**FEMS Microbiology Letters**
September 2005, Volume 250, Issue 1 : Pages 117-120
http://dx.doi.org/10.1016/j.femsle.2005.06.055




# Molecular characterization of penaeidins from two Atlantic Brazilian shrimp species, Farfantepenaeus paulensis and Litopenaeus schmitti

Margherita Anna Barracco[a]; Julien de Lorgeril[b], Yannick Gueguen[b, *], Evelyne Bachère[b]

[a]Departamento de Biologia Celular, Embriologia e Genética, Universidade Federal de Santa Catarina, Florianópolis/SC, Brazil.
[b]CNRS-Université de Montpellier II-Ifremer; UMR 5171 – Génome Populations Interactions Adaptation, Place E. Bataillon, CC80, F-34095 Montpellier cedex 5, France.

*: Corresponding author : Gueguen Y. Tel.: +33 4 67 14 47 07; fax: +33 4 67 14 46 22., email address : ygueguen@ifremer.fr

**Abstract:**

We report here the molecular cloning of new members of the penaeidin family from two Atlantic penaeids from Brazil, L. schmitti and F. paulensis. The presence of penaeidins in the granular hemocytes of both shrimps was first evidenced by immunofluorescence, using polyclonal antibodies raised against L. vannamei penaeidin Litvan PEN3-1. cDNAs from the hemocytes of both Brazilian species were obtained by reverse transcription and the sequences encoding penaeidins were amplified by PCR, using primers based on penaeidin consensus sequences. Five penaeidin clones were obtained. According to the international penaeidin classification (PenBase, http://www.penbase.immunaqua.com), the deduced amino acid sequences of two clones from L. schmitti and two from F. paulensis belong to the PEN2 subgroup and one clone from L. schmitti to the PEN4 subgroup of penaeidins. Surprisingly, no penaeidin from the PEN3 subgroup was obtained in both shrimp species, even though this subgroup appears to be the most commonly expressed in the hemocytes of penaeids.

**Keywords:** penaeidins, antimicrobial peptides, hemocytes, Brazilian penaeid shrimps, Farfantepenaeus paulensis; Litopenaeus schmitti, molecular cloning; immuno-detection.

## 1. Introduction

Antimicrobial peptides (AMPs) are major components of the innate immunity conserved in evolution and present in all phyla of the living kingdom including vertebrates, invertebrates, plants and bacteria [1]. In penaeid shrimps, to date, three kinds of antimicrobial peptides have been fully characterized, namely the penaeidins and the anti-lipopolysaccharide factor (ALF) from hemocytes [2-3] and anionic hemocyanin-derived peptides from plasma [4]. Penaeidins constitute an original peptide family, whose molecular structure is unique, composed of an N-terminal proline–rich region and a C-terminal domain containing six cysteines forming three intramolecular disulphide bridges [5]. They were first isolated from the hemolymph of the Pacific white shrimp *Litopenaeus vannamei* [6]. The functions of the penaeidins in defense reactions were fully characterized in terms of biological activity *in vitro* and gene expression and shrimp tissue distribution in response to microbial challenges *in vivo* [7-10]. More recently, penaeidins were also detected in other penaeid species [11-16].

Most of the currently identified penaeidins come from Pacific penaeid shrimps and to our knowledge only *L. setiferus* from Atlantic was examined so far [11]. The objective of the present study was to identify gene encoding penaeidins in two Atlantic native shrimps from Brazil with economical interest for aquaculture, the white shrimp *Litopenaeus schmitti* being found along all the Brazilian coast, and the pink shrimp *Farfantepenaeus paulensis*, which has a more restricted distribution, occurring only in colder waters (up to14°C) in South Brazil.

## 2. Material and Methods

### *2.1. Animals, hemolymph withdrawal and immuno-dectection of penaeidins*

The hemolymph of juveniles (n=6) of the pink shrimp *Farfantepenaeus paulensis* (Pérez-Farfante and Kensle, 1997) and the white shrimp *Litopenaeus schmitti* (Pérez-Farfante and

Kensle, 1997) was withdrawn from the ventral sinus under an anticoagulant solution (27 mM Na-citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0) and centrifuged at 800 g for 10 min (4ºC) to separate the hemocytes from plasma.

Immunofluorescence analyses were carried out using polyclonal antibodies specific for *Litvan* PEN3-1 of *Litopenaeus vannamei* according to the method of Destoumieux et al. [17].

*2.3 RT-PCR and cloning*

Total RNA was extracted from the hemocytes using Trizol reagent kit (GIBCO-BRL). Following heat denaturation (70°C for 5 min) of RNA samples, the cDNA was generated using Superscript$^{TM}$ reverse transcriptase for RT-PCR (Invitrogen). The PCR primers for penaeidin amplification in the cDNAs were designed based on the published sequences of penaeidins: sense primer 5´-CGCTCCGAGCCCGGGTTCCCTC-3´ from a consensus untranslated (UTR) sequence located before the signal peptide and anti-sense primer 5´-GGTTTYCATYGTCTTCTCCATCT-3´, located in a consensus 3´-UTR region of penaeidin gene. Amplified products were analyzed on 2% agarose gels, cloned into pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequenced from both directions with T7 and T3 primers.

**3. Results and Discussion**

In this study, we investigated the presence of members of the penaeidin family in two Atlantic native species of Brazil, the pink shrimp *F. paulensis* and the white shrimp *L. schmitti.* The first is a cold-tolerant shrimp, cultivated in the southern part of Brazil, whereas the second is especially farmed in Cuba. Penaeidins are antimicrobial peptides isolated and characterized from penaeid shrimps where they appear to be ubiquitous [6, 11-16]. All these peptides share amino acid sequence similarities and according to the international penaeidin classification

(PenBase, http://www.penbase.immunaqua.com), three distinct subgroups of penaeidins are now recognized, named PEN2, -3 and -4. Additionally, in *L. vannamei* and *L. setiferus*, the three subgroups of penaeidins were shown to be expressed in one single individual [11]. Most of the identified penaeidins originate from Pacific penaeid shrimps (except *L. setiferus*) and nothing is known about Brazilian shrimp species.

In both Brazilian native shrimps, it was possible to evidence by immunofluorescence the presence of penaeidins within the granular hemocytes using anti-penaeidin antibodies raised against *Litvan* PEN3-1 (Fig. 1). This immunohistochemistry (IHC) approach was already shown to be a good tool to evidence the presence of penaeidins and recently it led to the characterisation of the role of penaeidins in the defence reaction of *L. stylirostris* [2]. Then, to identify penaeidins encoding sequence, two PCR primers were designed, based on conserved region of penaeidin 3'-UTR and 5'-UTR. In both shrimps, a single product of around 220bp was amplified by PCR. After cloning and sequencing, the deduced amino acid sequences showed evident homology with the penaeidin antimicrobial peptide family. In both shrimp species, clones with high similarity to the PEN2 (two clones from both penaeids shrimps) and PEN4 (one clone from *L. schmitti*) subgroups were found (Fig. 2). The amino acid structure of these peptides was similar to that described for other penaeidins and was in agreement with the proposed signature for the penaeidin family (Fig. 2). According to the penaeidin nomenclature (PenBase, http://www.penbase.immunaqua.com) [18], the isolated peptides were named: *Farpau* PEN2-1, *Farpau* PEN2-2, *Litsch* PEN2-1, *Litsch* PEN 2-2 and *Litsch* PEN 4-1. *Litsch* PEN4-1 shows very high level of identity (>95%) with the three already described penaedins from the PEN4 subgroup. The two isolated PEN2 from *L. schmitti* show 70% or more identity with their counterpart from *Litopenaeus* shrimp species whereas *Farpau* PEN2-1 and PEN2-2, the two first penaeidins from the PEN2 subgroup that do not come from a *Litopenaeus* shrimp

species, possess less than 60% of identity with the others PEN2. Surprisingly, even though PEN3 appears to be the most abundant penaeidin subgroup commonly expressed in the hemocytes of most shrimp described so far [2], penaeidins for the PEN3 subgroups were not isolated from both Brazilian species according to this approach.

In conclusion, we described here the cloning of the complete gene sequence of novel members of the penaeidin family (PEN2 and -4) in two Atlantic species from the Brazilian coast. The study of such AMP functions and expression in shrimp is particularly important for fundamental understanding of shrimp immunity and for further establishment of disease control in shrimp aquaculture [19]. Moreover, AMP represents a new generation of therapeutic agents with potential applications in aquaculture.

**Acknowledgements**

This work was supported by the European Community, INCO-DEV IMMUNAQUA project contract ICA4-CT-2001-10023. Dr. M.A. Barracco was supported by a post-doctoral scholarship given by CAPES (Brazil).

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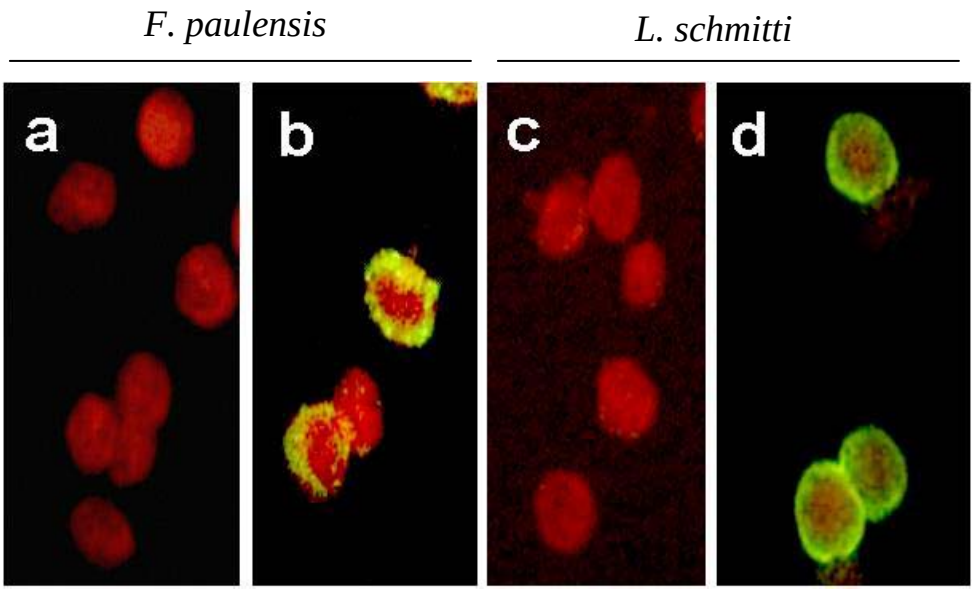
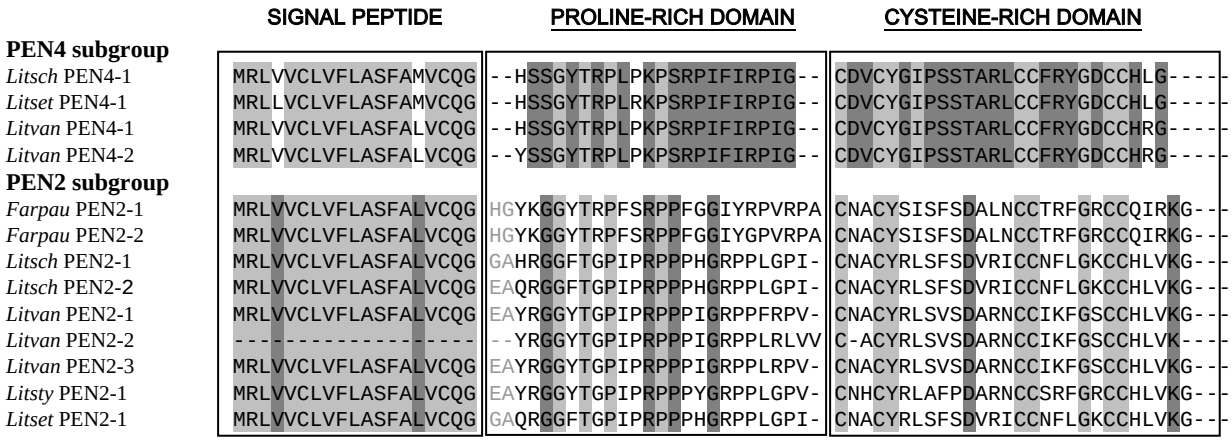
**Figure legends**

**Figure 1**: Detection of penaeidins in the granular hemocytes of *F. paulensis* (b) and *L. schmitti* (d) by immunofluorescence using polyclonal antibodies specific for *Litvan* PEN3-1. In controls (a and c), the anti-penaeidin 3 was replaced by a pre-immune serum.

**Figure 2**: Multiple alignment of amino acid sequences of penaeidins of the Altantic species *L. schmitti* (*Litsch*) and *F. paulensis* (*Farpau*) with penaeidins from the subgroups PEN4 and PEN2 from shrimps *Litopenaeus vannamei*, *L. setiferus*, *L. stylirostris* and *Penaeus monodon.* Sequences were selected from PenBase (http://www.penbase.immunaqua.com). Conserved residues of all the peaneidins are highlighted in grey, whereas conserved residues within a subgroup are highlighted in dark. The amino acids corresponding to the penaeidin signature are shown below the alignment.

**Figure 1**






| | | |
|---|---|---|
| **Penaeidin Signature** | (Y,F)**T**(R,G)**P**(X)$_2$(R,K)**P** | **C**(X)$_{1->3}$ **C**(X)$_2$(I,L)(X)$_7$**CC**(X)$_3$(G,R)X**CC** |