µm) and aerated seawater. Four replicates were performed for the infected group. Two tanks with infected animals were left untouched for the mortality determination, and the other two parallel tanks contained infected shrimps taken for the analyses. Non-infected animals were kept in a separate tank as a control. Mortalities were recorded twice a day over 5 days. Animals were sampled at 22 and 48 h after contact with *V. penaeicida* for in situ hybridization (ISH) and immunohistochemistry (IHC).

Hemolymph was collected in an equal volume of anticoagulant-modified Alsever solution [20] and the hemocytes were separated by centrifugation (800 g, 15 min). Supernatants were eliminated and the cell pellets were kept at 4 °C until use.

RT-PCR

Total RNAs were extracted from *L. stylirostris* hemocytes using the Trizol reagent kit (Gibco-BRL, Life Technologies). Following heat denaturation of RNA samples at 70 °C for 5 min, reverse transcription (RT) was performed using 1 µg RNA primed with 50 µg/µl oligo$(dT)_{15}$ in a 50-µl reaction volume containing 1 mM dNTPs, 1 unit/µl of RNasin (Promega), 100 µg/ml bovine serum albumin (BSA); New England BioLabs, and 2 units/µl M-MLV reverse transcriptase (New England BioLabs) in RT buffer. The PCR primers used were: sense 5′-GCCTCACCTGCAGAGACCGAC-3′ (PVJ4R sens from *Litvan* PEN3-1, -3-2, -3-3, and -2 conserved 5′UTR sequences; GenBank accession numbers, Y14926, Y14927, Y14928 and Y14925, respectively), and antisense 5′-CCAGGTTTCCATTGTCTTCTCC-3′ (PVJ5 Ranti; nucleotides 292–313 of 3′UTR sequence of penaeidin-2) (fig. 1). PCR conditions were as follows: 2 µl of hemocyte cDNA of *L. stylirostris*, 1 × PCR buffer (Promega), 200 µM of each dNTP, 0.2 µM of each primer, 2.5 U Taq DNA polymerase (Promega). The PCR profile was: 30 cycles of 95 °C denaturation for 1 min, 63 °C annealing for 1 min, and 72 °C extension for 1 min, followed by a final extension at 72 °C for 10 min. RT-PCR products were cloned into a pPCR vector (PCR-Script Amp Cloning Kit; Stratagene) and then sequenced with T7 and T3 primers. Sequence reactions were performed in both directions. A search for sequence homologies in SwissProt databases was done using BLASTX and by PSI- and PHI-BLASTP.

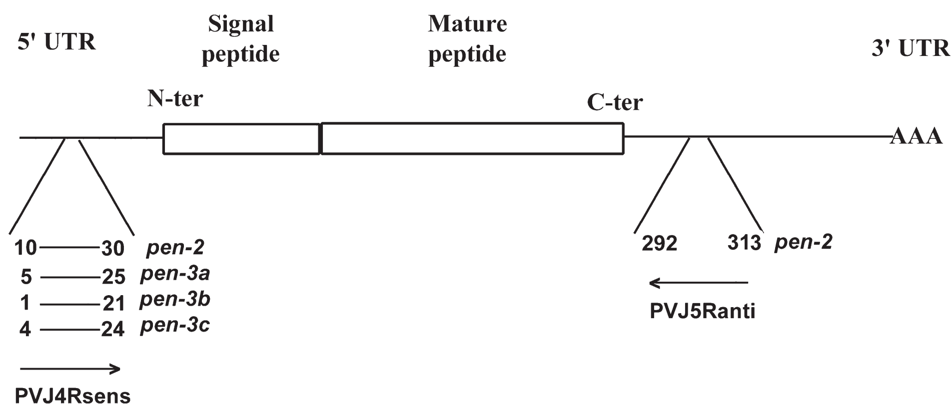

Figure 1. PCR strategy used to clone penaeidins in *L. stylirostris*. PCR primers were designed from the *L. vannamei* penaeidin cDNA sequences: one was based on conserved portions of the 5′-untranslated regions (PVJ4Rsens) of the *Litvan* PEN3-1, *Litvan* PEN3-2, *Litvan* PEN3-3, and *Litvan* PEN2 cDNAs, and the second was based on the 3′-UTR of *Litvan* PEN-2 (PVJ5Ranti).

Rapid amplification of cDNA ends

The full-length cDNA sequences of *L. stylirostris* penaeidins were obtained by rapid amplification of cDNA ends (RACE). First, for 3′-RACE, total RNA was reverse-transcribed as described above, followed by second-strand cDNA synthesis using PVJ4Rsens and oligo(dT)$_{15}$ primers. The PCR profile was: 95 °C denaturation for 5 min followed by 40 cycles of 95 °C denaturation for 1 min, 54 °C annealing for 1 min, and 72 °C extension for 1 min, followed by a final extension at 72 °C for 10 min. Amplification of the 5′ cDNA end was performed using a 5′-RACE PCR Kit (Boehringer Mannheim). Briefly, reverse transcription was performed using oligo(dT)$_{15}$ according to the manufacturer's procedures. The 5′-RACE products were obtained by PCR amplification using an oligo(dT)-anchor adapter primer (Boehringer Mannheim) and gene-specific primer PVJ5Ranti with the same PCR profile as above. The RACE products were cloned into the pCR2.1 TOPO vector using the TOPO TA cloning Kit (Invitrogen) and sequenced. Analyses of sequences were performed using the same softwares as described above.

Northern blot analyses

Hemocyte total RNAs (1 µg) were subjected to Northern blot analysis as previously described [14]. The *L. stylirostris* penaeidin cDNA probe was generated by PCR amplification on a 340-bp cDNA clone using primers as described above. A *Litopenaeus* sp. 18S rRNA-specific probe was prepared as described elsewhere [14]. The probes were ^{32}P-radiolabeled using the Ready-to-go DNA labeling kit (Amersham Pharmacia Biotech). Labeled probes were hybridized for 12 h to immobilized RNA in 50% (v/v) formamide, 5 × SSC, 8 × Denhardt's, 0.05 M sodium phosphate, pH 6.5, 0.1% SDS and 100 µg/ml denatured salmon sperm at 42 °C. The filters were washed twice in 2 × SSC, 0.1% SDS at room temperature and twice in 1 × SSC, 0.1% SDS first at room temperature and then at 65 °C, followed by autoradiography. After stripping, the membranes were hybridized under identical conditions with the ^{32}P-labeled 18S rRNA probe and further subjected to autoradiography. Resulting signals were quantified using the STORM system (Molecular Dynamics).

Matrix-assisted laser desorption/ionization-time of-flight mass spectrometry

To ascertain the presence, in *L. stylirostris,* of the mature forms of the penaeidins identified by the RT-PCR approach, mass spectrometry analysis was performed on hemocytes under the following conditions. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis was carried out on a Bruker BIFLEX III mass spectrometer in a positive linear mode with external calibration. Samples were prepared according to the sandwich method [22]. Briefly, 0.5 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA; Sigma) in acetone was placed on the probe tip. When dried, 0.5 µl of hemocyte sample was deposited and immediately covered with 0.5 µl of a saturated solution of HCCA in 50% acetonitrile. After drying under vacuum, the sample preparation was washed with 1.5 µl of 0.1% TFA and dried under vacuum before analysis.

In situ hybridization

Tissues from juvenile shrimps were prepared for histology as described previously [15]. Briefly, shrimp cephalothoraxes were fixed for 72 h in Davidson's AFA solution containing 22% formalin, 31.5% ethanol and 11.5% glacial acetic acid, pH 3.5–4. After tissue embedding in Paraplast, serial 8-µm sections were cut, mounted on poly-L-lysine-coated slides and stored at 4 °C until use. Digoxigenin (DIG)-UTP-labeled sense and antisense riboprobes were generated by in vitro transcription using a DIG-RNA labeling kit and T3 polymerase (Roche). DNA templates were produced by PCR from *Litsty-pen3*

cDNA using different primer couples, T3 and oligo(dT)$_{15}$, and T7 and PVJ4Rsense for sense and antisense probes, respectively, under the same conditions as described above for 3′-RACE PCR experiments. *V. penaeicida* probes were obtained by PCR from a pBluescript plasmid carrying a fragment of the *V. penaeicida* 16S rRNA gene, using the primer couples VecoF and T7, and VxhoR and T3 to produce sense or antisense probes, respectively. VecoF and VxhoR primers were designed as described elsewhere [16]. RNA probes were ethanol precipitated, reconstituted in DEPC water and kept at –20 °C until use. DIG-UTP probes were hybridized on tissue sections according to a previously developed protocol [15, 23]. As an additional control, sections were submitted to RNAase treatment prior to hybridization.

Immunohistochemistry

Immunodetection of penaeidins in shrimp tissues and hemocytes was performed as described elsewhere [15] using rabbit polyclonal antibodies specific to *Litvan* PEN3-1 [14]. Immunoreactivity controls were performed by pre-adsorbing the anti-penaeidin IgG overnight at 4 °C with pure penaeidin (10 µg of recombinant peptide [13] per microgram of purified IgG).

Protein extraction from hemocytes and Western blot analyses

Hemocyte extracts were prepared as previously described [12]. Briefly, the hemocyte homogenate was acidified to pH 4 by addition of 1 M HCl. After centrifugation (8000 g, 20 min), the supernatants were pre-purified by solid-phase extraction onto 35-cm^3 Sep-Pak C_{18} Vac cartridges (Waters Associates) equilibrated in acidified water (0.05 % trifluoroacetic acid). After washing with acidified water, three stepwise elutions were performed with 5, 40, and 80 % acetonitrile in acidified water.

The 40 % acetonitrile fractions were lyophilized, reconstituted in MilliQ water, and incubated in a 1:1 mixture of Laemmli buffer (25 mM Tris base, 0.19 M glycine; 0.1 % SDS) and 3 × denaturing sample buffer (170 mM Tris, pH 8.8, 6 % SDS, 21 % glycerol, 150 mM dithiothreitol) for 5 min at 65 °C. Denatured samples were analyzed by Tris-Tricine SDS-polyacrylamide gel electrophoresis (Tris-Tricine SDS-PAGE) containing 16.5 % acrylamide according to a standard protocol [21]. After migration, proteins were transferred (30 min, 10 V) to 0.2-µm nitrocellulose membranes (Bio-Rad) in a semidry electrophoretic transfer cell (Trans-blot SD; Bio-Rad Laboratories). The membranes fixed in 2.5 % glutaraldehyde and washed in PBS were then incubated with 3 µg/ml rabbit anti-penaeidin IgGs for 2 h at room temperature. Incubation of anti-penaeidin IgGs pre-adsorbed overnight at 4 °C with purified recombinant penaeidin was used as a control. After extensive washes in PBS containing 0.1 % Tween 20 at pH 7.4 (PBS-T), the membranes were incubated with a 1:50,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgGs (Jackson Immunoresearch). Antibodies were diluted in PBS-T supplemented with 0.5 % skimmed milk. The membranes were then developed using SigmaFast nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate tablets as chromogenic substrate.

In vitro bacteria agglutination assays

The marine bacteria strains *V. alginolyticus*, *V. penaeicida*, and *V. vulnificus* were grown in saline peptone water (1.5 % peptone, 1.5 % NaCl, w/v, pH 7.2) at 25 °C. *Escherichia coli* 363 and *Micrococcus luteus* were grown in Luria-Bertani broth (Bactotryptone 1 %, yeast extract 0.5 %, NaCl 1 % w/v, pH 7.5) at 37 °C and 30 °C, respectively. The bacterial suspensions were washed twice by centrifugation (1000 g for 10 min) in PBS, and 50 µl of each bacterial strain (10^8 bacteria/ml) was incubated in triplicate in a microtiter plate with purified penaeidin-3 [20] at 2, 5, and 10 µM for *Vibrio spp*. and at 1 and 2 µM (concentrations below the MIC values, 1.25 – 2.5 µM and 5 – 10 µM) for *M. luteus* and *E. coli*, respectively. In controls, the peptide was substituted by PBS. Incubations were performed at room temperature for the *Vibrio* strains, and at 37 °C for *M. luteus* and *E. coli*. Agglutination was examined after 2-h incubation with an Olympus inverted microscope.

Results

Cloning and sequencing of penaeidin cDNAs in *L. stylirostris*

A PCR strategy was used to identify and clone putative penaeidin sequences in *L. stylirostris*. PCR primers were designed from the *L. vannamei* penaeidin cDNA sequences: one was based on conserved portions of the 5′UTR of *Litvan* PEN3-1, *Litvan* PEN3-2, *Litvan* PEN3-3, and *Litvan* PEN2 cDNAs, and the second was based on the 3′UTR of *Litvan* PEN2 (fig. 1). Two PCR products 300 and 350 bp in length were obtained from *L. stylirostris* cDNA, cloned into pPCR script vector and three different clones were sequenced for each of them. They were shown to present sequence homologies with the penaeidins characterized in *L. vannamei*. cDNA sequences were then obtained by RACE (see Materials and methods). A first 321-bp cDNA sequence encompassed a 225-bp open reading frame (ORF) flanked by 72-bp 5′UTR codes for a 75-amino-acid sequence. The deduced sequence shares 86 % identity and 88 % homology with the precursor form of penaeidin-2 from *L. vannamei* and was named *Litsty* PEN2 (fig. 2A). The second 368-bp cDNA sequence presents a 261-bp ORF encoding an 87-amino-acid peptide. The deduced peptide sequence shares 64 % identity and 74 % homology scores with pe-

```
A
1   ccgctgggctctccctcgagcctcacctgcagacaccgacgctccgagcccgggttccct
61  cctccgtccgccatgcgcctcgtggtctgcctggtcttcttggcctccttcgccctggtc
                M  R  L  V  V  C  L  V  F  L  A  S  F  A  L  V
121 tgccaaggcgaagcgtacaggggcggttacacaggcccgatacccaggccaccaccctat
     C  Q  G  E  A  Y  R  G  G  Y  T  G  P  I  P  R  P  P  P  Y
181 ggaagaccaccgttaggacctgtttgcaaccattgctacagactcgccttcccagatgcc
     G  R  P  P  L  G  P  V  C  N  H  C  Y  R  L  A  F  P  D  A
241 cgcaattgctgcagcaggttcggacgttgttgtcacttagtaaaaggataaagaaattga
     R  N  C  C  S  R  F  G  R  C  C  H  L  V  K  G  *
301 tggagaagacaatggaaacct

B
1 ctggccactcggcgcttggctctccctcgagcctcacctgcagagaccgacgctccgagccc
63  gggttccctcctccgtccgccatgcgcctcgtggtctgcctggtcttcttggcctccttc
                         M  R  L  V  V  C  L  V  F  L  A  S  F
123 gccctggtctgccaaggccaaggctacaagggcggttacacaggcccggtagtcaggccc
     A  L  V  C  Q  G  Q  G  Y  K  G  G  Y  T  G  P  V  V  R  P
183 ttcgtgagaccgataggcaggcccttcgtgacaccgataggcaggcctgttgtttcgggc
     F  V  R  P  I  G  R  P  F  V  T  P  I  G  R  P  V  V  S  G
243 aacgtgtgccctctatcgtgccggggcattaccaccttacaagcgcgttcttgctgcagc
     N  V  C  P  L  S  C  R  G  I  T  T  L  Q  A  R  S  C  C  S
303 cgcttggggcgctgctgtcgcgaggccaaggggtactccggctgatggagaagacaatgg
     R  L  G  R  C  C  R  E  A  K  G  Y  S  G  *
363 aaacct

Litsty-Pen3     MRLVVCLVFLASFALVCQG(Q)GYKGGYTGPVVRPFVRPIGRPFVTPIGRPVVSGNVCPLSCRGITTLQARSCCSRLGRCCREAKGYS(G)
Litvan-Pen3-a   MRLVVCLVFLASFALVCQG(Q)VYKGGYTRPIPRP--PPFVRPLPGGPIGP---YNGCPVSCRGISFSQARSCCSRLGRCCHVGKGYS(a)
Litvan-Pen3-b   MRLVVCLVFLASFALVCQG(Q)VYKGGYTRPVPRP--PPFVRPLPGGPIGP---YNGCPVSCRGISFSQARSCCSRLGRCCHVGKGYS(G)
Litvan-Pen3-c   MRLVVCLVFLASFALVCQG(Q)VYKGGYTRPIPRP---PFVRPVPGGPIGP---YNGCPVSCRGISFSQARSCCSRLGRCCHVGKGYS(G)

Litsty-Pen-2    MRLVVCLVFLASFALVCQG--EAYRGGYTGPIPRP--PPYGRPPLGP---------VCNH-CYRLAFPDARNCCSRFGRCCHLVKG
Litvan-Pen-2    MRLVVCLVFLASFALVCQG--EAYRGGYTGPIPRP--PPIGRPPFRP---------VCNA-CYRLSVSDARNCCIKFGSCCHLVK(a)
C                                       **** *  **   *  **                  *   *      ** **   * **    *
```

Figure 2. *Litsty* PEN2 (*A*) and *Litsty* PEN3-1 (*B*) cDNA sequences and deduced amino acid sequences cloned from *L. stylirostris* hemocytes and alignment with penaeidins from *L. vannamei* (*C*). (*A*, *B*) Nucleotides in boldface indicate the position of the PCR primers (PVJ4Rsens and PVJ5Ranti) used to clone *L. stylirostris* penaeidins. The double-headed arrow indicates the putative cleavage site by a signal peptidase. An asterisk indicates the stop codon. (*C*) A solid underline indicates the putative signal sequence; dashes were created to optimize the alignment. Asterisks indicate conserved residues in boldface. Post-translational modifications are in parentheses: (Q) for pyroglutamic acid resulting from cyclization of glutamine residue; [a] for C-terminal amidation. Arrows indicate the position of predicted signal peptides by signal peptidase.

naeidin-3 from *L. vannamei* and was consequently named *Litsty* PEN3-1 (fig. 2B).

Detection of penaeidins in hemocytes of *L. stylirostris* by MALDI-TOF MS

To identify the putative mature forms of *L. stylirostris* penaeidins, hemocytes were analyzed by MALDI-TOF MS. MS analysis revealed the presence of several molecular ions at masses corresponding to the predicted molecular masses of the mature forms of *Litsty* PEN2 and *Litsty* PEN3-1 (fig. 3A). The predominant single charged ion at 7164.26 in m/z (average molecular mass) and the double-charged ion at 3584.81 in m/z corresponded approximately to the predicted calculated molecular masse (MassLynx v3.5) of *Litsty* PEN3-1 ($[M+H]^+$ at 7164.46 and $[M+2H]^{2+}$ at 3582.73), assuming that *Litsty* PEN3-1 has a pyroglutamic acid as an N terminus, an amidated C terminus, and the six cysteine residues form three disulfide bridges. Moreover, the mass spectrum revealed the presence of a single charged ion at 5617.01 in m/z and a doubly charged ion at 2809.93 in m/z corresponding to the predicted calculated molecular mass of *Litsty* PEN2 with a predicted C-terminally amidated lysine residue ($[M+H]^+$at 5614.75 in m/z and $[M+2H]^{2+}$ at 2807.87 in m/z).

Detection of penaeidins in hemocytes of *L. stylirostris* by Western blot analysis

To confirm the identity of these molecules, an acidic extract of hemocytes was analyzed by Western blot using a rabbit antibody directed against *Litvan* PEN3-1 [14]. No peptide ranging around 5.6 kDa and corresponding to *Litsty* PEN2 was detected by the antibody. A clear band, in the size range of penaeidin-3 and consistent with the measured molecular weight of *Litsty* PEN3-1, was detected with anti-penaeidin IgG (fig. 3B). This cross-immunoreactivity allowed us to use this antibody for further studies on peptide localization and distribution in different tissues of *L. stylirostris*.

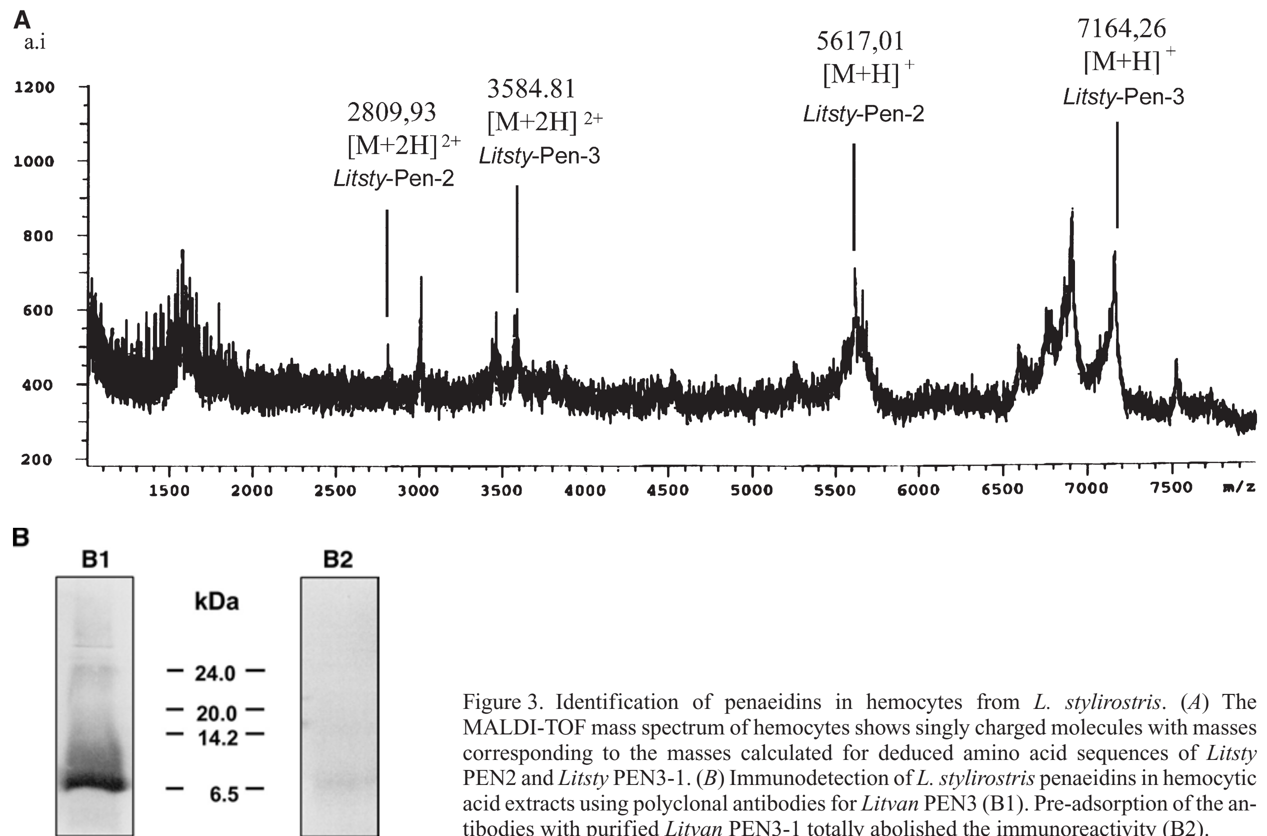

Figure 3. Identification of penaeidins in hemocytes from *L. stylirostris*. (*A*) The MALDI-TOF mass spectrum of hemocytes shows singly charged molecules with masses corresponding to the masses calculated for deduced amino acid sequences of *Litsty* PEN2 and *Litsty* PEN3-1. (*B*) Immunodetection of *L. stylirostris* penaeidins in hemocytic acid extracts using polyclonal antibodies for *Litvan* PEN3 (B1). Pre-adsorption of the antibodies with purified *Litvan* PEN3-1 totally abolished the immunoreactivity (B2).

Detection of penaeidin transcripts in hemocytes of *L. stylirostris*

Northern blot analyses on hemocyte total RNAs (1 µg) using comparative *Litsty* PEN2 or *Litsty* PEN3-1 coding region sequence probes showed positive signals but did not allow us to distinguish the corresponding transcripts. The probes hybridized to mRNAs of approximately 850 nucleotides. As the hybridization intensities obtained with *Litsty* PEN3-1 cDNA probes were very strong, we assumed that *Litsty* PEN3-1 was the most abundantly expressed and it was studied further (fig. 4A). In addition, Northern blot analyses on total RNAs (1 µg) from hemocytes collected from eight individual shrimps revealed that *Litsty* PEN3-1 is constitutively expressed in all tested shrimps (fig. 4B).

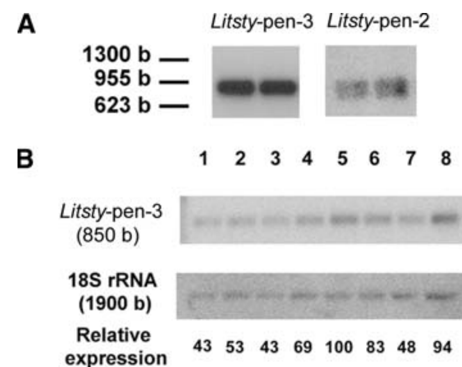

Figure 4. Expression of penaeidin mRNAs in *L. stylirostris* hemocytes. (*A*) Northern blot hybridization of penaeidin mRNA in shrimp hemocytes. One microgram per lane of hemocyte total RNA was fractionated by denaturing electrophoresis in a 1% formaldehyde gel. The RNA was probed with *Litsty* PEN2 and *Litsty* PEN3-1 coding-sequence probes. (*B*) One microgram of hemocyte total RNAs from individual shrimps was used and hybridized successively with radiolabeled *Litsty* PEN3-1 probe and with an 18S rRNA probe as control. Signals were quantified by the STORM system and the ratio of penaeidin/18S rRNA calculated.

Localization of *V. penaeicida* in infected shrimps

Following *V. penaeicida* infection by immersion, the first shrimp mortalities were observed at about 22 h and continued until 48 h. After 48 h, no massive death of shrimps was observed. The percentage of cumulative mortalities after 5 days was of 65% and 60% of shrimps in both infected tanks and the cumulative mortality observed in the non-infected tank was only 5% (table 1). Similar mortality kinetics were observed in two other batches of infected shrimps (data not shown). In tissues from three infected shrimps, *Vibrio* was detected by ISH using a DIG-labeled 16S RNA antisense riboprobe and alkaline phosphatase detection. No signal was observed on tissue sections from non-infected animals (data not shown). In contrast, at 22 h post-infection, hybridization signals were seen mainly in the gills (fig. 5A), while after 48 h,

Table 1. Record of shrimp mortalities after infection with *V. penaeicida*.

Time after infection (h)	Infected tank	Infected tank	Non infected
0	0	0	0
22	2	3	0
30	4	4	0
47	7	4	0
54	0	0	1
71	0	1	0
78–126	0	0	0
Dead shrimps	13	12	1
Surving shrimps	7	8	20
Percentage of mortalities	65	60	5

The procedures of infection were as described in Material and methods. The data only consider the record of mortalities observed in control tanks not harvested for histological analysis.

V. penaeicida was clearly observed not only in gills but also in the lymphoid organ (fig. 5B, inset C), the heart, and muscle. The specificity of the signal was confirmed by hybridization with sense 16S RNA riboprobe (fig. 5D, E).

Penaeidin expression and peptide distribution

To follow penaeidin expression in response to the *Vibrio* infection, penaeidin distribution was studied by both ISH and IHC. For ISH analyses, a plasmid containing full-length *Litsty* PEN3-1 cDNA was used to generate sense and antisense riboprobes. In non-infected shrimps, *Litsty* PEN3-1 mRNA was detected in hemocytes infiltrating most tissues or located in blood vessels irrigating tissues (fig. 6A). A hybridization signal was observed neither with the sense riboprobe (fig. 6B) nor after RNAse pretreatment of sections (data not shown). In parallel, immunodetection using rabbit polyclonal IgG directed against *Litvan* PEN3-1 revealed the presence of penaeidins in hemocytes located in most of the tissues and vessels (fig. 6C). In contrast, no immunostaining was observed when anti-penaeidin antibodies were pre-incubated with purified penaeidin (fig. 6D), confirming the specificity of the peptide detection.

In the context of *Vibrio* infection, striking reactivity was seen at 22 h post-infection both at the mRNA and peptide levels in gills where *Vibrio* was also detected as demonstrated above. Increased numbers of penaeidin-expressing hemocytes were observed in gills, compared to non-infected animals (fig. 7A–C). Simultaneously, on adjacent cuts, strong penaeidin immunostaining was also seen throughout this tissue, showing the release of the peptide

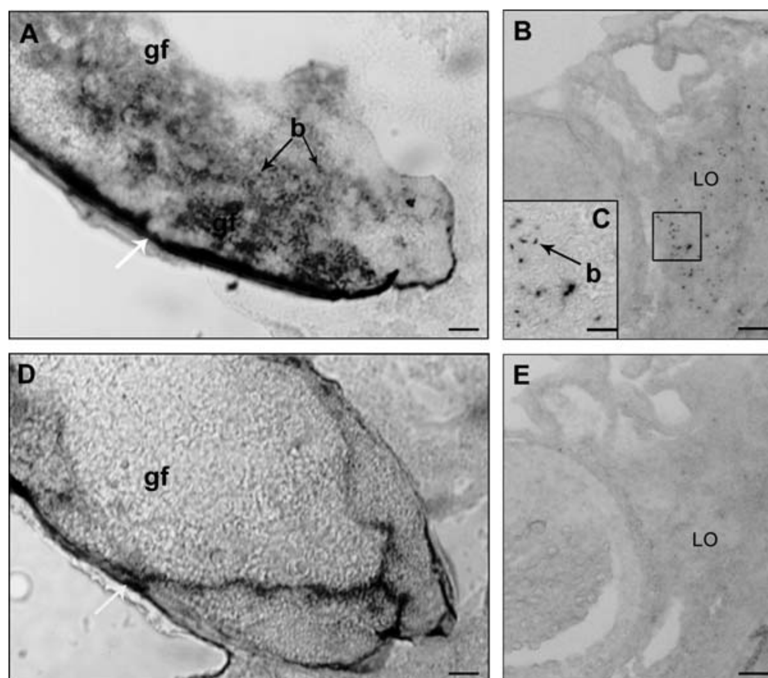

Figure 5. Detection of *V. penaeicida* by in situ hybridization in shrimp tissues infected by immersion challenge. Paraffin-embedded sections of *L. stylirostris* shrimps infected with *V. penaeicida* were hybridized with *Vibrio* 16S rRNA DIG-UTP antisense or sense probes, then revealed using alkaline phosphatase-conjugated antibodies. Relatively intense labeling was detected in gills (*A*) and the lymphoid organ (*B*, *C*) using the antisense probe. Hybridization using the 16S rRNA sense riboprobe did not present labeling in gills (*D*) or the lymphoid organ (*E*), confirming the specificity of the signal. Note that the cuticle is stained by the chromagen used to reveal the phosphatase-conjugated antibodies (white arrows). Abbreviations: b, bacteria; LO, lymphoid organ; gf: gill filament. Bars, *A*, *C*, *D*: 5 µm; *B*, *E*: 20 µm.

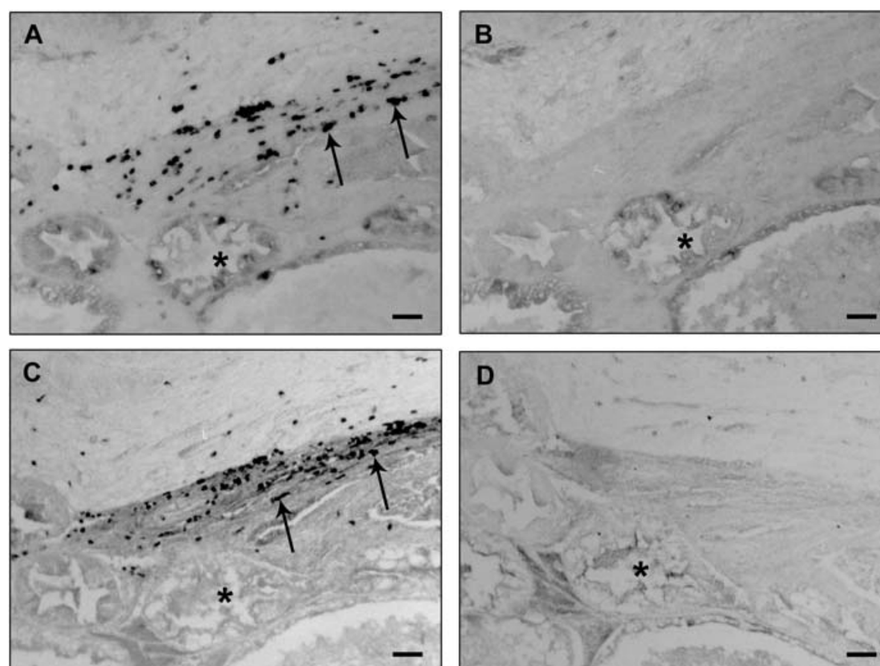

Figure 6. Detection of *L. stylirostris* penaeidin mRNAs and peptides in non-infected shrimps by ISH and IHC, respectively. Adjacent paraffin-embedded sections of *L. stylirostris* were hybridized with *Litsty* PEN3-1 DIG-UTP antisense (*A*) or sense (*B*) probes. Similar sections were immune stained for penaeidin (*C*, *D*). ISH using the antisense probe and immunohistological analyses show positive signals only in hemocytes present in tissues (*A*, *C*). Adjacent control sections were totally devoid of labeling (*B*, *D*). Arrows, hemocytes; * blood vessels. Bars, 50 µm.

(fig. 7D). In parts of the gills distant from *Vibrio* localization sites, a few hemocytes expressing penaeidin mRNA (fig. 7E) but immunonegative for penaeidins (fig. 7F) were observed.

At 48 h post-infection, the number of hemocytes in most tissues, both expressing and storing penaeidins, appeared to be similar to that observed in non-infected shrimps (fig. 7G, H). However, scattered nodular bodies reactive to anti-penaeidin antibody were observed in gill sinuses (fig. 8A, inset B) and lymphoid organ (fig. 8C).

Penaeidin expression in hematopoietic tissue

In non-infected shrimps, few penaeidin-expressing cells were seen in hematopoietic tissue (fig. 9A). At 22 h after bacterial infection, strong hybridization signals of the *Litsty* PEN3-1 antisense probe were observed in this tissue (fig. 9B). As a control, ISH analyses using a sense probe on adjacent sections of infected shrimps (fig. 9D) confirmed the specificity of the reaction. At 48 h, penaeidin mRNA contents appeared to be similar to that observed in non-infected shrimps (fig. 9C). In contrast, no modification in the pattern of penaeidin immunodetection was seen in the hematopoietic tissue whatever the infection time. Penaeidin labeling was restricted to a few hemocytes infiltrating the tissue (fig. 9E–G).

In vitro agglutinating activity of penaeidin-3 against *Vibrio* bacteria

In vitro agglutination assay was performed against different bacterial strains using recombinant *Litvan*-Pen3 from *L. vannamei* [13]. *Litvan* PEN3-1 at 5 µM was found to agglutinate *V. alginolyticus, V. vulnificus,* and *V. penaeicida*. No agglutination of *M. luteus* or *E. coli*, used as test organisms for Gram-positive and Gram-negative bacteria, respectively, was observed at the peptide concentration tested.

Discussion

Penaeidin antimicrobial peptides were initially isolated from hemocytes of *L. vannamei* [12] and their presence in *L. stylirostris* was suggested by Northern blot analyses using a *Litvan* PEN3-1 probe (data not shown). Subsequently, we used a PCR approach to clone and characterize penaeidin sequences in the *L. stylirostris* shrimp species. Comparison of full cDNA sequences of the different penaeidins characterized in *L. vannamei* revealed conserved regions in 5′UTR and 3′UTR regions, which allowed us to identify two new members of the penaeidin family in *L. stylirostris*. The two deduced peptide sequences of the cloned PCR products revealed a high level of homology with penaeidin-2 and -3 from *L. vannamei* (see fig. 2C) and were named *Litsty* PEN2 and

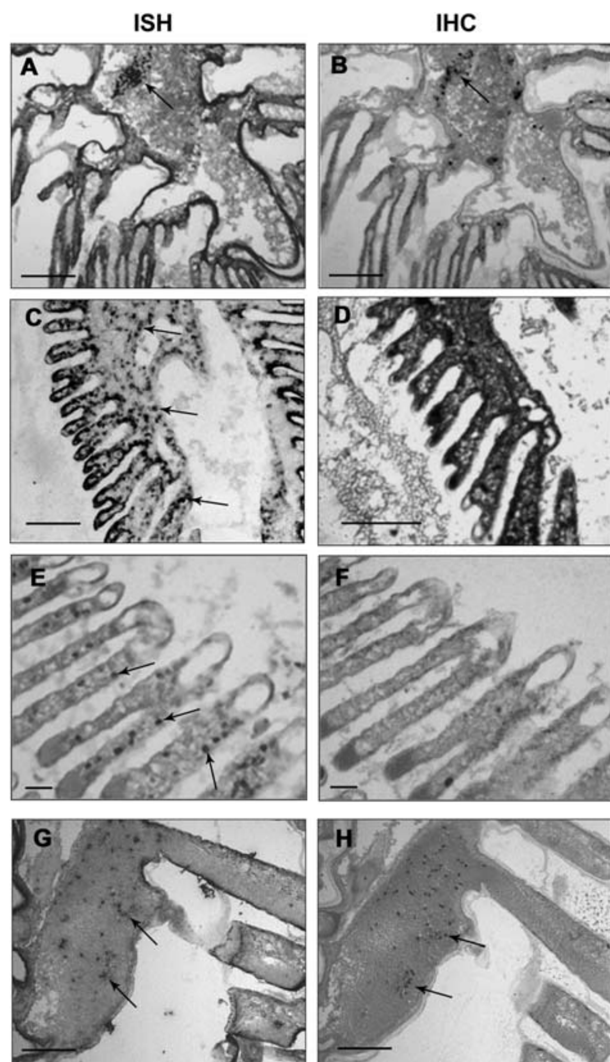

Figure 7. Analyses of penaeidin expression in *L. stylirostris* hemocytes after *V. penaeicida* infection by ISH and IHO. Paraffin-embedded sections of infected and non-infected *L. stylirostris* shrimps were hybridized with *Litsty* PEN3-1 DIG-UTP antisense probes (*A*, *C*, *E*, *G*). Penaeidin immunostaining was performed on adjacent sections using anti-penaeidin antibodies, a 1-nm gold particle-labeled secondary antibody and revealed by silver amplification. (*B*, *D*, *F*, *H*). A hemocytes. Arrows, Bars, *A*, *B*: 80 µm; *C*, *D*, *G*, *H*: 160 µm; *E*, *F*: 40 µm. (*A*, *B*) Non-infected shrimps. (*C*) In parts of the gills where *Vibrio* was detected at 22 h after infection, great numbers of hemocytes expressing penaeidins were concentrated. (*D*) In adjacent cut sections strong immunoreactivity distributed throughout the tissue was observed. (*E*, *F*) In other parts of gill tissue where *vibrios* were not detected, penaeidin expressing hemocytes were observed (*E*) but most did not contain penaeidins (*F*). (*G*, *H*) In gill vessels 48 h after infection, the number of hemocytes containing penaeidin mRNAs (*G*) became equivalent to the number of hemocytes storing penaeidins (*H*).

Litsty PEN3-1, respectively. The two translation products are composed of a putative 19-residue signal sequence, with 100% homology with those of *L. vannamei* penaeidins, followed by a mature peptide of 55 or 67 residues, respectively. The peptide sequences contain the pattern

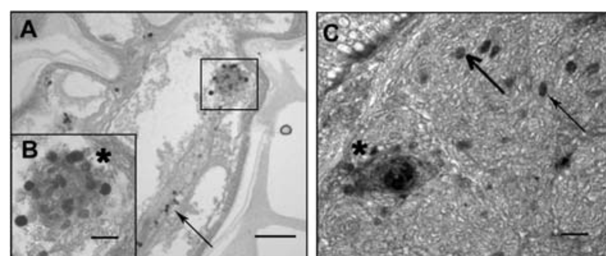

Figure 8. Immunohistological detection of penaeidin in *L. stylirostris* gills and epigastric nodules at 48 h after infection. Numerous hemocytes containing penaeidin were observed in nodular structures located in vessels of gills (*A*, *B*). Nodular structures containing immunoreactive cells appeared also in epigastric hematopoietic nodules (*C*). Arrows, hemocytes; *, nodular structure. Bars, *A*: 200 µm; *B*, *C*: 20 µm.

of the six cysteines in their C-terminal segment and of the proline-rich N-terminal domain. To date, this unique chimeric structure is characteristic of the penaeidin family [9, 12]. The primary structure of these two novel forms of penaeidin in *L. stylirostris* hemocytes was confirmed by direct analysis of an acidic extract of hemocytes by MALDI-TOF MS. In fact, two molecular masses at 5617 Da and 7167 Da were detected in the extract. These two masses correspond to the calculated molecular masses of the deduced sequences of *Litsty* PEN2 and *Litsty* PEN3-1, respectively. These analyses not only demonstrated the presence of the penaeidin peptides in hemocytes but also confirmed the post-translational modifications already shown in *L. vannamei* penaeidins, i.e., a C-terminal amidation of the peptides, an N-terminal blockage with pyroglutamic acid of the penaeidin-3 peptides [12], and the bridging of the six cysteine residues.

Like its homologue in *L. vannamei*, *Litsty* PEN2 displays two additional amino acids (Glu-Ala) in its C terminus and two predicted cleavage sites by signal peptidase were also obtained according to SignalP IV.1 software [24]. Moreover, *Litsty* PEN2 also contains in the N-terminal domain, eight proline residues, some of them involved in the conserved motif PIPRP common to *Litvan* PEN2 and *Litvan* PEN3 [9] and present in other antimicrobial peptides such as bactenecin-7 from cattle (three times, P19661) and sheep (only once, P50415) neutrophils [25]. *Litsty* PEN3-1, even if it contains many proline residues, differs from all the other known penaeidins by the absence of this PIPRP motif in the N-terminal proline-rich region. Like penaeidins from *L. vannamei, Litsty* PEN3-1 contains in its C-terminal domain the chitin-binding motif identified in plant lectins and various antifungal peptides [26]. Additionally, whereas *Litvan* PEN3 isoforms are composed of 62 amino acid residues, *Litsty* PEN3-1 is longer (67 residues) due to the presence of additional residues in the medial sequence linking the two conserved regions of the molecule [26].

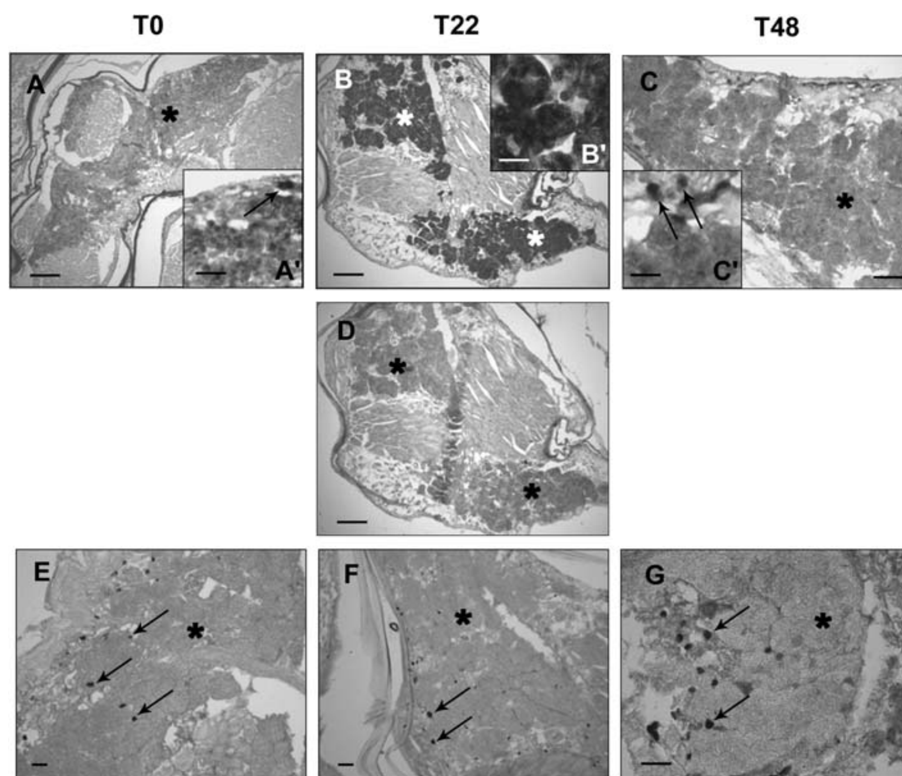

Figure 9. ISH and immunohistological analysis of penaeidin expression in hematopoietic tissues of *L. stylirostris* after *V. penaeicida* infection. Paraffin-embedded sections of non-infected and infected shrimps were hybridized with *Litsty* PEN3-1 DIG-UTP antisense (*A–C*) and sense (*D*) probes. Immune detection of penaeidins was performed using anti-penaeidin antibodies revealed by silver amplification (*E–G*). In non-infected shrimps, no hybridization signal was seen within hematopoietic tissue (*A*), but penaeidin-expressing hemocytes could be observed in the periphery of hematopoietic tissue (*A*). Twenty-two hours after infection, a strong hybridization signal was detected with *Litsty* PEN3-1 antisense probe in hematopoietic tissues (*B*, white stars; *B′*) but not with the sense probe confirming the specificity of the reaction (*D*). Fourty-eight hours after infection, the same hybridization pattern as observed in non-infected shrimps was seen (*C*) with positive hemocytes (*C′*). Penaeidin immunostaining was seen in some infiltrating hemocytes in hematopoietic tissues of non-infected (*E*), 22-h-infected (*F*), and 48-h-infected (*G*) shrimps. Arrows, hemocytes; *, hematopoietic tissues. Bars, *A–D*: 200 µm; *A′*, *B′*, *C′*: 20 µm; *E–G*: 40 µm.

In previous work, analyses of penaeidin expression and distribution following injection of heat-killed microorganisms [14, 15] provided new insights into the immune responses of shrimp to a microbial challenge, confirming the importance of the hemocytic response. In the present paper, we considered the shrimp defense responses following an infection with the pathogenic strain *V. penaeicida* [17]. Numerous *Vibrio* species have been reported as causal agents of diseases in penaeid species [1–4]. However, most *Vibrio* species are now widely accepted as being opportunistic and may cause severe mortalities in larvae or juveniles, which suffer from stress or any deficiency. *V. penaeicida* disease is unique since this bacteria has been shown to be predominantly pathogenic for juvenile and adult shrimps, whereas post-larvae stages remain unaffected [16, 27]. Indeed, vibrioses are mainly known to affect shrimp larvae and post-larvae. Since, to date, nothing is known about the immune capability of shrimp at early developmental stages, the host/pathogen model juvenile *L. stylirostris/V. penaeicida* appears particularly well adapted to study the shrimp immune response to a true infection. Events of infection remission are observed among the shrimps and understanding this phenomenon will be important for further development of prophylactic measures based on shrimp health monitoring [5].

Upon experimental *V. penaeicida* infection, shrimp mortalities appear from 22 to 48 h post-infection. Therefore, these periods were selected to detect and localize both *Litsty* PEN3-1 and pathogens within the shrimp tissues. This is particularly useful to study the penetration route of *V. penaeicida* in the shrimp. We saw by ISH that *Vibrio* is predominantly located in the shrimp gills where, at the same time, penaeidin detection was mainly observed, i. e., penaeidin-expressing hemocytes and free penaeidin in the tissues. Later, *Vibrio* was also detected in more internal tissues, especially the lymphoid organ. The localization of *Vibrio* in these tissues has already been reported in *Penaeus japonicus* naturally infected by *V. penaeicida* [28] and more recently in *P. monodon* upon an immersion challenge with *V. vulnificus* [29]. Accumulation of bacteria may indicate that gills and the lymphoid organ are natural routes for either bacteria entry or for

elimination. They also may be target sites for infection. Several studies based on bacteria injection have shown the gills to be involved in the clearance and elimination of the microorganisms [30, 31]. The massive accumulation of hemocytes expressing penaeidins observed in gills at 22 h, with their concomitant decrease in the other shrimp tissues illustrated significant hemocyte recruitment and a massive release of peptides to the infection sites. This accumulation appears to be concomitant with the triggering of a proliferative process. Indeed, a striking penaeidin mRNA content was observed in hematopoietic tissues, whereas no peptide immunoreactivity was seen in this tissue at the same time. This suggests intense hematopoietic activity and production of cells with a high level of penaeidin expression, as a consequence of *Vibrio* infection. Young hemocytes with high transcriptional activity would be released into the hemolymph where they maturate, i.e., translate and store penaeidins. According to the kinetics of mortality for this disease, the shrimps analyzed at 48 h after the immersion challenge, were those that survived the infection. We can assume they developed an efficacious response against *V. penaeicida*. At that time, the proportion of hemocytes expressing and storing mature penaeidins observed in the shrimp tissues appeared to be similar to that observed in non-infected animals. This strongly suggests possible restoration to apparently homeostatic conditions resulting from hematopoiesis. Proliferative processes may provide a systemic response, contributing to rapidly replace the hemocytes lysed in response to the microbial infection. This is in agreement with previous studies that have demonstrated an intense penaeidin release resulting from the lysis of granular hemocytes [14]. However, in the case of *V. penaeicida* infection, we cannot exclude the possibility that the hemocytes have been massively lysed due to the pathogenic effect of *Vibrio* extracellular products (ECPs) or toxins. Proteases have been suggested to be important virulent factors, causing tissue damage in the host and thus contributing to the bacterial entry [32]. ECPs and toxic cysteine proteases from *V. harveyi* were shown to impair shrimp homeostasis [33]. For *V. penaeicida*, an ECP toxic effect was suggested by in vivo injection in *L. stylirostris*, since cytopathic effects were shown in vitro on hemocytes and ovarian primary cultured cells [19]. Such toxic effects would result in the massive release of penaeidins observed in the present study. However, according to our observations, such putative *Vibrio* virulence factors would not impair the hemocyte proliferative activity of the shrimps.

At 48 h post-infection, numerous nodular bodies containing penaeidins were observed within gill filaments and the lymphoid organ. These are thought to be related to the isolation and elimination of the pathogens, participating in the successful circumvention of the disease. The formation of aggregates of hemocytes and bacteria in gills has been described in shrimps [14] and crabs [30] following an injection of bacteria. In crabs, these hemocytic clumps have been shown to display antibacterial activity, and microbial death was associated with an encapsulation phenomenon [31]. In a previous study, opsonic activity against *V. alginolyticus* was shown for penaeidin-3, which may contribute to phagocytosis and elimination of *Vibrio* [15]. Additionally, penaeidins would be involved in bacteria agglutination and encapsulation processes. To investigate such properties of penaeidins, we have shown that purified peptides are able, in vitro, to agglutinate *Vibrio* strains. Other bacteria such as the Gram-positive *M. luteus* or the Gram-negative *E. coli* are not agglutinated but are killed after incubation with penaeidins. Shrimp may have developed with penaeidins a defense mechanism which can discriminate between bacterial species. Foreign microorganisms such as Gram-positive bacteria or filamentous fungi appeared to be sensitive to the penaeidins [13]. Vibrionacea, which generally belong to the shrimp natural flora, would be agglutinated. When *Vibrio* strains reach a high concentration in shrimp, penaeidins may contribute to their elimination by opsonization and phagocytosis, as well as by agglutination and encapsulation. High penaeidin concentrations are reached in the nodules where the *Vibrio* can be consequently killed.

Our findings indicate that gills are the preferential route for entry of *Vibrio*. We can also speculate that the ability of shrimps to surmount an infection and restore homeostasis may depend on their physiological ability to trigger hematopoiesis. This is in agreement with previous studies on *L. stylisrostris* challenged with *V. penaeicida*, where shrimp survival was correlated with a higher number of circulating hemocytes, according to the molting cycle [34]. A more thorough understanding of penaeid shrimp immunity will require considering the molecular mechanisms underlying the activation of hematopoiesis, a process that may contribute to the ability of the shrimps to surmount infections. In the same way, progress may be achieved by studying host-pathogen interactions and the effect of physiological or environmental parameters that can impair the defense capability of the shrimps.

Acknowledgements. 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 No. ICA4-CT-2001-10023 (IMMUNAQUA). It is also supported by the Centre National de la Recherche Scientifique, the Institut Français de Recherche et d'Exploitation de la Mer, and the University of Montpellier 2. The authors thank S. Fish and C. Zatylny for their contribution and Dr M. Barracco for critical reading of the manuscript.

1 Sano T. and Fukuda H. (1987) Principal microbial diseases of mariculture in Japan. Aquaculture **67:** 59–69
2 Lightner D. V. (1992) Shrimp pathology: major diseases of concern to the farming industry in the Americas. Mem. Congr. Ecuat. Acuicult. **1992:** 177–195
3 Ishimaru K., Akagawa-Matsushita M. and Muroga K. (1995) *Vibrio penaeicida* sp. nov., a pathogen of Kuruma prawns (*Penaeus japonicus*). Int. J. Syst. Bact. **45:** 134–138
4 Saulnier D., Haffner P., Goarant C., Levy P. and Ansquer D. (2000) Experimental infection models for shrimp vibriosis studies: a review. Aquaculture **191:** 133–144
5 Bachère E. (2000) Shrimp immunity and disease control. Aquaculture **191:** 3–11
6 Hose J. E., Martin G. G. and Gerard A. S. (1990) A decapod classification scheme integrating morphology, cytochemistry and function. Biol. Bull. **178:** 33–45
7 Söderhäll K. and Cerenius L. (1998) Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Opin. Immunol. **10:** 23–28
8 Sritunyalucksana K. and Söderhäll K. (2000) The proPO and clotting systems in crustaceans. Aquaculture **191:** 53–69
9 Destoumieux D., Muňoz M., Bulet P. and Bachère E. (2000) Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). Cell. Mol. Life Sci. **57:** 1260–1271
10 Destoumieux-Garzon D., Saulnier D., Garnier J., Jouffrey C., Bulet P. and Bachère E. (2001) Crustacean immunity: antifungal peptides are generated from the C-terminus of shrimp hemocyanin in response to microbial challenge. J. Biol. Chem. **276:** 47070–47077
11 Gross P. S., Bartlett T. C., Browdy C. L., Chapman R. W. and Warr G. W. (2001) Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific white shrimp, *Litopenaeus vannamei*, and the Atlantic white shrimp, *L. setiferus*. Dev. Comp. Immunol. **25:** 565–577
12 Destoumieux D., Bulet P., Loew D., Van Dorsselaer A., Rodriguez J. and Bachère E. (1997) Penaeidins: a new family of antimicrobial peptides in the shrimp *Penaeus vannamei* (Decapoda). J. Biol. Chem. **272:** 28398–28406
13 Destoumieux D., Bulet P., Strub J.-M. and Bachère E. (1999) Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. Eur. J. Biochem. **266:** 335–346
14 Destoumieux D., Muňoz M., Cosseau C., Rodriguez J., Bulet P., Comps M. et al. (2000) Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. J. Cell Sci. **113:** 461–469
15 Muňoz M., Vandenbulcke F., Saulnier D. and Bachère E. (2002) Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimps. Eur. J. Biochem. **269:** 2678–2689
16 Saulnier D., Avarre J. C., Le Moullac G., Ansquer D., Levy P. and Vonau V. (2000) Evidence that *Vibrio penaeicida* is the putative etiological agent of syndrome 93 in New Caledonia and development of a rapid and sensitive PCR assay for its detection in shrimp and sea water. Dis. Aquat. Org. **40:** 109–115
17 Costa R., Mermoud I., Koblavi S., Morlet B., Haffner P., Berthe F. et al. (1998) Isolation and characterization of bacteria associated with *Penaeus stylirostris* disease (syndrome 93) in New Caledonia. Aquaculture **164:** 297–309
18 Mermoud I., Costa R., Ferré O., Goarant C. and Haffner P. (1988) 'Syndrome 93' in New Caledonian outdoor rearing ponds of *Penaeus stylirostris*: history and description of three major outbreaks. Aquaculture **164:** 297–309
19 Goarant C., Herlin J., Brizard R., Marteau A. L., Martin C. and Martin B. (2000) Toxic factors of *Vibrio* strains pathogenic to shrimp. Dis. Aquat. Org. **40:** 101–107
20 Rodriguez J., Boulo V., Mialhe E. and Bachère E. (1995) Characterisation of shrimp haemocytes and plasma components by monoclonal antibodies. J. Cell Sci. **108:** 1043–1050
21 Schägger H. and Jagow G. von (1987) A tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:** 368–379
22 Kussmann M., Lassing U., Sturmer C. A., Przybylski M. and Roepstorff P. (1997) Matrix-assisted laser desorption/ionization mass spectrometric peptide mapping of the neural cell adhesion protein neurolin purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis or acidic precipitation. J. Mass Spectrom. **32:** 483–493
23 Mitta G., Vandenbulcke F., Noel T., Romestand B., Beauvillain J. C., Salzet M. et al. (2000) Differential distribution and defence involvement of antimicrobial peptides in mussel. J. Cell Sci. **113:** 2759–2769
24 Nielsen H., Engelbrecht J., Brunak S. and Heijne G. von (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:** 1–6
25 Gennaro R., Skerlavaj B. and Romeo D. (1989) Purification, composition and activity of two bactenecins, antibacterial peptides of bovine neutrophils. Infect. Immun. **57:** 3142–3146
26 Bachère E., Destoumieux D. and Bulet P. (2000) Penaeidins, antimicrobial peptides of shrimp: a comparison with other effectors of innate immunity. Aquaculture **191:** 71–88
27 Goarant C., Régnier F., Brizard R. and Marteau A. L. (1998) Acquisition of susceptibility to *Vibrio penaeicida* in *Penaeus stylirostris* postlarvae and juveniles. Aquaculture **169:** 291–296
28 Takahashi Y., Shimoyama Y. and Momoyama K. (1985) Pathogenicity and characteristics of *Vibrio* sp. isolated from cultured kuruma prawn *Penaeus japonicus* Bate. Bull. Jpn. Soc. Sci. Fish. **50:** 721–730
29 Alday-Sanz V., Roque A. and Turnbull J.-F. (2002) Clearing mechanisms of *Vibrio vulnificus* biotype I in the black tiger shrimp *Penaeus monodon*. Dis. Aquat. Org. **48:** 91–99
30 Smith V. J. and Ratcliffe N. A. (1980) Cellular defense reactions of the shore crab, *Carcinus maenas*: in vivo hemocytic and histopathological responses to injected bacteria. J. Invert. Pathol. **35:** 65–74
31 White K. N., Ratcliffe N. A. and Rossa M. (1985) The antibacterial activity of haemocyte clumps in the gills of the shore crab, *Carcinus maenas.* J. Mar. Biol. **65:** 857–870
32 Milton D. L., Norqvist A. and Wolf-Watz H. (1992) Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum.* J. Bacteriol. **174:** 7235–7244
33 Lee K. K., Chen Y. L. and Liu P. C. (1999) Hemostasis of tiger prawn *Penaeus monodon* affected by *Vibrio harveyi*, extracellular products, and a toxic cysteine protease. Blood Cells Mol. Dis. **25:** 180–192
34 Le Moullac G., Le Groumellec M., Anquer D., Froissard S., Levy P. and Aquacop (1997) Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: protection against vibriosis. Fish Shellfish Immunol. **7:** 227–234