On the wave of the crustin antimicrobial peptide family: From sequence diversity to function

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A B S T R A C T

Crustins represent the largest and most diverse family of antimicrobial peptides (AMPs) found in crustaceans. They are classically defined as disulfide-rich peptides/polypeptides holding a typical Whey Acidic Protein (WAP) domain at the C-terminal end. This WAP domain has eight cysteine residues forming a tightly packed structure, the four-disulfide core (4DSC) motif, that is also found in other proteins displaying protease inhibitory properties. Crustins are highly diverse in terms of primary structure, size and biochemical features, thus exhibiting a series of biological functions beyond their antimicrobial properties. In order to better categorize the distinct crustin members, different classification systems have been proposed. In this review, we discuss the current classification systems and explore the biological implication of the impressive molecular diversity of this unique AMP family. We also summarize the recent findings on the role of these effectors in crustacean immunity and homeostasis as well as in host-microbe interactions.

Introduction

Antimicrobial host-defense peptides (AMPs) are widespread in nature and represent a major component of metazoan immunity, especially for invertebrates that lack adaptive immune system [39]. In addition to the quick and efficient neutralizing action against invading microorganisms (eg, Gram-positive and Gram-negative bacteria, yeasts, filamentous fungi, and enveloped viruses), these natural antibiotics may play multifunctional roles in host-pathogen interactions by regulating important components of immunity upon infections. The multifaceted nature of AMPs as immune effectors and/or regulatory elements underlines their importance in animal physiology as well as biotechnological candidates for new drug development [13]. In the ongoing scenario of emergence of microbial resistance to multiple drugs, AMPs have shown to be promising molecules, mostly due to their broad antimicrobial spectrum, high efficiency, low toxicity to eukaryotic cells and the lower rate of bacterial resistance when compared to conventional antibiotics [81].

AMPs are typically short-chain peptides composed of fewer than 50 hydrophobic and cationic amino acid residues. This basic design usually confers amphiphilic properties, allowing AMPs to equally diffuse in both aqueous and lipidic environments [47]. Indeed, the presence of cationic residues provides a net positive charge at physiological pH, whereas the hydrophobic residues allow their partition in non-polar hydrocarbon environments through hydrophobic interactions. Nevertheless, some AMPs do not fit this canonical structure and exhibit an anionic character due to the overrepresentation of negatively charged residues [16]. Interestingly, some AMPs are rather small fragments derived from proteolytic cleavage of larger proteins whose functions can be unrelated to the immune system. This is the case of the antifungal PvHCt, a short fragment originated from the C-terminal cleavage of the respiratory protein hemocyanin from the Pacific white shrimp Litopenaeus vannamei [18]. Because AMPs are mostly constituted from the ordinary 20 amino acids found in both single-celled and multicellular organisms, their remarkable antimicrobial properties prompted extensive research towards the understanding of the biochemical mechanisms associated with their biological activity.

Functional studies have shown that AMPs act generally at the membrane level through insertion and destabilization of the microbial membrane, leading to the release of cytoplasmic contents. This ability derives from the combination of their elementary physicochemical properties and the chemical composition of microbial surfaces. The

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membranes of many microbes display negatively charged phospholipid headgroups to the outer face, whereas cells from multicellular organisms expose zwitterionic phospholipids on that face and segregate those with a negative nature on the intracellular leaflet [46]. This important contrast between surfaces of these two cell types underlies the classic mechanism of AMPs: the negatively charged surface of bacteria attracts AMPs through electrostatic forces, which is followed by AMP insertion into lipid bilayers mediated by hydrophobic portions, ultimately compromising irreversibly membrane functions. Although it represents a classic mechanism of microbial killing, other mechanisms of action, including activity on intracellular targets, have also been proposed. In this case, AMPs are translocated through the microbial membrane and act on specific targets, promoting the inhibition of essential metabolic functions, such as cell wall production or synthesis of proteins and nucleic acids [1].

AMPs emerge as functionally plastic molecules, since substitutions in amino acid residues can alter their antimicrobial spectrum without drastically changing their overall biophysical properties. The mechanism of action of AMPs also offers a great evolutionary advantage: most microorganisms have a lower capacity to evade the attack of these molecules. Given that AMPs act mainly at the cell membrane level, the development of resistance becomes costlier, since a wide range of mutations associated with the structural reorganization of the microbial cell membrane is required. In addition, the absence of unambiguous AMP molecular signatures prevents the emergence of microbial proteases that act specifically on them [81].

Currently, different families of AMPs have been identified in crustaceans [44]. For economic reasons, the best characterized AMPs come from cultivated species, such as marine decapods (e.g., penaeid shrimp, lobsters, and crabs). In decapods, the best described AMP families are crustins, penaeidins and anti-lipopolysaccharide factors (ALFs) [17]. Crustins are the most representative, occurring widely in the class Malacostraca, the largest class among crustaceans which gathers about 30,000 living species and have been shown to be involved in multiple roles in host immunology and physiology.

**Crustins: definition, history, and taxonomic distribution**

Crustins are disulfide-rich secreted antimicrobial peptides/poly-peptides (6-22 kDa) holding a conserved Whey Acidic Protein (WAP) domain at the C-terminal end (Fig. 1A). Historically, crustins were one of the first families of gene-encoded AMPs identified in crustaceans. Its history begins with the identification of a cationic 11.5-kDa peptide isolated from the granular hemocytes of the shore crab *Carcinus maenas* [56] (Fig. 1B). Although only a partial characterization of this peptide had been performed at the time, these molecules exhibited a primary structure distinct from any immune-related protein described in crustaceans and showed antibacterial activity against salt-tolerant marine Gram-positive bacteria [56].

The so-called ‘11.5-kDa peptide’ was conveniently named as ‘carcinin’ after the genus *Carcinus* by Smith and Chisholm [63] and showed to be active even after boiling. In 2002, Bartlett and colleagues [7] identified homologous sequences in two penaeid shrimp species (*L. vannamei* and *Litopenaeus setiferus*) and then coined the term ‘crustins’, a nomenclature that became universally used for the description of similar molecules in other crustaceans (Fig. 1B). Indeed, subsequent studies identified crustin-like sequences in other shrimp species along following years [55,59,70,82], and to date, more than 200 sequences were described in the literature or submitted to public databases.

Crustins represent the largest and most diverse family of AMPs in crustaceans [44]. They have been broadly identified amongst the order Decapoda with representatives in both Pleocyemata and Dendrobranchiata suborders [44,64]. At the time of writing, 215 amino acid sequences and 245 nucleotide sequences from 36 species of invertebrates are available in the NCBI database under the description of the term ‘crustin’ (https://www.ncbi.nlm.nih.gov/search/all/?te

![Fig. 1.](image-url) (A) Schematic representation (not to scale) of the structural organization of crustins: a signal (leader) peptide followed by a mature peptide/polypeptide containing an N-terminal multidomain region and a conserved C-terminal Whey Acidic Protein (WAP) domain. In the WAP motif, X represents any residue, X_n denotes a stretch of n residues and (X_m, X_n) represents a variable length from m to n residues. (B) The image illustrates the main events related to the crustin family over a timeline from 1999 to 2022.
rm=crustin). The vast majority of sequences was identified and characterized in decapod crustaceans such as penaeid shrimp, freshwater prawns, lobsters, crayfishes, and crabs. However, crustin sequences were also identified in non-decapod crustaceans, especially those belonging to the orders Amphipoda and Isopoda. Recently, Becking and collaborators [8] identified crustin-coding transcripts in transcriptomic databases of 21 species of isopods belonging to six different families. Similar approaches led Lai and Aboobaker [40] to identify crustin transcripts in 55 crustaceans, including 37 non-decapod species. In addition, crustin homologues were also identified in ants (hymenopteran insects) based on comparative genomics studies, revealing that this AMP family is present in different species within Pancrustacea [83] (Fig. 1B).

The Whey Acidic Protein (WAP) domain: a hallmark of crustins

Unlike other AMPs, crustins have the unique feature of holding a conserved Whey Acidic Protein (WAP) domain at the C-terminal end (Fig. 1A). The WAP domain is a 50 amino acid protein motif (https://pfam.xfam.org/family/PF00095) that has eight conserved cysteine residues involved in four intramolecular disulfide bonds. The internal disulfide bonds create a tightly compacted structure that ultimately forms a typical three-dimensional arrangement described on PROSITE as ‘four-disulfide core’ or 4DSC [65].

Although all crustins share this protein motif, the WAP domain is not exclusive to this AMP family. In fact, the term ‘WAP’ was coined by Piletz and colleagues [51] to describe a new major whey protein present in the milk of rodents. The transcripts encoding the WAP proteins present in murine models were cloned and characterized and their deduced amino acid sequences were shown to exhibit canonical 4DSC motifs previously described in non-milk proteins, such as snake venom neurotoxins and wheat germ agglutinins [28]. Since the terms ‘WAP domain’ and ‘4DSC’ denote the same protein motif, they have come to be used interchangeably and, for the purposes of this review, no distinction will be made between them.

The 4DSC protein superfamily is widespread across nearly all forms of life, although ironically a disproportionately small fraction has been reported from arthropods, the most abundant animals on the planet. Out of the over 5500 WAP protein sequences deposited on the PFAM database (ID: PF00095), around 12% (646 sequences) come from this group of metazoans. Amongst WAP proteins present in invertebrates are pearlwaps, a small group of proteins from mollusks. These proteins are composed of three WAP domains and have been associated with the formation of the shell by regulating the deposition of calcite crystals in abalones [75]. Interestingly, the most basal organisms that encode proteins belonging to the WAP superfamily are bacteria, suggesting that the WAP coding genes emerged early in nature and, most likely, due to its involvement in multiple biological functions, reached a wide range of organisms throughout the evolutionary time course [62].

Functionally, the WAP superfamily joins biologically diverse proteins, including members with either one or more WAP domains. In fact, with the exception of the conserved cysteine residues, the amino acid composition of the WAP domain can be quite divergent, and such diversity is reflected in its biological properties. Examples of WAP proteins include caltrin-like protein II, a calcium transport inhibitor for sperm in guinea pigs [12]; SPAI-1, an inhibitor of sodium/potassium ATPase in the intestine of pigs [4]; ANOS1, a protein involved in the formation of several organs during embryonic development and potentially associated with Kallman’s syndrome in humans [14]; WDNM1, a protein involved in the metastatic potential of adenocarcinoma in rats [15]; and the milk WAP proteins themselves, whose biological function has not yet been clarified. Interestingly, some WAP domain-containing proteins, such as SWAM1 and SWAM2 from mice and the omwaprins isolated from the venom of the snake Oxyuranus microlepidotus display antibacterial activities [27,49]. Despite this broad functional diversity, WAP proteins are mainly associated with antiproteolytic activity, as evidenced by the mammalian anti-leukoproteinases (ALPs) that play a key role during inflammation, preventing the degradation of host tissues by proteases secreted by neutrophils [29]; elafins, a group of inhibitors of elastase-type serine proteases [60]; and papillins, a group of metalloproteinase inhibitors involved in the regulation of embryonic development in invertebrates [38].

The antiproteolytic capacity of the most studied protease inhibitors belonging to the WAP superfamily (eg, elafin and ALPs) has been associated with a specific region called ‘primary contact region’ [50]. This region lies around the second cysteine residue in the WAP domain and usually contains a conserved methionine adjacent to the scissile peptide bond [35]. Curiously, in those proteins that exhibit antimicrobial activity, the corresponding region does not contain any homologous sequence, but is rather substituted by cationic and hydrophobic residues [27]. The substitution of residues in this region is thought to contribute to the shifting towards antimicrobial function since the appropriate positioning of cationic and hydrophobic amino acids tends to render the protein more amphipathic, an important characteristic to increase its detergent capacity and make it capable of inserting and destabilizing microbial membranes. Typically, crustins lack the region associated with protease inhibition but do possess the cationic and hydrophobic residues that confer the antimicrobial properties. Indeed, phylogenetic reconstructions performed with a small group of crustins along with vertebrate WAP domain-containing proteins suggest that crustins are more similar to proteins that exhibit antimicrobial activity than those with antiproteolytic properties [77].

Although some diversity is observed in WAP domain’s amino acid composition, the arrangement of the cysteine residues seems to be precisely conserved. Ranganathan and colleagues [54] carried out an extensive analysis of the amino acid sequences of several WAP proteins present in invertebrates in order to identify specific protein motifs that compose the 4DSC. The alignment of 84 members of the 4DSC family, including milk WAP proteins and several other proteins with confirmed or putative protease inhibitory activity, led authors to propose a new trustworthy identifying signature for the WAP motif (Fig. 1A).

The presence of a conserved WAP domain at the C-terminal end places crustins as a unique group of AMPs in invertebrates and, more importantly, differs them from other crustacean multiple domain cysteine-rich AMP families, such as arasins, hyastatins, penaeidins and stylicins [58]. Notably, the WAP domain of crustins is thought to be an essential motif for their activity, since crustins that lack the WAP-type four-disulfide core domain exhibit impaired biological functions. Full-length cDNA sequences coding for a naturally-occurring crustin with an incomplete WAP domain were isolated from hemocytes of the Chinese shrimp Fenneropenaeus chinensis and the correspondent recombinant peptides were shown to be devoid of antimicrobial activity. By contrast, a WAP-complete crustin which differed by the presence of 30 additional amino acid residues at the N-terminal end of the WAP motif was shown to exhibit a clear antimicrobial action [82]. These results highlight the importance of the amino acid composition of the WAP domain in the biological function of crustins and underscore the functional impact of structural changes in this domain.

Considering the long-term evolutionary history of the WAP domain-containing proteins, it is not surprising that such functional diversity exists in the WAP superfamily. During the time course of evolution, the WAP motif seems to have provided a molecular scaffold suitable for the emergence of proteins engaged in diverse functions, including participation in the immune system as antimicrobials. Therefore, crustins may represent a distinct lineage of WAP domain-containing molecules from invertebrates that exhibit antimicrobial and/or protease inhibitory activities.

The current classification systems

The massive publication of crustin-related sequences from diverse crustaceans throughout the 2000s, along with the absence of functional
characterizations, has led to misconceptions about what should be considered as an authentic member of the crustin family. This increasing complexity was systematically organized for the first time in 2008, when Smith and colleagues proposed an accurate system for crustin classification. In this system, crustins were divided into three groups, referred to as ‘Types’ (Types I to III), which differ based on the presence/absence of structural domains lying at the N-terminal end of the mature peptide/polypeptide (Fig. 2).

Type I crustins are characterized by the presence of a signal peptide followed by a variable size domain containing four conserved cysteine residues (the ‘cysteine-rich domain’) arranged in specific positions upstream of the WAP domain (Fig. 2). Although these cysteine residues are invariably present among Type I crustins, no evidence supports their engagement in disulfide bonds. Type I crustins gather sequences similar to the C. maenas carcinin (11.5-kDa peptide) and are found predominantly in crustaceans from the suborder Pleocyemata, such as crabs, lobsters, freshwater prawns, and crayfishes. A few studies have also reported them in penaeid shrimp, decapods from the suborder Dendrobranchiata [19,33].

Type II crustins, on the other hand, have a highly polymorphic hydrophobic domain ahead of the cysteine-rich domain also found in Type I crustins (Fig. 2). This N-terminal domain usually spans between 20 and 160 residues and is enriched in glycine residues (the ‘glycine-rich domain’). The number of glycine residues can vary considerably between different species and usually appear in blocks of four to five amino acids (tetra- or penta-peptides, such as VGGGLG) that are tandemly repeated [65]. Despite being an easily detectable feature, no function has yet been assigned to this structural domain. Type II crustins have been widely found among penaeid shrimp (suborder Dendrobranchiata), but they can also be found in some crustaceans from the suborder Pleocyemata [36,66]. Type II crustins represent the best-characterized crustin group and are by far the most diverse within the family. In addition, they can be further divided into two subgroups according to specific amino acidic signatures: Type IIa crustins, referred to as ‘Crustins’ and Type IIb crustins referred to as ‘Crustin-like peptides’ [6].

The third group of crustins (also known as Single WAP Domain-containing peptides or SWDs) gathers peptides composed of a single C-terminal WAP domain. Type III crustins might contain short N-terminal domains enriched in proline/arginine residues but, markedly, they lack the typical glycine-rich domain of Type II crustins and the cysteine-rich domain found in both Type I and Type II crustins [3,31,35].

Fig. 2. The current systems proposed for crustin classification.
After proposing what would be the first crustin classification system, Smith and colleagues [62] reviewed the molecular diversity and functional roles of WAP proteins in lower vertebrates and invertebrates and addressed the recent description of proteins containing two WAP domains in the peneaid shrimp species *L. vannamei* [34] and *Marsupenaeus japonicus* [10]. These proteins were initially described based on their similarity with Secretory Leukocyte Proteinase Inhibitors (SLPI) present in vertebrates and were named SLPI-like proteins. Despite their markedly different structural profile in comparison to crustins from Types I to III, SLPI-like proteins were included as a fourth group of crustins and designated as Type IV (also known as Double WAP Domain-containing proteins or DWD) (Fig. 2). Finally, Tassanakajon and colleagues [74] adapted the classification system by including a fifth group, Type V. Crustins from Type V were identified in ants (hymenopteran insects) and displayed a molecular architecture similar to that of Type I crustins, differing due to the presence of a structural domain rich in aromatic residues prior to the cysteine-rich domain [83] (Fig. 2). To date, this is the only group of crustins found outside the Subphylum Crustacea.

To unravel the evolutionary relationships between the different types of crustins, phylogenetic reconstructions have been persistently performed based on the increasing number of sequences reported in the literature or deposited in public databases [6, 65, 74, 76, 84]. Crustins from Types I, II and V consistently showed to form a monophyletic group, suggesting a common evolutionary origin, while Type III and Type IV crustins form a separate and distant group (Fig. 3). This phylogenetic unit of crustins from Types I, II and V correlates with the striking common feature that they hold the 12 conserved cysteine residues (four from the cysteine-rich domain and eight from the WAP domain) comprising the ‘crustin signature’ [6, 84] (Fig. 3).

Although the crustin classification system proposed by Smith and colleagues [62] and adapted by Tassanakajon and colleagues [74] is central in the current literature, alternative systems for crustin classification have also been proposed, addressing inconsistencies that emerged with the growing description of new sequences. Zhao and Wang [84] proposed a system where crustins were classified into three classes: Class I, Class II and Class III. Class I crustins (type A and type B) are characterized by the presence of a single WAP domain and a cysteine-rich domain. Class II crustins (type C and type D) are characterized by the presence of two WAP domains and a cysteine-rich domain. Class III crustins (type E and type F) are characterized by the presence of a single WAP domain and a cysteine-rich domain. The 12 conserved cysteine residues are highlighted in black. The ‘12-cysteine crustin signature’

![Diagram of crustin types](image-url)

**Fig. 3.** Crustins from Types I, II and V (but not Types III and IV) cluster in a common clade due to the presence of a conserved 12-cysteine crustin signature. The phylogenetic tree was constructed using the Neighbor-Joining method and bootstrap sampling was reiterated 1000 times. The sequences included in analyses were:

(i) Type I crustins (‘Carcinins’): CrustHa1 and CrustHa2 from *Hya araneus* (GenBank: ACJ06763.1, respectively), CrustSp from *Scylla paramamosain* (GenBank: ABY20727.1), PtCrustin from *Portunus trituberculatus* (GenBank: ACM9167.1), carcinin (11.5-kDa peptide) from *Carcinus maenas* (GenBank: CAD20734.1), MJCrust-1 and MJCrust-2 from *Marsupenaeus japonicus* [33], carcinninp2 from *Penaeus monodon* [19] and PCrustin2 from *Pacifastacus leniusculus* (GenBank: ABP88043.1); (ii) Type II crustins (‘Cruins’): CrustEs from *Eriocheir sinensis* (GenBank: ACR77767.1), crustinPM1 and crustinPM4 from *P. monodon* (GenBank: ACQ66004.1 and ACQ66005.1, respectively), M. japonicus crustin-like peptide 1 (GenBank: AB121740.1), crustin L1-6 from *Penaeus japonicus* (GenBank: AAL36890.1, AAL36891.1, AAL36892.1, AAL36893.1, AAL36894.1 and AAL36895.1, respectively), crustin P from *Penaeus setiferus* (GenBank: AAS9736.1) and crustin I from *Penaeus japonicus* (GenBank: AAS9734.1) from *L. vannamei*, crustina from *Farfantepenaeus brasiliensis* (GenBank: ABQ096197.1, crustina from *F. subtilis* (GenBank: ABQ093223.1), crustina from *F. paulensis* (GenBank: ABM63361.1) and crustinCh from *L. schmitti* (GenBank: ABM63362.1); (iii) Type III crustins (‘CruRins’): CrustF from *Pteropurpurina chilensis* (GenBank: AAZ76017.1), crustinPM2 from *P. monodon* (GenBank: ABY25094.1), crustin L1 from *L. vannamei* (GenBank: AFV77524.1); (iv) Type IV crustins (‘Single WAP domain-containing proteins or SWD’): SWDPM1 from *P. monodon* (GenBank: ACF28464.1), SFD from *F. chinensis* (GenBank: ABN09668.1), MJSD from *M. japonicus* (GenBank: AU176270) and SWDL from *L. vannamei* (GenBank: AAS17722.1); (v) Type IV crustins (‘Double WAP domain-containing proteins or DWD’): LVSLPI from *L. vannamei* (GenBank: ABR19819.1), SWDPC from *Procambarus clarkii* (GenBank: ACY64753.1), PnDW from *P. monodon* (GenBank: BI784457), FcWD from *F. chinensis* (GenBank: ACY64754.1) and MDW from *M. japonicus* (GenBank: ABW88999.1); (vi) Type V crustins [83]: Navicrustin from *Nasonia vitripennis*, Bicrustin from *Bombus impatiens*, Botcru from *B. terrestris*, Merocrustin from *Megachile rotundata*, Hascrustin from *Harpegnathos saltator*, Pobcru from *Pogonomyrmex barbatus*, Cafcrustin from *Camponotus floridanus*, Lhcrustin from *Linepithema humile*, Sicrustin from *Solenopsis invicta*, Atacrustin from *Atta cephalotes* and Acrustin from *A. echinatum*. Alignment of amino acid sequence of the C-terminal region holding the 12 conserved cysteine residues (the ‘12-cysteine crustin signature’) present in crustins from Types I, II and V. The conserved cysteine residues are highlighted in black.
regulation

The diversity of crustins in terms of spatial gene expression and regulation

The participation of crustins as immunological effectors has not been assumed considering only their antimicrobial properties, but also because in the vast majority of crustaceans, crustins are primarily expressed by and stored in circulating hemocytes, cells specialized in the immune response [65] (Fig. 4). Notably, studies support hemocytes as the main site of crustin expression based on the detection of highly abundant crustin transcripts in these cells by conventional molecular approaches, such as reverse-transcribed PCR (RT-PCR) [6,71]. This statement was corroborated by studies using immunofluorescence and confocal microscopy. Indeed, Suleiman and colleagues [68] have recently shown that the Type I carcinnin is primarily produced by granule-containing hemocytes. These results are in accordance with previous findings on the spider crab Hyas araneus, in which Type I crustins are over 2000-fold more expressed in granular hemocytes than in hyaline cells [66]. Additionally, crus Fpau, a Type Ia crustin from the Brazilian pink shrimp Farfantepenaeus paulensis, showed to be prominent in subsets of granular hemocytes, as demonstrated by a strong fluorescent signal in cytoplasmic granules [5].

However, although some authors argue for an unquestionable hemocytic origin, crustin transcripts have also been found in several other tissue/organs (eg, gills, intestines, hepatopancreas, hematopoietic organs, and gonads) of both challenged and unchallenged animals, suggesting that crustin genes can be constitutively expressed in a wide range of tissues (Fig. 4). These reports include those on shrimp [69,78], lobsters [52,67], crabs [48,80], and crayfishes [36,79]. In fact, some crustin genes were demonstrated to be exclusively expressed in alternative tissues and absent in hemocytes. For instance, crustinPm5 transcripts, a Type II crustin hitherto found only in the tiger shrimp Penaeus monodon, was shown to be expressed in the epipodite and eyestalk of healthy animals and at very low or undetectable levels in hemocytes [78]. Additionally, the carcinnin-like Fc-crus 3 gene from F. chinensis showed high transcript abundance in the heart of unchallenged animals, in stomach of bacterially challenged shrimp and a remarkable expression in ovaries of both challenged and unchallenged animals, but no signal in hemocytes [69]. Recently, Tandel and colleagues [73] reported two Type II crustins in M. japonicus that were mainly expressed in gills, but poorly detected in hemocytes.

The occurrence of crustin transcripts in several tissues is commonly interpreted as evidence that the organ or tissue itself is primarily responsible for crustin biosynthesis. However, two points should be taken into account: (i) the open circulatory system of crustaceans which allows the hemolymph to flood hemocoel, and (ii) the ability of hemocytes to penetrate into highly vascularized tissues, resulting in hemocytic infiltration. In those cases, crustin-expressing hemocytes would give a positive signal in non-expressing tissues which prompts a misinterpretation of crustin spatial distribution. Then, techniques of localization (eg, immunohistochemistry and/or in situ hybridization) are imperative to accurately determine the potential sites in which crustins are expressed. To address this question, Suleiman and colleagues [68] have performed immunohistochemical staining of various

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\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Type I} & \text{Type II} & \text{Type III} & \text{Type IV} \\
\hline
\text{Hemocytes} & \text{Gills} & \text{Digestive} & \text{Lymph} & \text{Lymph} \\
\text{Gill} & \text{Digestive} & \text{Tissues} & \text{Lymph} & \text{Tissues} \\
\text{Lymph} & \text{Lymph} & \text{Lymph} & \text{Lymph} & \text{Lymph} \\
\text{Gill} & \text{Gill} & \text{Gill} & \text{Gill} & \text{Gill} \\
\text{Digestive} & \text{Digestive} & \text{Digestive} & \text{Digestive} & \text{Digestive} \\
\text{Tissues} & \text{Tissues} & \text{Tissues} & \text{Tissues} & \text{Tissues} \\
\text{Lymph} & \text{Lymph} & \text{Lymph} & \text{Lymph} & \text{Lymph} \\
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Fig. 4. Distribution of gene expression, transcriptional response and biological functions of Types I to IV crustins from decapod crustaceans (classification system proposed by [65] and adapted by [74]).
tissues/organs excised from *C. maenas* to track for carcinin potential producing sites. Curiously, the positive signs of carcinins across the analyzed tissues were product of infiltrating hemocytes, although a strong signal was observed in ovaries due to high expression levels in oocytes. Up to now, this is the only experimental demonstration of crustins outside hemocytes.

The patterns of expression of crustin genes during crustacean development are not well established as most studies have been conducted in larval post-larvae or juveniles (Fig. 4). Crustin expression could be detected at high levels in all stages of *P. monodon* development from early larvae (nauplii IV) up to juveniles [36]. Likewise, both Type II crustins *Fc-crus 1* and *Fc-crus 2* showed similar expression profiles during *F. chinesis* development, which could be detected in early developmental stages (eg. nauplii, mysis, and post-larvae) as well as in juveniles and adults. By contrast, the *Fc-crus 3* member was shown to be prominent in ovaries of adults, but could be barely detected in any of the larval stages [69]. Barreto and colleagues [6] reported the first characterization of the transcriptional profile of a Type Ib crustin (*Crustin-Lv*) during shrimp development. The transcript abundance was quantified across 12 developmental stages of *L. vannamei* and could be detected in all shrimp ontogenesis, including fertilized eggs. Interestingly, a quite similar gene expression profile was also observed for the *Crustin Lv* gene (Type IIA) [53]. Both *L. vannamei* Type II crustins were transcribed to different extents during shrimp development, with hemocytes of juveniles showing the highest mRNA levels.

The contribution of crustins as immune effectors has also been inferred from studies of their transcriptional response following experimental infections with distinct classes of microorganisms (Fig. 4). It is well established that most crustin genes are responsive to both Gram-positive and Gram-negative bacteria. The crustin *PmPm7* gene (Type Iib) from *P. monodon* was up-regulated 24 h post-infection (hpi) with the pathogenic Gram-negative *Vibrio harveyi*, returning to basal levels at 48 and 72 hpi [3]. In *M. japonicus*, the transcriptional levels of four crustins were surveyed in a time-course experiment after bacterial and viral infections in both hemocytes and gills. The crustin members *MjCru I-3* and *MjCru I-4* were shown to be up-regulated in hemocytes upon both Gram-positive or Gram-negative challenges, whereas *MjCru I-2* did not show any response after bacterial infection but was significantly induced by the White spot syndrome virus (WSSV) at 24 to 48 hpi. Conversely, the expression patterns of *MjCru* I-2, -3, and -5 in gills were up-regulated after Gram-positive and Gram-negative bacterial challenges and a slight up-regulation after WSSV infection [33]. In crabs, lobsters and crayfishes crustin genes are also responsive to microbial infections. The expression levels of *CrusEs* from the Chinese mitten crab *Eriocheir sinensis* were shown to be down-regulated after a challenge with the Gram-positive *Micrococcus luteus*, although no differences could be found between the control group and animals experimentally challenged with the Gram-negative *Listonella anguillarum* [48].

Pisutharachai and colleagues [52] investigated the expression levels of four Type II crustins in the phyllosomas of the Japanese lobster *Panulirus japonicus* and higher transcriptional levels of the crustins *PjC2* and *PjC3* in diseased animals, in detriment of *PjC1* and *PjC4* were reported. In the freshwater crayfish *Pacifastacus leniusculus*, the expression levels of *Pcrustin1* were shown to be higher in hemocytes of animals injected with both pathogenic and non-pathogenic bacteria than in those of controls. By contrast, *Pcrustin3* was induced to a lower extent by non-pathogenic *Acinetobacter sp.* in hemocytes, whereas *Pcrustin 2* did not show an obvious difference in expression among control and challenged animals [37].

Vatanavicharn and colleagues [78] provided evidence that crustins could also participate in the regulation of homeostasis in *P. monodon*. Type II crustin *PmPm5* transcripts were shown to be hardly detected in most tissue/organs of non-manipulated shrimp, including immune-associated tissues such as hemocytes or lymphoid organ, but were expressed in these and other sites when animals were subjected to a heat-shock treatment. This phenomenon could be also observed with the hemocytic Type II crustin *PmPm1*. Heat-shock induction is consistent with the findings of the presence of heat-shock elements (HSEs) in the 5′ upstream regulatory region of the crustin *PmPm5* gene. Similarly, the expression of both crustin *PmPm1* and crustin *PmPm5* genes was significantly up-regulated in shrimp epipodites after hyperosmotic stress. The marked induction of expression in shrimp subjected to abiotic challenges in contrast to its modest antimicrobial activity against a narrow range of Gram-positive bacteria may suggest that crustin *PmPm5* acts in shrimp physiology as a modulator of immune responses or tissue damage and injury-related stresses following bacterial infection.

Overall, the distinct patterns of gene expression after experimental infection and abiotic challenges along with a broad variability in the distribution of transcripts in different tissues and developmental stages reinforce the hypothesis that sustain crustins as players engaged in alternative functions in crustacean physiology beyond immune response.

### The multifunctional role of crustins

Functionally, crustins represent diverse molecules exhibiting multiple biological functions [17] (Fig. 4). In vitro studies using recombinantly expressed proteins have shown that crustins are mainly active against Gram-positive bacteria and, to a lesser extent, against Gram-negatives and yeasts [44] (Fig. 4). Indeed, the first crustin member, the Type I carcinin 11.5-kDa peptide, showed a narrow antimicrobial spectrum, being effective only against marine Gram-positive or salt-tolerant bacteria, including some pathogens for fish and lobsters [56]. Particularly, only a few crustin members have shown to be active against Gram-negative bacteria, such as *crusPau* from *P. paulensis* (Type IIA), *crustinPm7* (Type Iib) from *P. monodon*, and *SWDfc* from *F. chinesis* (Type III) [3,5,31] (Fig. 4). The mechanism of action and the spectrum of crustins against viruses and protozoans are hitherto unknown.

In *in vivo* studies using RNA interference (RNAi)-mediated post-transcriptional gene silencing (PTGS) have confirmed the participation of crustins in host immunity. The knockdown of shrimp Type II crustins causes an increase in mortality after infections with the bacterial pathogen *Vibrio peneaicaida*, but not in response to the filamentous fungus *Pusarium oxycorum* or the WSSV [30,61]. Similarly, the depletion of *Pc-crustin* 4 (Type I) transcripts in the red swamp crayfish *Procambarus clarkii* resulted in a higher mortality rate after bacterial infections [20]. Likewise, a higher survival rate was observed in *M. japonicus* shrimp injected with synthetic *MjCru* I-1 and subsequently infected with lethal doses of *Vibrio anguillarum* or *Staphylococcus aureus* [43], indicating that Type I crustins can also play a key role in crustacean antibacterial defenses (Fig. 4).

Evidence of the participation of crustins as protease inhibitors, on the other hand, has been described only for crustins from Types III and IV, and appears to be associated predominantly with the WAP domain (Fig. 4). For instance, the recombinant rSWD*Pm2* from *P. monodon* exhibited antiproteolytic activity against the bacterial protease subtilisin A [2]. Similarly, the recombinant rSWDfc from *F. chinesis* showed strong inhibitory activity against the proteases subtilisin A, trypsin, and proteinase K [31]. Interestingly, both rSWD*Pm2* and rSWDfc are anti-bacterial Type III crustins containing a short N-terminal region rich in proline and arginine residues. The recombinant Type III crustin rLSWD from *L. vannamei* showed antiproteolytic activities over bacterial proteases and adherent properties on both Gram-positive and Gram-negative bacteria [23]. By contrast, Type III crustins from *M. japonicus* and from *P. clarkii* have shown to display only anti- proteolytic activities, being devoid of antimicrobial action [21,22]. Interestingly, these crustin members lack the proline/arginine-rich region found in SWD*Pm2* and SWDfc, suggesting an important contribution of this region to the antimicrobial function of Type III crustins.

Unlike their crustin counterparts, all shrimp Type IV crustins reported to date have exclusively antiproteolytic activities, such as
MJ-DWD from *M. japonicus*, Fc-DWD from *F. chinensis*, and Pm-DWD from *P. monodon* [10,22,27]. However, the same is not true for Type IV crustins from other decapods. Li and colleagues [42] reported a Type IV crustin in *E. sinensis* (EsDWD) with evident antimicrobial activity against both Gram-positive and Gram-negative bacteria, and yeasts. Additionally, the recombinant rEsDWD showed strong adherence on both yeast and bacterial cells, and inhibitory activity against endogenous and commercial bacterial proteases.

In addition to their antimicrobial and antiproteolytic activities, crustins are also involved in other immune and non-immune functions (Fig. 4). More than an antimicrobial molecule, the Type I crustin MJ*Cru* I-1 from *M. japonicus* showed (i) to bind to bacterial surfaces, (ii) to promote bacterial agglutination, and (iii) to facilitate phagocytosis by hemocytes [43]. Moreover, Type I crustins showed to be expressed at sites of olfactory sensory neuron proliferation of the spiny lobster *Pandalus argus* [67] and in regenerating limbs of the fiddler crab *Carcinus maenas* [24], indicating a possible role in tissue regeneration. Crustins have also been shown to participate in the regulation of shrimp hematopoiesis (Fig. 4). In *P. monodon*, the Type IIa crustinPmP4 and the shrimp transglutaminase I form a non-conventional ribonucleoprotein complex that promotes a down-regulation in astakine protein expression, a cytokine involved in hematopoiesis [9]. These findings suggest that crustins, along with other immune-related proteins, may function as regulatory elements that provide a post-transcriptional gene regulation mechanism in crustaceans. Interestingly, the expression of crustins has also been spatio-temporally correlated with the establishment of ectosymbiotic microbial communities in the extremophile deep-sea hydrothermal vent shrimp *Rimicaris exoculata*, suggesting a key role in the regulation of natural microbiota [41] (Fig. 4).

Considering the broad molecular diversity found among crustin sequences, along with the distinct patterns of tissue distribution and gene expression regulation, it is not surprising that these molecules can perform multiple biological roles. In fact, given the vast amount of crustins described so far, we argue that antimicrobial activity might represent just one aspect of the biological relevance of these peptides in crustaceans. Moreover, we strongly encourage the development of functional studies with the aim to describe not only their relevance for application in marine biotechnology as a strategy to mitigate the impacts of infectious outbreaks, but also to understand the selective pressures that might have shaped the functional properties of this unique AMP family.

Concluding remarks

The demands for new tools to combat and control infectious diseases highlight the importance of studies focused on the exploitation of molecules with potential for use in shrimp farming and mitigation of the severe impacts caused in the productive sector. Antibiotics have been used to combat and prevent disease outbreaks, since there are no concrete alternatives to deal with this problem. However, this practice has become strongly discouraged due to (i) its severe impacts on the environment, (ii) the selection of antibiotic-resistant bacteria, and (iii) the contamination of the animals, making them unfit for human consumption. For these reasons, the discovery of environmentally friendly molecules naturally produced by living organisms and capable of reducing the viability of pathogenic microorganisms without inducing resistance are of great value in worldwide aquaculture and for application in marine biotechnology. In this context, studies on the diversity of immunological effectors in crustaceans, especially crustins, are important to expand knowledge about their molecular defense mechanisms and, also, to expand the repertoire of candidate immune effectors for use in different therapies. Additionally, considering the biotechnological potential of AMPs, understanding the diversity of these multifunctional molecules can pave the way for new research directions aimed at application in human and animal health.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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