Antimicrobial peptides in crustaceans

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Abstract

Crustaceans are a large and diverse invertebrate animal group that mounts a complex and efficient innate immune response against a variety of microorganisms. The crustacean immune system is primarily related to cellular responses and the production and release of important immune effectors into the hemolymph. Antimicrobial proteins and/or peptides (AMPs) are key components of innate immunity and are widespread in nature, from bacteria to vertebrate animals. In crustaceans, 15 distinct AMP families are currently recognized, although the great majority (14 families) comes from members of the order Decapoda. Crustacean AMPs are generally cationic, gene-encoded molecules that are mainly produced by circulating immune-competent cells (hemocytes) or are derived from unrelated proteins primarily involved in other biological functions. In this review, we tentatively classified the crustacean AMPs into four main groups based on their amino acid composition, structural features and multi-functionality. We also attempted to summarize the current knowledge on their implication both in an efficient response to microbial infections and in crustacean survival.

Key Words: antimicrobial peptides; innate immunity; crustacean defense; crustaceans; decapods

Introduction

Crustaceans compose a large, ancient and diverse animal group that includes many well-known, commercially exploited members, such as shrimp, crab, crayfish and lobster. Together with insects, they are by far the most numerous, diverse and widespread animals on Earth. Crustaceans are primarily marine organisms. They are the most abundant animals inhabiting the world oceans, but there are also freshwater, terrestrial and semi-terrestrial species. During their long-standing existence, crustaceans have confronted a broad variety of challenges to their self-integrity because their natural habitats are typically overloaded with infectious organisms, such as viruses, bacteria, fungi and other parasites. Their evolutionary success confirms the efficient strategies they use for survival in such a potentially hostile and microbe-enriched environment. Part of this coexisting microbial biota is beneficial and can establish advantageous associations with the crustacean in a commensal or mutualistic manner, whereas another part of this biota is potentially harmful. Indeed, crustaceans can harbor specific microbial communities in their surface and internal epithelia that have important roles in nutrition and defense (Gil-Turnes et al., 1989).

In normal conditions, crustaceans maintain a healthy state and keep infections under control. Externally, they are covered by a hard, rigid exoskeleton that functions as an efficient physico-chemical barrier against mechanical injury and microbe invasion. Their gastrointestinal tract, another important route for pathogen invasion, is also protected almost entirely by chitinous membranes. This cuticular coat, in combination with an acid environment rich in digestive enzymes, is able to inactivate and degrade most viruses and bacteria (Jiravanichpaisal et al., 2006). However, once the cuticle barriers are disrupted, pathogenic and/or opportunist microorganisms can penetrate into the hemocoel and thus activate the internal immune defenses of the crustacean.

Crustaceans lack the complex and highly specific adaptive immune system of vertebrates, which is based on lymphocytes, immunoglobulins and immunological memory. Their internal defenses rely only on innate immune responses that are relatively less specific, but are fast and efficient defenses against microbes. The innate immune system of crustaceans is primarily related to their
blood or hemolymph and is comprised of cellular and humoral responses. Humoral defenses include pattern-recognition receptors/proteins that recognize pathogen-associated molecular patterns (PAMPs), the production of toxic oxygen and nitrogen metabolites, complex enzymatic cascades leading to melanization, clotting proteins and antimicrobial peptides. Conversely, cellular immune responses are mediated by circulating blood cells or hemocytes (hyaline and granular hemocytes) that are capable of neutralizing and/or eliminating pathogens by phagocytosing microbes and/or trapping them in hemocyte aggregates or nodules or by encapsulating larger microorganisms and parasites (see review of Jiravanichpaisal et al., 2006).

Antimicrobial peptides or proteins (AMPs) are one of the major components of the innate immune defense and are ubiquitously found in all kingdoms from bacteria to mammals, including fungi and plants. AMPs are primarily known as “natural antibiotics” because of their rapid and efficient antimicrobial effects against a broad range of microorganisms, including Gram-positive and Gram-negative bacteria, yeast, filamentous fungi and, to a lesser extent, protozoans and enveloped viruses (Bulet et al., 2004; Yount et al., 2006; Guaní-Guerra et al., 2010). Recent evidence has shown that in addition to their antimicrobial activity, AMPs may encompass a number of other diverse biological roles and are, in fact, multifunctional molecules. It was demonstrated that these peptides have antitumor effects, mitogenic activity and, most importantly, participate in immunoregulatory mechanisms by modulating signal transduction and cytokine production and/or release (Kamysz et al., 2003; Bowdish et al., 2005; Brown and Hancock, 2006; Yount et al., 2006; Easton et al., 2009; Lai and Gallo, 2009; Guaní-Guerra et al., 2010). Thus far, more than 1,500 AMPs sequences have been identified and are accessible on databases (http://aps.unmc.edu/AP/main.php) or in journal publications (see for review Thomas et al., 2010).

AMPs are classically described as small cationic, amphipathic, gene-encoded molecules (<10 kDa, 15-100 amino acids) that differ considerably in amino acid sequence and structural conformation. They are commonly found in the blood or epithelial (mucosal) surfaces that are most exposed to microorganisms. More recently, other groups of antimicrobial molecules have been identified that do not fit into this classical definition. These are anionic peptides, proteins larger than 10 kDa and multifunctional proteins that contain antimicrobial sub-domains that are cleaved under certain conditions and generate fragments that resemble classical antimicrobial peptides (Brogden, 2005; Yount et al., 2006).

The AMP mode of action is basically determined by structural conformation, cationic charge and amphipathicity. It is generally agreed that AMPs predominantly act by disrupting the membrane integrity of the cell target. The cationic portion of the peptide is first attracted to the negatively-charged bacterial and fungal cell walls and/or membranes, and following this first electrostatic interaction, the peptide inserts into and permeabilizes the microbial cell membranes through its hydrophobic portion. The microorganisms are then destroyed via membrane destabilization and/or pore formation (Brogden, 2005; Yount et al., 2006). The detailed mechanism of pore formation (barrel-stave, toroidal and carpet-like models) has been described in detail elsewhere (Brogden, 2005; Salditt et al., 2006). Beyond this direct interaction with microbial membranes, a growing body of evidence suggests that AMPs may have additional mechanisms to inactivate pathogens. Numerous recent studies have documented that AMPs can be translocated into the cytoplasm of the microorganism where they act on specific intracellular targets. Once inside, the peptides interfere with several essential metabolic functions, such as protein, nucleic acid and cell wall syntheses, leading to bacterial cell death (Kamysz et al., 2003; Brogden, 2005; Yount et al., 2006; Hale and Hancock, 2007; Nicolas, 2009). Moreover, it appears that many AMPs may be multifunctional microbicides, are priming simultaneously at the cell membrane and internal sites (Yount et al., 2006).

Different families of AMPs have been identified in crustaceans. For economic reasons, the best characterized peptides come from cultivated species, such as marine shrimp. The cultivation of penaeid shrimp is a potent industry worldwide, which generates significant profits for the producing countries. This industry is consistently threatened by devastating disease outbreaks caused by infectious agents that usually lead to massive losses in shrimp production. To overcome this constraint, the study of shrimp immune system, including the antimicrobial peptides, is essential in order to better comprehend their internal defenses and open new perspectives for developing novel strategies to prevent and control infections in aquaculture. Besides marine shrimp, AMPs have been also identified and characterized in a number of other crustaceans, primarily decapods, including marine crabs and lobsters, freshwater crayfishes and prawns and amphibious terrestrial species, such as isopods. The AMPs of penaeid shrimp have been very recently reviewed in great detail (Zhao and Wang, 2008; Tassanakajon et al., 2010).

In this review, we discuss the current information on the different antimicrobial peptide families identified to date in all crustacean groups. Special emphasis is given to their structure, biological properties and involvement in host defense and survival. We also draw attention to the fact that crustaceans possess a significant number of single-gene encoded AMPs that are composed of distinct domains and are larger molecules (>10 kDa) than the classic antimicrobial peptides in other organisms.

**Crustacean AMPs: structure, classification and biological properties**

The earliest studies on isolation and characterization of AMPs in crustaceans date back to mid-1990s. The first isolated AMPs, including Bir-like, crustulins and pen cepelins, were purified from the hemolymph of crab and shrimp by biochemical approaches. Since then, novel AMP families have been identified in crustaceans using
Table 1 Summarized characteristics of crustacean AMPs

<table>
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<tr>
<th>AMPs</th>
<th>Molecular mass (kDa)</th>
<th>Charge</th>
<th>Crustacean Order</th>
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<td><em>Single-domain linear α-helical AMPs and peptides enriched in certain amino acids</em></td>
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<tr>
<td>Bac-like</td>
<td>6.5</td>
<td>cationic</td>
<td>Decapoda (crab)</td>
<td>Schnapp et al., 1996</td>
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<td>Callinectin</td>
<td>3.7</td>
<td>cationic</td>
<td>Decapoda (crab)</td>
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<td>Astaciscin 2</td>
<td>1.8</td>
<td>cationic</td>
<td>Decapoda (crayfish)</td>
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<td>Armadillidin</td>
<td>5.2</td>
<td>cationic</td>
<td>Isopoda (woodlouse)</td>
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<td>Homarin</td>
<td>4-6</td>
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<td><em>Single-domain peptides containing cysteine residues engaged in disulfide bonds</em></td>
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<td>Defensin</td>
<td>6.7-7.1</td>
<td>cationic</td>
<td>Decapoda (lobster)</td>
<td>Pisuttharachai et al., 2009b</td>
</tr>
<tr>
<td>Anti-LPS factor</td>
<td>7-11</td>
<td>cationic</td>
<td>Decapoda (various)</td>
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<td>Scygonadin</td>
<td>10.8-11.4</td>
<td>anionic</td>
<td>Decapoda (crab)</td>
<td>Huang et al., 2006</td>
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<td><em>Multi-domain or chimeric AMPs</em></td>
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<tr>
<td>Penaeidin</td>
<td>5.5-6.6</td>
<td>cationic</td>
<td>Decapoda (penaeid shrimp)</td>
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</tr>
<tr>
<td>Crustin</td>
<td>7-14</td>
<td>cationic</td>
<td>Decapoda Amphipoda</td>
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<tr>
<td>Hyastatin</td>
<td>11.7</td>
<td>cationic</td>
<td>Decapoda (crab)</td>
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<tr>
<td>Arasin</td>
<td>4.3-4.8</td>
<td>cationic</td>
<td>Decapoda (crab)</td>
<td>Stensvåg et al., 2008</td>
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<tr>
<td>Stylicin</td>
<td>8.9</td>
<td>anionic</td>
<td>Decapoda (penaeid shrimp)</td>
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<td><em>Unconventional AMPs</em></td>
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<tr>
<td>Histones and derived fragments</td>
<td>11-15</td>
<td>cationic</td>
<td>Decapoda (penaeid shrimp)</td>
<td>Patat et al., 2004</td>
</tr>
<tr>
<td>Hemocyanin-derived peptides</td>
<td>2.7-8.3 (shrimp)</td>
<td>anionic</td>
<td>Decapoda (shrimp)</td>
<td>Destoumieux-Garzón et al., 2001</td>
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<tr>
<td></td>
<td>1.9 (crayfish)</td>
<td>cationic</td>
<td>Decapoda (crayfish)</td>
<td>Lee et al., 2003</td>
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Different molecular approaches. At the present time, 15 AMP families or single peptides sharing common molecular features with the currently known AMP families have been recognized in crustaceans. All crustacean AMP families display antimicrobial activities against a number of specific microorganisms (with the exception of two putative β-defensin-like peptides identified in a spiny lobster species by a genomic approach). However, the high diversity of the variants (subgroups and isoforms) found in most crustacean AMP classes can presumably confer a broad spectrum of activity to a single AMP family.

In terms of structure, crustacean AMPs can be primarily defined as small, cationic, amphipathic molecules that are encoded by a single gene, as commonly described in other organisms. However, more recently, this definition was expanded to include less common AMPs, such as anionic peptides, multifunctional proteins that are primarily implicated in other cellular functions and protein-derived fragments that display antimicrobial activities. Interestingly, an important number of gene-encoded AMPs in crustaceans are composed of different structural domains. Each of these domains possess singular features found in other AMP groups, such as the overrepresentation of specific amino acids or the presence of cysteine residues that form disulfide bonds. It has been proposed that these chimeric peptides could act as multifunctional proteins in different physiological systems in addition to their role in innate immunity (Bachère et al., 2004).

Based on amino acid composition and structure, we tentatively clustered the families of antimicrobial peptides found in crustacean species into four main groups: (1) single-domain linear α-helical AMPs and peptides enriched in certain amino acids, (2) single-domain peptides containing cysteine residues engaged in disulfide bonds, (3) multi-domain or chimeric AMPs and (4) unconventional AMPs including multifunctional proteins and protein-derived fragments that exhibit antimicrobial functions (Table 1). These groups
encompass the major characteristics of crustacean AMPs, although using this classification, some families could be categorized into more than one group.

**Single-domain linear α-helical AMPs and peptides enriched in certain amino acids**

Linear amphipathic peptides comprise a large group of AMPs that lack cysteine residues engaged in disulfide linkages. These peptides are usually unfolded in solution, but can adopt an amphipathic α-helical structure in the presence of lipid bilayers (Brogden, 2005). They are largely found in many vertebrate and invertebrate species, displaying a broad spectrum of antimicrobial activities against bacteria, fungi and protozoa. Additionally, some linear peptides are enriched with a high proportion of select residues, particularly arginine, proline, glycine, tryptophan or histidine (Tossi and Sandri, 2002). To date in crustaceans, five linear AMPs have been described, including homarin, armadillidin and three proline/arginine-rich peptides (Bac-like, callinectin and astacin 2).

**Proline/arginine-rich peptides**

Historically, the first antimicrobial peptide characterized in crustaceans was a 6.5-kDa proline/arginine-rich (or PRP-rich) cationic peptide isolated from the hemocytes of *Carcinus maenas* (Schnapp et al., 1996). Partial sequence analysis of the amino (N)-terminal region revealed that this peptide contains three repetitions of the motif PRP (Proline-Arginine-Proline), which are usually found in other insect antimicrobial peptides (Bulet and Stöcklin, 2005) (Fig. 1A). Moreover, due to significant amino acid similarities to bactenecin-7, a cathelicidin antimicrobial peptide from bovine neutrophils (Frank et al., 1990), this AMP is also known as Bac-like. Similar to the bovine AMP, the 6.5-kDa peptide was active against both Gram-positive (*Micrococcus luteus*) and Gram-negative (*Psychrobacter immobilis*) bacteria in inhibition zone assays. Interestingly, it also shares high sequence similarities with the PRP-rich domain of penaeidins (see section 3.1), an AMP family found exclusively in penaeid shrimp. Unfortunately, this first-identified crustacean AMP was not further characterized, and it remains unclear if its full-length sequence contains only PRP-rich motifs or if it includes additional unidentified molecular domains. New information on its complete amino acid sequence will reveal if Bac-like is related to the penaeidin family or if it is a distinct class of single domain antimicrobial peptides.

A few years later, another PRP-rich peptide, named callinectin, was isolated from the hemocytes of the blue crab *Callinectes sapidus* (Khoo et al., 1996). In contrast to Bac-like, the partially characterized N-terminal sequence of callinectin showed no significant homology with any other known antimicrobial peptide (Fig. 1A). This novel cationic peptide of 3.7 kDa was only tested against *Escherichia coli* D31, and information on its full-length sequence and spectrum of antimicrobial activity are currently lacking. Similar to Bac-like, it is uncertain if callinectin is a genuine single-domain PRP-rich AMP.

Another peptide belonging to the PRP-rich family was isolated from the hemocytes of the freshwater crayfish *Procambarus clarkii* and was fully characterized (Fig. 1A). This original peptide, named astacin 2, is composed of 14 amino acids and displays strong antimicrobial activity against both Gram-positive (Minimal Inhibitory Concentration [MIC] of about 5.5-10.3 μM: *Bacillus sp.*, *Staphylococcus aureus* and *M. luteus*) and Gram-negative bacteria (MIC of about 0.5-4.3 μM: *Shigella flexneri*, *Proteus vulgaris*, *E. coli* and *Pseudomonas aeruginosa*) (Jiravanichpaisal et al., 2007a). Unlike astacin 1, a crayfish antimicrobial peptide derived from the carboxyl (C)-terminus of hemocyanin (see section 4.2), astacin 2 is a gene-encoded molecule that is synthesized as a prepropeptide and then processed at both N- and C-terminal regions. This highly cationic peptide of 1.8 kDa (isoelectric point or pI of 11.8) is constitutively expressed in crayfish hemocytes and shows significant homology to metalnikowin-1 from the insect *Palomena prasina* (Chernysh et al., 1996). Recently, an astacin 2 homolog was also identified in the crayfish *Procambarus clarkii* (Shi et al., 2009).

**Armadillidin: a glycine-rich peptide**

Antimicrobial peptides rich in glycine residues have been isolated from a variety of arthropod species, including insects and spiders (Bulet et al., 1991; Lorenzini et al., 2003). In crustaceans, the first glycine-rich antimicrobial peptide, named armadillidin, was purified from the hemocytes of the terrestrial isopod *Armadillidium vulgare* (Herbinère et al., 2005). Armadillidin is a linear cationic peptide (pI of 12.1) that is characterized by the presence of a high content of glycine residues (about 47%). This peptide displays antibacterial activity against the Gram-positive bacteria *Bacillus megaterium* (MIC: 0.5 μM). The mature peptide (5.2 kDa) is amidated at the C-terminus and encloses a unique five-fold repeated motif GGGFH(R/S) (Fig. 1B). This glycine-rich peptide is the unique crustacean AMP currently characterized in a non-decapod species. Interestingly, armadillidin was found only in the hemocytes of the woodlouse A. vulgar, but not in other isopod species such as *Oniscus asellus*, *Porcellio dilatatus petiti* and *Armadillo officinalis* (Herbinière et al., 2005).

**Homarin: a linear α-helical peptide**

Homarin (also designated as Cationic Antimicrobial Peptide or CAP) is a short linear peptide isolated from the hemocytes of the American lobster *Homarus americanus* and does not contain any specific abundant amino acid residues (Battison et al., 2008). This cationic peptide shares evident amino acid similarities with temporins (Mangoni, 2006) (Fig. 1C). Temporins are small amphipathic α-helical AMPs from amphibian skin that exhibit antimicrobial activity against Gram-positive bacteria (*Simmaco et al.*, 1996). In contrast, two synthetic homarin variants (sCAP-1.1 and sCAP-1.4) were shown to be effective against *Vibrio sp.* isolated from the lobster intestinal tract and also exhibited protozoacidal activity against an
(A) Proline/arginine-rich peptides

Bac-like*  

Callinectin*  
WNSN[X][FHVGR[PP][V][G][X][F][P][A]

Astacidin 2  
RP[RN][X][P][I][Y][R]

(B) Glycine-rich peptide

Amadillidin  
GH[LR][P][Y][GG][G][F][N][R][GG][F][H][R][G][F][H][S][G][G][F][H][S][G][S][F][Y][R]

(C) Linear α-helical peptide

Homarin 1.1 (CAP 1.1)  
QYGN[L][S][L][N]GYR

Fig. 1 Primary structure of crustacean single-domain linear α-helical AMPs and peptides enriched in certain amino acids. (A) The proline and arginine residues are shadowed with a black background (where X is any amino acid residue). (B) The fivefold repeated motif GGGFH(R/S) is shown in bold and underlined. (C) The amino acid sequence of the linear α-helical homarin, which is identical to the amphibian temporins, is represented in bold type. The asterisks indicate the non-yet fully characterized crustacean AMPs.

Important ciliate pathogen for lobsters and crabs (Battison et al., 2008).

Single-domain peptides containing cysteine residues engaged in disulfide bonds

This group of single domain AMPs is characterized by the presence of pairs of cysteine residues that are capable of forming intramolecular disulfide bridges. The number of cysteine residues (generally 2 to 12) and their binding result in the formation of cyclic or open-ended cyclic stabilized peptides. Cysteine-containing AMPs are found in many species, from bacteria to eukaryotes, including fungi, plants and both vertebrate and invertebrate animals (Tossi and Sandri, 2002). This AMP group includes thanatin, tachyplesins, gomesin, protegrins and the well-known defensins (plant-, invertebrate-, α-, β- and θ-defensins) (for review, see Bulet et al., 2004). In decapod crustaceans, only three cysteine-containing AMPs have been characterized (defensins, anti-lipopolysaccharide factors and scygonadins), although a significant number of cysteine-containing domains are also present in crustacean multi-domain antimicrobial peptides.

Defensins

The defensin family appears to be the best characterized and the most widespread family of antimicrobial peptides, occurring in most plants and invertebrate and vertebrate animals (Bulet et al., 2004). Curiously, in crustaceans, defensin members were only very recently identified in the Japanese spiny lobster Panulirus japonicus using an expressed sequence tag (EST) approach (Pisuttharachai et al., 2009a). In this species, two different isoforms, PJD1 (7.1 kDa) and PJD2 (6.7 kDa), were detected in various tissues through reverse transcription-polymerase chain reaction (RT-PCR), including hemocytes, heart, gills and hepatopancreas (Pisuttharachai et al., 2009b). Both isoforms possess an N-terminal signal peptide and a C-terminal defensin-like domain. Interestingly, the cysteine pattern found in P. japonicus defensins (C-X₄₋₈-C-X₃₋₅-C-X₉₋₁₃-C-X₄₋₇-C-C) is distinct from those present in invertebrate defensins, but identical to that of β-defensins found in vertebrates (Taylor et al., 2008) (Fig. 2A). Unfortunately, to date, defensins are merely considered to be “putative AMPs” in crustaceans because their spectrum of antimicrobial activity has not yet been determined.

Anti-lipopolysaccharide factors

Anti-lipopolysaccharide factors (anti-LPS factors or ALFs) were first purified from the hemolymph cells (amebocytes) of two marine chelicerate arthropods, the horseshoe crabs Limulus polyphemus and Tachypleus tridentatus (Tanaka et al., 1982). Limulus and Tachypleus anti-LPS factors (LALF and TALF) have been initially identified as potent inhibitors of LPS-mediated hemolymph coagulation. The coagulation system of horseshoe crabs can be activated by two distinct pathways (triggered by LPS or 1,3,β-D-glucan) that mediate the activation of the procoagulant enzyme,
resulting in the formation of hemolymph clots, a defense reaction against microbial invasion (Kawabata et al., 2009). However, in addition to inhibiting the LPS-mediated coagulation pathway, the horseshoe crab anti-LPS factor also exhibits a strong antibacterial activity against R-types of Gram-negative bacteria, such as Salmonella minnesota R595 (Morita et al., 1985), thus acting as a multifunctional protein. Structurally, both LALF and TALF are large cationic peptides of approximately 100 amino acids that contain two conserved cysteine residues and a hydrophobic N-terminal region (Aketagawa et al., 1986; Muta et al., 1987). A cluster of positively-charged residues is comprised between the two cysteine residues that form the disulfide-bond loop, which has been defined as the LPS-binding domain (Hoess et al., 1993). Horseshoe crab anti-LPS factors are stored in the large granules of amebocytes, although no signal sequence has been recognized in the cDNA sequence of TALF (Wang et al., 2002).

In crustaceans, homologues to horseshoe crab ALFs were first identified from the hemocytes of the penaeid shrimp species Litopenaeus setiferus (Gross et al., 2001) and Penaeus monodon (Supungul et al., 2002) using an EST approach. To date, the genes encoding ALFs have been only identified in decapod crustaceans, such as penaeid shrimps (Tassanakajon et al., 2010), freshwater prawns (Rosa et al., 2008; Lu et al., 2009), crayfish (Liu et al., 2006), lobster (Beale et al., 2008) and crabs (Imjongjirak et al., 2007; Li et al., 2008; Yedery and Reddy, 2009a; Yue et al., 2010). These peptides are part of a very well-characterized family of crustacean AMPs composed of different subgroups and variants that are either encoded by distinct genes or generated by alternative mRNA splicing (Thamtada et al., 2008). In the tiger shrimp P. monodon, the most expressed variants, ALFPm2 and ALFPm3, are transcribed by different genes with distinct genomic organization, designated as group A and B genes (Fig. 2B). A genomic structure similar to the shrimp group A gene was also found in the crabs Scylla paramamosain and Eriocheir sinensis (Imjongjirak et al., 2007; Li et al., 2008). These peptides are constitutively expressed in circulating granular hemocytes, although cis-regulatory elements, which are involved in transcriptional regulation, were also recognized in their gene promoters (Thamtada et al., 2008).

ALF sequences from decapod crustaceans are encoded as precursor molecules. These precursors are composed of a leader sequence followed by a large mature peptide (of about 11 kDa) containing a highly hydrophobic N-terminal region and the two characteristic cysteine residues. Recently, the three-dimensional structure of shrimp ALF heterologously expressed in yeast cells (rALFPm3) was resolved by nuclear magnetic resonance (Yang et al., 2009) (Fig. 2B). The ALF structure consists of three α-helices (one at the N-terminus and two at the C-terminus) packed against a four-stranded β-sheet, as found in limulids. Based on amino acid sequence alignments, the ALF family was tentatively classified into three main groups: ALF Cluster I, II and III (Zhao and Wang, 2008). Later, another classification was proposed, but considered only penaeid shrimp ALFs: Groups I, II and III ALFs (Tassanakajon et al., 2010). Although an increasing number of studies have focused on the characterization of this AMP family, a consensus nomenclature and classification regarding structural features and phylogenetic relationships among different crustacean species and limulids are not still accessible.

Notably, ALFs exhibit a potent (MIC < 6.25 μM) and broad spectrum of antimicrobial activity against a large number of both Gram-positive and Gram-negative bacteria, including several opportunistic/pathogenic Vibrio species, fungi and human enveloped virus (Somboonwiiwat et al., 2005; Carriel-Gomes et al., 2007; Yedery and Reddy, 2009a). Interestingly, rALFPm3 showed a bactericidal effect against a large number of bacterial strains, including shrimp pathogens (MIC < 1.56 μM) (Somboonwiiwat et al., 2005). Moreover, it was very recently shown that rALFPm3 also has the ability to bind to both negatively-charged lipopolysaccharide (LPS) and lipoteichoic acid (LTA), the major cell wall components of Gram-negative and Gram-positive bacteria, respectively (Somboonwiiwat et al., 2008). Synthetic peptides corresponding to the ALF putative LPS-binding domain of the kuruma prawn Marsupenaeus japonicus displayed an efficient LPS neutralizing activity in vitro (Nagoshi et al., 2006), suggesting that crustacean ALFs are also multifunctional proteins.

Interestingly, in their recent review, Smith et al. (2010) do not classify ALFs as conventional AMPs, but as binding proteins. This classification is due to their ability to bind to LPS (lipid A portion) (Somboonwiiwat et al., 2008) in addition to their strong antimicrobial property. Moreover, the authors also point out that horseshoe crab ALFs are not segregated into the same granular population (small granules) as the classic antimicrobial peptides (tachylesin, polyphemusin, tachycitin and tachystatin), but into the large amebocyte granule population together with the components of the coagulation system (Iwanaga and Lee, 2005). This separate compartmentalization apparently suggests that limulid ALFs are primarily implicated in the regulation of the coagulation cascade. However, in crustaceans, the coagulation process is distinct from that of horseshoe crabs and is more similar to the insects (Theopold et al., 2004), and ALFs do not seem to take part in this process.

Scygonadins

In contrast to the cationic hemocyte-produced AMPs, scygonadin is an anionic (pI of 6.09) sex-specific large antimicrobial peptide of 10.8 kDa that was originally purified from the seminal plasma of the mud crab Scylla serrata (Huang et al., 2006). The scygonadin gene is composed of three exons and two introns, and its expression is restricted to the ejaculatory duct of adult males (Wang et al., 2007). The purified peptide only showed antibacterial activity against the Gram-positive bacteria M. luteus (Huang et al., 2006). Later, a scygonadin homolog of 11.4 kDa, named SSAP (for S. serrata antimicrobial protein), was purified from granular hemocytes of the same crab species.
Fig. 2 Crustacean single-domain peptides containing cysteine residues engaged in disulfide bonds. (A) Amino acid sequence alignment between two defensins isoforms (PJD1 and PJD2) from the Japanese spiny lobster *Panulirus japonicus*. (B) Amino acid sequence comparison between two ALF variants encoded by distinct genes (ALF<sub>Pm</sub>2 and ALF<sub>Pm</sub>3) from the shrimp *Penaeus monodon* and the three-dimensional structure of ALF<sub>Pm</sub>3. (C) Amino acid sequence alignment between the male-specific peptide scygonadin and the non-gender specific *Scylla serrata* antimicrobial protein (SSAP). Identical residues are marked with an asterisk (*) and the cysteine residues are shadowed with a black background.

(Yedery and Reddy, 2009b) (Fig. 2C). Both anionic scygonadin and SSAP (pI of 5.7) contain two cysteine residues that are arranged as in ALFs. However, a phylogenetic relationship between both anionic peptides from *S. serrata* and ALFs was not clearly evidenced. In contrast to scygonadin, SSAP is expressed in multiple tissues (as determined by RT-PCR and northern and western blot analyses) of both male and female crabs (Yedery and Reddy, 2009b). SSAP displayed antibacterial activity mainly against Gram-positive bacteria (MIC of about 7.5-30 µM: *M. luteus*, *Streptococcus pyogenes* and *S. aureus*), but not against yeast and filamentous fungi (Yedery and Reddy, 2009b; Peng et al., 2010).

**Multi-domain or chimeric AMPs**

Antimicrobial molecules with at least two distinct domains comprise a remarkable group of crustacean AMPs. Each domain may exhibit particular characteristics of classical single-domain AMPs, such as PRP- or cysteine-rich peptides. Multi-domain AMPs are not common in all living kingdoms, and apart from crustaceans, they have been identified in insects and arachnids (Wicker et al., 1990; Saito et al., 1995). In some cases, chimeric constitution is essential for establishing cationicity and amphipathicity. A cationic domain can be committed in electrostatic interactions with negatively-charged microbial walls, while a hydrophobic domain can be responsible for membrane destabilization. The presence of different structural arrangements in a single molecule can also provide multifunctional and/or synergic properties in addition to its antimicrobial activities. Additional biological functions have been shown for the crustacean multi-domain penaeidins, crustins and hyastatin as discussed hereafter.
**Penaeidins**

Penaeidins are unquestionably the most well-characterized family of antimicrobial peptides described in crustaceans so far and have been the subject of many review articles (Bächère et al., 2000, 2004; Destoumieux et al., 2000a; Cuthbertson et al., 2008; Tassanakajon et al., 2010). They are chimeric cationic peptides composed of an unstructured N-terminal PRP-rich domain and a C-terminal region containing six cysteine residues that are engaged in three intramolecular disulfide bridges (Destoumieux et al., 1997; Yang et al., 2003; Cuthbertson et al., 2005) (Fig. 3A). Interestingly, the N-terminal domain shares high sequence similarities with PRP-enriched peptides, in particular with crab Bac-like (Schnapp et al., 1996). On the other hand, the C-terminal cysteine-rich domain does not correspond to any other “cysteine motifs” previously described in cysteine-containing peptides. Penaeidins were originally isolated from the hemolymph of the Pacific white shrimp *Litopenaeus vannamei* (Destoumieux et al., 1997) and appear to be ubiquitous only in the family Penaeidae. All penaeidin precursors comprise a highly conserved signal peptide followed by a cationic mature peptide (5.48-6.62 kDa) with a calculated isoelectric point above 9 (Bächère et al., 2004). Because the signal peptide is cleaved, the mature peptides can be post-translationally processed by the formation of a pyroglutamic acid in the N-terminus and/or by a C-terminal amidation involving the elimination of a glycine residue (Destoumieux et al., 1997, 2000a). Based on amino acid sequence comparisons and the position of some precise residues in both the N- and C-terminal regions, four distinct subgroups of penaeidins have been classified: PEN2, PEN3, PEN4 and PEN5 (Cuthbertson et al., 2002; Gueguen et al., 2006; Kang et al., 2007) (Fig. 3A). The subgroup PEN1, which was initially purified from shrimp hemolymph, was later classified as a PEN2 variant (Cuthbertson et al., 2002). Each penaeidin subgroup possesses a characteristic amino acid signature and common biochemical features. Gueguen et al. (2006) have proposed and developed a specific nomenclature as well as a specialized database for the penaeidin family (PenBase, available at http://www.penbase.immunaqua.com).

The genomic structure of the penaeidin genes is variable according to each subgroup. Both PEN2 and PEN4 genes from *L. vannamei* (O’Leary and Gross, 2006), the PEN3 gene from *P. monodon* (Choit et al., 2007) and the PEN5 gene from the fleshy prawn *Fenneropenaeus chinensis* (Kang et al., 2007) are encoded by two exons interrupted by one intron. In all of these cases, a single intron of variable length divides the PRP- and cysteine-rich domains. Conversely, the PEN3 gene from *L. vannamei* lacks this typical intron sequence (O’Leary and Gross, 2006). Interestingly, in two species of *Litopenaeus*, transcripts of PEN2, PEN3 and PEN4 were all found to be expressed in a single individual (Cuthbertson et al., 2002). In naïve (unchallenged) shrimp, penaeidin precursors are constitutively expressed, processed and addressed to cytoplasmic granules of both granular and semi-granular hemocytes (Destoumieux et al., 2000b). The production of penaeidins is restricted to granule-containing hemocytes that can be free in the hemolymph or infiltrating shrimp tissues (Bächère et al., 2004). Interestingly, no PEN2 and PEN4 members were described in Asiatic shrimps, while the PEN5 subgroup seems to be absent in “occidental” species (Tassanakajon et al., 2010).

The spectrum of the antimicrobial activity of penaeidins has been studied in detail using native peptides purified from shrimp hemolymph, synthetic peptides and recombinant variants produced in heterologous expression systems (Destoumieux et al., 1997, 1999; Cuthbertson et al., 2004, 2005, 2006; Li et al., 2005; Kang et al., 2007). These peptides have been shown to be particularly effective against Gram-positive bacteria (MIC of about 0.3–2.5 μM: *Aerococcus*, *Micrococcus*, *Bacillus*, *Staphylococcus*) and filamentous fungi (MIC of about 1.25–2.5 μM: *Fusarium*, *Nectria*, *Alternaria*, *Neurospora*, *Botrytis*, *Penicillium*), but poorly or not active against Gram-negative bacteria and marine vibrios (with the exception of *Fench PEN5*, which appears to be active against *Klebsiella pneumoniae*). Moreover, recent studies have confirmed that penaeidin subgroups can display distinct antimicrobial activities (for review, see Cuthbertson et al., 2008).

Interestingly, besides their antimicrobial properties, penaeidins are also able to bind to chitin. A conserved chitin-binding motif is recognized in the cysteine-rich domain, whereas the PRP-rich domain is preferentially involved with the antimicrobial activities (Destoumieux et al., 2000a; Cuthbertson et al., 2004). According to Destoumieux et al. (2000b), the chitin-binding motif of penaeidins could have additional functions, such as the ability to bind to shrimp carapace upon injury and participate in wound healing and/or molting processes.

**Crustins**

Crustins are generally defined as multi-domain cationic antibacterial polypeptides (7–14 kDa) containing one whey acidic protein (WAP) domain at the C-terminus (Smith et al., 2008). The first identified crustin member is an 11.5-kDa protein purified from the granular hemocytes of the shore crab *C. maenas* that exhibits specific activity towards Gram-positive marine or salt-tolerant bacteria (Reif et al., 1999). The term crustin was later proposed by Bartlett et al. (2002) to describe transcripts found in two penaeid shrimp species (*L. vannamei* and *L. setiferus*) with high sequence similarity to the crab 11.5-kDa protein, which was later designated carcinin (Brockton et al., 2007). Since the first characterization of crustin from *C. maenas*, over 50 crustins and crustin-like sequences have been identified in numerous crustacean species, including crayfish, shrimps, freshwater prawns, crabs and lobsters, and also in non-decapod crustaceans, such as amphipods, (through EST-based approaches) (Smith et al., 2008).

In terms of structure, all known crustin precursors possess a leader sequence at the N-terminus and a C-terminal region containing a WAP domain. "WAP" is a general family of proteins usually found in the whey fraction of mammalian
milk that contains eight conserved cysteine residues in a conserved arrangement, forming a single four disulfide core (4DSC). This molecular motif seems to exert a protease inhibitory activity, in addition to other biological functions (Ranganathan et al., 1999). It is well established that protease inhibitors can play an essential role in crustacean immunity, such as inhibiting microbial proteases or regulating immune-protease cascades (Cerenius and Söderhäll, 2004).

Based on their structural features, Smith et al. (2008) categorized crustins into three main types: Types I, II and III (Fig. 3B). Type I crustins comprise the members most related to carcinin and possess a cysteine-rich region of variable length between the leader sequence and the WAP domain. This type of crustins is mainly present in crabs (Reff et al., 1999; Sperstad et al., 2009a; Imjongjirak et al., 2009; Mu et al., 2010; Yue et al., 2010), lobsters (Stoss et al., 2004; Hauton et al., 2006; Christie et al., 2007; Pisutharachai et al., 2009c) and crayfish (Jiravanichpaisal et al., 2007a; Shi et al., 2009), shrimp (Sun et al., 2010) and freshwater prawn (Dai et al., 2009). On the other hand, Type II crustins are characterized by the presence of a hydrophobic region containing an overrepresentation of glycine residues upstream of the cysteine-rich and WAP domains found in Type I crustins. This group is frequently documented in penaeid shrimps (Bartlett et al., 2002; Rattanachai et al., 2004; Supungul et al., 2004; de Lormeri et al., 2005; Rosa et al., 2007; Zhang et al., 2007; Antony et al., 2010) and crayfish (Jiravanichpaisal et al., 2007a). Conversely, Type III crustins possess a short PRP-rich region between the leader sequence and the single WAP domain, but do not contain the characteristic cysteine-rich domain present in both Type I and II crustins nor the glycine region motif. These peptides are usually known as single-whey domain (SWD) proteins, chelonianin-like proteins or antileukoprotease-like proteins with antibacterial activity (Hagiwara et al., 2007; Zheng et al., 2009) and crustin-like CshFc from Vibrio harveyi (Amparyup et al., 2008b). The antimicrobial activity of crustins appears to be related to the tertiary structure of the 4DSC (tightly constrained by three disulfide bonds and containing a small α-helix). Zhang et al. (2007) have shown that the crustin-like CshFc from F. chinensis, which lacks the authentic WAP domain, did not affect any bacteria tested, in contrast to the WAP-containing Type II crustin CruFc, which inhibited the growth of Gram-positive bacteria in vitro.

Interestingly, another classification for crustins was also proposed by Zhao and Wang (2008). These authors consider that the ‘crustin signature’ should not be restricted to the sole presence of a WAP domain at the C-terminal region of the molecule. In their classification, the crustin domain comprises the arrangement of the 12 conserved cysteine residues found in Type I and II crustins, which includes the WAP domain (eight cysteines). Accordingly, the SWD (Type III crustins) and DWD-containing proteins, which only contain WAP domains (one for SWD and two for DWD), cannot be considered as authentic crustin molecules. However, as mentioned above, shrimp SWDs combine antiprotease and antimicrobial properties. In addition, regarding the spacing of the cysteine residues within the crustin domain, Zhao and Wang (2008) proposed that shrimp crustins could be recognized as either crustin I or crustin II.

Although crustin antimicrobial peptides have been extensively reviewed and categorized into distinct types, a consensus nomenclature and classification as established for penaeid crustins (Gueguen et al., 2006) has not yet been proposed. In this review, we adopted the nomenclature used by Smith et al. (2008) to facilitate comparisons between the different crustin subgroups already reported in different crustaceans.

The genomic organization of crustins is distinct among the different groups. The genes of two Type I crustins are encoded by four exons interrupted by three introns (Brockton et al., 2007; Imjongjirak et al., 2009), in contrast to the SWDPm2 gene from P. monodon, which belongs to the Type III crustin group and contains three exons and two introns (Amparyup et al., 2008a). Furthermore, two crustin variants from the shrimp P. monodon are encoded by different genes; crustinPm5 contains four exons separated by three introns, and crustin-likePm contains only two exons and one intron (Amparyup...
et al., 2008b; Vatanavicharn et al., 2009). Expression of most crustin-encoding genes has mainly been found in circulating hemocytes, although some reports point to the possibility of expression in other tissues (as determined by RT-PCR), such as heart, ovary and intestines (Smith et al., 2008). Surprisingly, through RT-PCR analysis, transcripts of different crustin types, such as crustinFm5, Pcrustin2, Fc-crus 3 and PET-15, were essentially identified in the epipodite, hematopoietic tissue, ovary and ofalfactory organ, respectively (Stoss et al., 2003; Jiravanichpaisal et al., 2007a; Vatanavicharn et al., 2009; Sun et al., 2010).

Recent findings from our laboratory indicate that the Type II crustin crusFpau from the pink shrimp *Farcantopenaeus paulensis* (Rosa et al., 2007) is constitutively produced and stored in the granules of some populations of the shrimp granular hemocytes. Moreover, monospecific polyclonal antibodies raised against the recombinant rcrusFpau were able to cross-react with corresponding crustins in the hemocyte granules of other penaeids (*L. vannamei* and *Litopenaeus schmittii*), freshwater prawn (*Macrobrachium potiuna*) and crab (*C. sapidus*), suggesting that these antimicrobial peptides are produced and stored in the hemocyte granules, as described for penaeidins and ALFs (unpublished data).

**Hyastatin**

Hyastatin is a novel multi-domain antimicrobial peptide that was purified and characterized from the hemocytes of the small spider crab *Hyas araneus* (Sperstad et al., 2009b). The mature cationic molecule (pl of 9.84) is a large polypeptide of 11.7 kDa that is composed of a noteworthy glycine-rich N-terminal domain, a short PRP-containing portion and a C-terminal region with six cysteine residues (Fig. 3C). Interestingly, the glycine-rich domain consists of about 27 % of glycine residues and is very similar to that of Type II crustins. On the other hand, both the PRP- and cysteine-containing domains are comparable to those of penaeidins. Interestingly, the arrangement of the six cysteine residues of hyastatin is identical to the cysteine pattern found in all penaeidin groups (Gueguen et al., 2006). Moreover, hyastatin has chitin-binding abilities as described for penaeidins (Destoumieux et al., 2000b). Recently, gene sequences encoding hyastatin peptides were also discovered in EST libraries of other crab species, such as *C. sapidus*, *E sinensis*, *Cancer magister*, *Portunus trituberculatus* and *Celula pugilator*. To date, information about gene arrangement and organization is not yet available for this peptide.

Hyastatin was assayed against a reduced number of microorganism species but showed a broad spectrum of activity. It is capable to inhibit the growth of yeast (MIC of about 6.3-12.5 µM: *Saccharomyces cerevisiae* and *Candida albicans*), Gram-positive bacteria (one species: *Corynebacterium glutamicum* - MIC of 0.4 µM) and Gram-negative bacteria (one species: *E. coli* - MIC of 12.5 µM), thus differing from penaeidins and crustins that possess a more restricted antimicrobial activity to Gram-positive bacteria. Curiously, the antimicrobial activity of hyastatin appears to be related to the cysteine-rich domain (Sperstad et al., 2009b) rather than to the PRP-rich domain as presumed for penaeidins (Cuthbertson et al., 2004). Moreover, the chitin-binding property of hyastatin is linked to its N-terminal region (PRP- and glycine-rich domains) instead of to the cysteine-rich domain as in penaeidins (Sperstad et al., 2009b). Therefore, even though hyastatin and penaeidins share sequence and structural similarities, the functional properties of their molecular domains appear to be distinct.

**Arasins**

Like hyastatin, arasins are cationic chimeric peptides (pl ~11) isolated from the hemocytes of *H. araneus* (Stensvåg et al., 2008). These peptides contain a leader sequence of 25 amino acids followed by a linear PRP-rich N-terminal region and a C-terminal portion containing four cysteine residues engaged in two disulfide linkages (Fig. 3D). The N-terminal PRP motif is similar to that of bactenecin-7, metalnikowin-1 and of crustacean PRP-containing AMPs, such as Bac-like and astacin 2 (Schnapp et al., 1996; Jiravanichpaisal et al., 2007a). Furthermore, the four cysteine residues from the C-terminal region are arranged similarly to vertebrate protegrins (Capone et al., 2010). Like hyastatin, arasin 1 (4.3 kDa) was only tested against a few microorganisms species and exhibited activity against both Gram-positive (*C. glutamicum* - MIC of 0.8 µM) and Gram-negative (*Listonella anguillarum* and *E. coli* - MIC of 6.3-12.5 µM) bacteria in vitro. Arasin 2 was only identified through sequencing of a hemocyte cDNA library, and its spectrum of antimicrobial activity has not yet been determined. Like most other crustacean antimicrobial peptides, transcripts of both arasin forms were mainly detected in circulating hemocytes (Stensvåg et al., 2008). Unfortunately, information about arasin encoding-genes is not currently available.

**Stylicins**

Similar to scygonadins, stylicins are anionic peptides with a theoretical pl of 5. They are composed of 82 amino acids (8.9 kDa) and are characterized by a proline-rich N-terminal region and a C-terminal portion containing 13 cysteine residues (Rolland et al., 2010) (Fig. 3E). This family of antimicrobial peptides shows homology to mouse cryptdin and was first evidenced in the shrimp *Litopenaeus stylirostris* that was able to survive an infection of the pathogenic *Vibrio penaeicida* (de Lortgeril et al., 2005). These peptides were also identified in EST libraries of two other penaeid species, *L. vannamei* and *P. monodon*. *Ls-Stylicin1* from *L. stylirostris* is encoded by two exons interrupted by one intron, and its expression is strictly limited to the hemocytes. *Ls-Stylicin1* did not display significant activity against either Gram-positive or Gram-negative bacteria, but exhibited strong antifungal effect on *Fusarium oxysporum*. Interestingly, *Ls-Stylicin1* showed a potent LPS-binding activity comparable to rALFPln3 and was able to agglutinate *V. penaeicida* in vitro in a lectin-like manner (Rolland et al., 2010).
Unconventional AMPs: multifunctional proteins and protein fragments with antimicrobial activities

This last group of crustacean AMPs is composed of multifunctional proteins that primarily serve other functions and protein fragments that display antimicrobial activity and are generated by the processing of larger proteins. These unconventional AMPs, so called by Smith et al. (2010), possess important molecular elements found in the structure of classical AMPs, such as charge, hydrophobicity and/or amphipathicity (Brogden, 2005), and have been isolated and characterized from many different invertebrate and vertebrate species. They include whole proteins, such as histones, ribosomal proteins and mammalian milk proteins, and peptide fragments derived from large precursors with no evident antimicrobial properties, such as hemoglobin, lactoferrin and hemocyanin (Bulet et al., 2004). In crustaceans, antimicrobial activities were reported for histones and histone fragments and also for peptides derived from the crustacean oxygen carrier hemocyanin.

Histones and derived fragments

Histones are major protein components of chromatin that are directly implicated in DNA packing and the regulation of gene expression. They are cationic proteins, highly conserved in all eukaryotic cells that might also be involved in antimicrobial defense (Cho et al., 2009). Antimicrobial activity has been reported for different types of histones (H1, H2A, H2B, H3 and H4) in many vertebrate and invertebrate species, confirming their multifunctional properties (Kashima, 1991; Park et al., 1996, 1998; Richards et al., 2001). Their high content of cationic residues and amphipathic secondary structure could be responsible for their antimicrobial property. In fish and amphibians, histones and histone-derived fragments with antimicrobial activity have been found in skin secretions and mucus (Park et al., 1998; Robinette et al., 1998; Fernandes et al., 2002, 2004). The antimicrobial property of histones seems to be related to their capacity of destabilizing bacterial membranes and not to their ability of forming stable pores (Fernandes et al., 2002). However, the mechanisms by which histones are recruited to sites outside the cell nucleus and the regulation of histone production in response to microbial infections is far from being clear.

In crustaceans, histones and histone-derived fragments displaying antimicrobial activities were recently identified in the shrimp *L. vannamei* (Patat et al., 2004). Using biochemical approaches, high levels of the four core histone proteins (H2A, H2B, H3 and H4) were detected in circulating hemocytes. Both full length histone H2A and its N-terminal fragment, which is similar to buforin I and parasin AMs, were strongly active (MIC < 4.5 µM) against *M. luteus* and two strains of *Bacillus*. Likewise, a chromatographic fraction containing both H2B/H4 histones inhibited *M. luteus* in liquid growth inhibition assays. Moreover, a shrimp H1-derived peptide was also found to be active against *M. luteus* (Patat et al., 2004).

Hemocyanin-derived peptides

Hemocyanin is a copper-containing oxygen transport protein in crustaceans that is produced in the hepatopancreas and then released into the plasma. Hemocyanin is the most abundant protein in the crustacean hemolymph, representing more than 95% of the total protein in the plasma. Arthropod hemocyanins are organized as multiples of hexamers, and each hexamer contains monomers of about 75 kDa (van Holde et al., 2001). In addition to its role as an oxygen carrier, hemocyanin appears as a multifunctional protein since it is also involved in osmoregulation, protein storage and some immune reactions (Decker and Jaenicke, 2004). In chelicerates, the N-terminal region of hemocyanin was suggested to have phenoloxidase activity after proteolytic cleavage (Decker and Rimke, 1998; Nagai and Kawabata, 2000). The phenoloxidase system (or proPO system) involves a complex cascade in which phenol compounds are oxidized and many toxic molecules (quinones and reactive oxygen intermediates) are generated in response to a microbial infection (Cerenius and Söderhäll, 2004; Cerenius et al., 2008).

In crustaceans, some antimicrobial peptides derived from the C-terminal part of hemocyanin were isolated and characterized from the plasma of the penaeids *L. vannamei* (*PvHCt*) and *L. stylostris* (*PsHCt1, PsHCt2*) (Destoumieux-Garzón et al., 2001) and the crayfish *P. leniusculus* (astacin 1) (Lee et al., 2003). The three peptides derived from shrimp hemocyanin exhibited strong antifungal activity against many filamentous strains (MIC of 3.15-12.5 µM) (Destoumieux-Garzón et al., 2001). By contrast, astacin 1 from crayfish was active against Gram-positive (MIC of 1.9-12.8 µM: *Bacillus* sp. and *M. luteus*) and Gram-negative bacteria (MIC of 15 µM: *S. flexneri* and *E. coli*).

All three shrimp hemocyanin-derived peptides are anionic molecules with molecular masses of 2.7 kDa (*PvHCt*), 7.9 kDa (*PsHCt1*) and 8.3 kDa (*PsHCt2*) and are induced or activated in the plasma of animals stimulated by microbial injection (Destoumieux-Garzón et al., 2001). These peptides are generated from the C-terminal region of hemocyanin, which lacks the copper-binding site. In the crayfish *P. leniusculus*, astacin 1 (about 1.9 kDa) appears to be released from the C-terminus of hemocyanin by a cysteine-like protease, and its production is enhanced by injection of LPS or glucan into the crayfish (Lee et al., 2003).

Involvement of AMPs in crustacean host defense and survival

Evidence of the implication of AMPs in innate host defenses has been reported in multiple species across different taxa, including crustaceans (Hancock and Scott, 2000; Pazgier et al., 2006; de Lurgeret et al., 2008; Conlon, 2010; Smith et al., 2010). In most decapod species, especially in penaeid shrimp, the modulation of gene expression of some AMPs in response to microbial challenge
Monodon transcripts in the circulating hemocytes of family and/or the crustacean species. ALF expression pattern of the well-characterized P. monodon (ALFpM3), F. chinensis and L. stylirostris increase in abundance in the first hours after a Vibrio challenge (Supungul et al., 2004; Liu et al., 2005; de Lorgeril et al., 2008). In contrast, the mRNA concentration of shrimp crustins, penaeidins and stylicin significantly decrease at this stage of infection (Muñoz et al., 2004; Supungul et al., 2004; de Lorgeril et al., 2008). Furthermore, the expression of these last AMPS returns to basal levels 24-72 h post-infection, followed by a subsequent remarkable increase when compared to unchallenged animals (Muñoz et al., 2004; de Lorgeril et al., 2008).

Conversely, the in vitro gene expression of three AMPS (arasin-1, hyastatin and a Type I crustin) in primary hemocyte cultures of the crab H. araneus were not affected neither by Gram-positive nor by Gram-negative bacteria stimulation (Sperstad et al., 2010). However, these observations cannot be extended to an in vivo situation, in which the conditions are considerably different. Interestingly, in the crayfish P. leniusculus, the modulation of crustin expression appears to vary according to the crustin type. The expression of Plcrustin1 (Type I crustin) was increased in circulating hemocytes and hematopoietic tissue after stimulation with both Gram-positive and Gram-negative bacteria, while Plcrustin3 (Type II crustin) expression was only induced after injection with a non-pathogenic Gram-negative bacteria (Acinetobacter sp.). In contrast, the expression of Plcrustin2 (another crayfish Type I crustin) and astacinid 2 were not modulated by microbial challenge (Jiravanichpaisal et al., 2007a).

According to Bachère et al. (2004), two main phases are recognized in shrimp (L. vannamei) immune response to a Vibrio infection, based on penaeidin expression: Phase I (local reaction) and Phase II (systemic reaction). During the first phase (12 h post-infection), there is a decrease in the mRNA abundance of penaeidins due to the migration of penaeidin-producing hemocytes to the sites of infection. In response to a presumed chemotactic effect, these hemocyte populations release large amounts of penaeidins in the infected tissues. The second phase occurs at about 48 h post-infection and is characterized by the activation of hematopoiesis and the appearance of penaeidin-expressing hemocytes in both hemolymph and shrimp tissues. A similar observation was also observed in ALF kinetics in P. monodon after a V. harveyi injection (Somboonwiwat et al., 2008).

Recently, the in vivo role of some crustacean AMPS was investigated in shrimp and crayfish using RNA interference (Liu et al., 2006; de la Vega et al., 2008; Shockey et al., 2009, 2010; Thamtada et al., 2009). A significant increase in mortality after silencing crustin gene transcripts was observed in L. vannamei infected with the Gram-negative pathogen V. penaeicida, but not with the virulent fungus F. oxysporum, as compared to crustin-expressing animals (Shockey et al., 2009). These results are curious because crustins do not seem to have an effect on marine vibrios, according to in vitro assays (see section 3.2). On the other hand, in the same shrimp species, ALF was shown to be involved in both bacterial and fungal infections (de la Vega et al., 2008).

In addition to bacterial infections, crustaceans are also attacked by different classes of viruses. To date, viral infections are the most serious constraint to shrimp farming worldwide, particularly the disease caused by the white spot syndrome virus (WSSV). In the crayfish P. leniusculus, injection of ALF-dsRNA resulted in an increased expression of the WSSV envelope protein VP28 in hematopoietic tissue cell cultures, indicating that this molecule is involved in antiviral defense (Liu et al., 2006). Moreover, preincubation of WSSV with rALFpM3 (from P. monodon) reduced virus propagation in both crayfish cell cultures (hematopoietic tissue) and infected P. monodon shrimp (Thamtada et al., 2009). Conversely, the ALF member LvALF1 does not seem to be responsible for direct virus protection in the shrimp L. vannamei (de la Vega et al., 2008). These results, together with differences in ALF antimicrobial activities according to species, suggest that this AMP family could be implicated differentially in the immune responses of each crustacean group.

Over the past few years a significant number of large-scale EST sequencing projects have successfully identified many immune genes, such as antimicrobial peptide family genes, in important cultivated shrimp species (Gross et al., 2001; Rotjinnakorn et al., 2002; Supungul et al., 2002; Tassanakajon et al., 2006; Dong and Xiang, 2007). Currently, over 170,000 sequences are available in the GenBank database from various tissues of different shrimp species (for review, see Robalino et al., 2009). In addition, the analysis of these EST libraries has confirmed that hemocytes are the main site for AMP synthesis. Sequences encoding AMPS in L. vannamei and L. setiferus comprise about 20 % of all sequenced transcripts in hemocyte cDNA libraries (Gross et al., 2001). In this context, it has been shown that penaeidins, crustins and ALFs appear to be the most highly expressed AMPS in shrimp hemocytes (Gross et al., 2001; Supungul et al., 2002; Dong and Xiang, 2007). Furthermore, in specific EST libraries of P. monodon, some AMPS were found to be up-regulated after both WSSV and V. harveyi challenge (Tassanakajon et al., 2006).

The developmental expression of the three most well-characterized crustacean AMPS was studied in detail in penaeid shrimp and crayfish. Significant mRNA expression levels of penaeidins, crustins and ALFs were detected in the early developmental stages (nauplius, zoea and mysis) of L. vannamei, P. monodon and F. chinensis (Muñoz et al., 2003; Chiou et al., 2007; Jiravanichpaisal et al., 2007b; Sun et al., 2010; Tassanakajon et al., 2010). Interestingly, two Type II crustins from F. chinensis (Fc-crus 1 and Fc-crus 2) are expressed in several shrimp developmental stages, while Fc-crus 3 (the sole Type I crustin member found in penaeid shrimp to date) is found only in the ovaries.
Fig. 3 Crustacean multi-domain or chimeric AMP families. (A) Sequence comparison of different penaeidin subgroups (PEN2 to PEN5) and the three-dimensional structure of Litvan PEN3-1 from Litopenaeus vannamei. (B) A not-to-scale schematic illustration of the three crustins types (Type I to III crustin). (C) Schematic comparison (not-to-scale) of hyastatin, penaeidins and Type II crustins. (D) Amino acid sequence alignment between the two arasins from the spider crab Hyas araneus. (E) Schematic representation (not-to-scale) of stylacin from the blue shrimp Litopenaeus stylirostris. In the amino acid alignments, the proline/arginine residues and conserved cysteines are highlighted in grey and black boxes, respectively.
the circulating hemocytes of naïve during distinct periods of the crustacean life cycle families of AMPs could be preferentially produced during distinct periods of the crustacean life cycle and highlight their importance during ontogenesis. Taken together, these results suggest that some families of AMPs could be preferentially produced during distinct periods of the crustacean life cycle. 

Recently, genes potentially associated with shrimp survival capacity were identified in *L. stylirostris* using the suppression subtractive hybridization (SSH) strategy (de Lorgeril et al., 2005). Among the genes differentially expressed in the circulating hemocytes of naïve versus *Vibrio*-infected shrimp, many were immune-related genes, including some crustacean AMP families. It was shown that the mRNA abundance of penaeidins (*L. stylirostris* PEN2 and *L. stylirostris* PEN3), crustins and stylicin (previously called cysteine-rich peptide or cryptdin-like) before a *V. penaeicida* infection could predict shrimp survival. These results evidenced for the first time a relationship between the gene expression profile and the capacity of shrimp to survive an infection (de Lorgeril et al., 2008). Additionally, it was shown that shrimp AMPs could display different expression kinetics after microbial stimulation.

It is noteworthy that a single animal can concomitantly express multiple AMP families, including many different subgroups and isoforms, and that this can confer a broad spectrum of antimicrobial responses to this animal. Unfortunately, at the present moment it is not known if different crustacean AMPs co-localize in the same vesicles of granular hemocytes or if each peptide family is segregated in different granules or cellular subpopulations. It would be also interesting to elucidate if crustacean AMPs co-localize with other immune effectors, such as the proteins of the proPO system and lysosomal degrading enzymes in the hemocyte granules, as shown in detail in limulids (Iwana, 2002; Iwana and Lee, 2005).

### Other considerations on crustacean AMPs

The knowledge acquired over the last two decades on the identification and characterization of antimicrobial peptides in crustaceans has revealed their essential role in the immune response and also in the capacity of these animals to survive infection. However, from the identified AMP families, only a few members have been tested against crustacean pathogens, as marine vibrios may cause severe infections to these animals (Table 2). From these, solely the ALFs displayed a consistent and potent effect against a variety of *Vibrio* species, including strains that are pathogenic to crustaceans (Somboonwiwat et al., 2005). In addition, ALFs are also implicated in crustacean antiviral defense against WSSV (Liu et al., 2006). These properties make the ALFs a particularly interesting anti-infective AMP family within crustaceans. To date, ALF members have been identified in several decapod species, but their occurrence and distribution in other crustacean orders remain unknown. Curiously, the complete genomic sequencing of the water flea *Daphnia pulex* (a basal crustacean lineage: Class Branchiopoda, Order Cladocera) did not reveal the presence of genes encoding ALFs or any other currently known AMP family identified in crustaceans (McTaggart et al., 2009). These findings are intriguing because gene sequences encoding ALFs are present in ancient chelicerates (horsehoe crabs) as well as in decapods (derived crustacean lineage), which are obviously more phylogenetically related to branchiopods than to chelicerates. Therefore, it remains unclear if ALF sequences were lost in *D. pulex* or if they have never been present.

With respect to crustins, EST sequences containing a putative WAP domain with characteristics of the crustin WAP domain were identified in the marine branchiopod *Artemia franciscana* and the copepod *Calanus finmarchicus* (Smith et al., 2008). However, in other molecular aspects, these partial sequences did not share significant similarities with the well-characterized crustin family. Conversely, two EST sequences were identified in the amphipod *Gammarus pulex* (a derived crustacean lineage), including many features of Type II crustins, such as a glycin-rich region similar to those of shrimp and a cysteine-rich region containing the WAP domain (Smith et al., 2008). The authors suggest that although incomplete, these sequences are strong candidates for being true members of the crustin family. Therefore, crustins appear to be the unique AMP family characterized in decapods that is also present in non-decapod species (at least in amphipods) to date. It would be interesting to elucidate if certain AMP families are widely distributed across crustaceans, as it appears to be the case for crustins, while others have a more restricted distribution, such as penaeidins, which are only present in penaeid shrimp.

Another important point concerns the in vitro assessment of the activity of the different AMP families of crustaceans (Table 2). In most studies, the physiological conditions of the animals are not taken into account, particularly of marine crustaceans (the most well-studied group in terms of AMPs), which have high salt concentrations in their body fluids. It is well known that AMPs lose their effect at high salinity due to alterations of their charge and structural conformation (Lee et al., 1997; Löfgren et al., 2009). It is therefore essential to confirm whether the AMPs of marine animals are stable under salt conditions to better comprehend their effect in vivo. Indeed, it was shown that the penaeidin variant Litvän PEN2-1 lost its activity against *M. luteus* at a salinity above 0.9 % (marine salinity is about 3.5 %). Conversely, the antimicrobial property of the ALF rALF Pm3 was not significantly affected by high NaCl concentration (Löfgren et al., 2009), and this is another interesting feature of this molecule. In contrast, carcinin (Type I crustin) requires high salt concentrations for its activity (Reif et al., 1999).

The great majority of AMPs was isolated and/or identified from crustacean hemocytes. It was shown that penaeidins, ALFs and Type II crustins from shrimp are synthesized and stored in the granules of granular hemocytes. However, it was also reported that some AMP families could be also expressed in
### Table 2 Antimicrobial properties of crustacean AMPs

<table>
<thead>
<tr>
<th>AMP families</th>
<th>Bacteria</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram-positive</td>
<td>Gram-negative</td>
</tr>
<tr>
<td>Bac-like</td>
<td>★★★</td>
<td>★★</td>
</tr>
<tr>
<td>Callinectin</td>
<td>NT</td>
<td>★★★</td>
</tr>
<tr>
<td>Astacidin 2</td>
<td>★★★</td>
<td>★★★</td>
</tr>
<tr>
<td>Armadillidin</td>
<td>★★★</td>
<td>NA</td>
</tr>
<tr>
<td>Homarin</td>
<td>NA</td>
<td>★★</td>
</tr>
<tr>
<td>Defensin</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>ALF</td>
<td>★★★</td>
<td>★★★</td>
</tr>
<tr>
<td>Scygonadin</td>
<td>★★</td>
<td>NA</td>
</tr>
<tr>
<td>Penaeidin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subgroup 2 (PEN2)</td>
<td>★★★</td>
<td>NA</td>
</tr>
<tr>
<td>Subgroup 3 (PEN3)</td>
<td>★★★</td>
<td>NA</td>
</tr>
<tr>
<td>Subgroup 4 (PEN4)</td>
<td>★★★</td>
<td>NA</td>
</tr>
<tr>
<td>Subgroup 5 (PEN5)</td>
<td>★★★</td>
<td>★★</td>
</tr>
<tr>
<td>Crustin&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>★★★</td>
<td>NA</td>
</tr>
<tr>
<td>Type II</td>
<td>★★★</td>
<td>★</td>
</tr>
<tr>
<td>Type III</td>
<td>★★★</td>
<td>★★★</td>
</tr>
<tr>
<td>Hyastatin</td>
<td>★★★</td>
<td>★★</td>
</tr>
<tr>
<td>Arasin</td>
<td>★★★</td>
<td>★★&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stylicin</td>
<td>NA</td>
<td>★</td>
</tr>
<tr>
<td>Histones and derived fragments</td>
<td>★★★&lt;sup&gt;1&lt;/sup&gt;</td>
<td>NT</td>
</tr>
<tr>
<td>Hemocyanin-derived peptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astacidin 1</td>
<td>★★</td>
<td>★★</td>
</tr>
<tr>
<td>PvHCt/PsHCt1/PsHCt2</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Minimal inhibitory concentration (MIC) values: ★★★ up to 10 µM; ★★ 10-20 µM; ★ 20-40 µM.

NA: not active (>40 µM).

NT: not tested.

<sup>1</sup>AMPs tested against a reduced number of microorganisms.

<sup>2</sup>Crustin classification proposed by Smith et al. (2008).

Other crustacean tissues, such as the defensins of the spiny lobster *P. japonicus* (Pisutharatrachai et al., 2009b) and certain crustin types (Smith et al., 2008). However, it happens that most of these results are based on RT-PCR and Northern blot analyses whose results can be misleading. Crustaceans possess an open circulatory system, and the circulating blood cells can infiltrate most of their tissues. Consequently, the presence of AMP transcripts in different tissues can actually originate from infiltrated hemocytes. Indeed, the expression of penaeidins was initially identified in a wide range of tissues in *L. vannamei* using Northern blot analysis (Destoumieux et al., 2000b). However, after *in situ* hybridization assays it was revealed that penaeidins were only expressed in shrimp granular hemocytes (Muñoz et al., 2002). Similar results were also obtained for the ALFs of *F. chinensis* and *P. monodon* (Liu et al., 2005; Soombowiwat et al., 2008). Hence, it is of prime importance to determine the gene expression sites of the other crustacean AMP families using more appropriate technical approaches (such as *in situ* hybridization and immunohistochemistry) to avoid misinterpretations.
Another relevant aspect that remains to be clarified concerns the co-localization of the different AMP families within the crustacean hemocytes. To date, it is unclear if the different AMP classes and/or isoforms are selectively or randomly segregated into different granules of distinct granular hemocyte populations. As previously mentioned, this specific subcellular compartmentalization was elegantly demonstrated in horseshoe crabs, in which the classic AMPs are stored within small granules, while ALF is segregated into large granules in association with the components of the coagulation cascade (Iwanaga and Lee, 2005), suggesting its primary role in horseshoe crab coagulation. In crustaceans, nothing is currently known about the co-localization of the different AMP families within immune cells. A
better elucidation of their storage in the same or different granules could help to predict their behavior during infections. It is generally assumed that crustacean AMPs are promptly released into the hemolymph from the granules upon cell stimulation by invading pathogens (Jiravanichpaisal et al., 2006), as it occurs in limulids (Iwanaga and Lee, 2005). Accordingly, crustacean AMPs should combat and control infections extracellularly in a systemic response. However, these molecules could also exert their activity intracellularly within phagosomes.

Interestingly, a significant number of crustacean AMPs are composed of distinct domains (penaeidins, crustins, hyastatin, arasins and stylicins). This structural feature contrasts with the AMPs commonly described in other organisms, even the phylogenetically related groups, such as limulids (Iwanaga et al., 2005) and insects (Bulet and Stöcklin, 2005), which mainly possess small single-domain peptides. It would be interesting to elucidate when and how the genes encoding these multi-domain proteins have been formed and rearranged during crustacean evolution. The implication of the different domains in different immune or physiological functions in crustaceans is uncertain. Perhaps the biological role of these proteins is primarily a reflex of their overall structural conformation (forming cationic amphipathic structures) and not of their independent intramolecular domains. However, the multifunctional role of some crustacean AMP is better documented. For example, the chitin-binding activity of penaeidin and hyastatin presumably participates in wound healing and molting (Destoumieux et al., 2000a; Sperstad et al., 2009b), the LPS-binding properties of ALFs and stylicins and the protease inhibition activity of Type III crustins block pathogen proteases and/or regulate endogenous protease cascades.

Concerning the unconventional AMPs, the antimicrobial activity of histones and histone-derived fragments was indeed an expected phenomenon in crustaceans because the antimicrobial property of these highly conserved proteins was already reported from fish to mammals. In shrimp, Patat et al. (2004) hypothesized that these proteins are stored in hemocyte granules and released into the hemolymph during microbial infection, together with other classical AMPs. However, the presence of histones in granules is unexpected because their transcripts do not possess a signal sequence to direct them to cytoplasmic granules. Expectedly, they contain nuclear localization signals within their N-terminal domain for translocation into the nuclear compartment. Consequently, their storage in hemocyte granules and their presumed release into the hemolymph in response to infection remain to be demonstrated.

Finally, maybe the most important aspect regarding crustacean AMPs concerns the complementary and synergistic antibiotic roles that these molecules probably encompass. It is reasonable to assume that the multiple classes of variants and isoforms of crustacean AMPs, which are concurrently present in the hemocytes of decapods, should act in a cooperative and complementary manner in order to circumvent infections and ensure host survival (Fig. 4). To date, synergistic experiments have only been reported for L. vannamei penaeidins. However, no synergistic effects were observed between Litvan PEN2-1 and Litvan PEN3-1 (Destoumieux et al., 1999). Also, in addition to AMPs, the immune cells of crustaceans produce several well-known immune effectors, such as lysozyme and other degrading enzymes and the components of the proPO system, to control infections. Smith et al. (2010) suggest that lysozyme, a widely distributed antimicrobial enzyme that cleaves the carbohydrate portion of the peptidoglycan from the bacterial cell wall, potential plays a synergistic role with the common antimicrobial peptides. After disrupting the bacterial cell wall, lysozyme could facilitate the access of classic AMPs to permeabilize the bacterial membrane. Therefore, lysozyme could simultaneously serve as a direct effector and synergistic agent for enhancing AMP activity.

**Concluding remarks**

In conclusion, as illustrated above, both in vitro and in vivo properties of AMPs confirm that they are indeed essential components of the crustacean immune system. Unfortunately, the vast majority of the current knowledge refers only to the order Decapoda and is far from representing the entire and highly diverse group of crustaceans. This is clearly evidenced by the apparent absence of gene sequences encoding for hitherto described AMPs in the complete genome of the branchiopod D. pulex. Perhaps a more refined analysis of its genome could reveal the presence of gene sequences with similarity to some AMP domains that have already been characterized in decapods. Moreover, in view of the exceptional diversity of crustaceans, it is expected that novel unknown AMP families with singular molecular structures and interesting biological properties are waiting to be discovered. On the other hand, concerning the already identified crustacean AMPs, it would be interesting to fill in gaps of missing knowledge, such as their precise mode of action, in vivo activity, co-localization with other AMP members and especially their synergistic effect with other AMP families and/or other immunologic effectors also present in the immune cells of crustaceans. This information would certainly significantly contribute to a more comprehensive and integrated vision of the essential role that these molecules exert in crustacean immunity.

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