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MINIREVIEW

Antimicrobial Peptides from Marine Invertebrates

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Marine invertebrates lack an acquired, memory-type immunity based on T-lymphocyte subsets and clonally derived immunoglobulins (72). This differs from the vertebrate immune system, which is characterized by somatic gene rearrangement, clonal selection, and expansion and a discriminative ability that includes lymphocytes, among other factors, which impart specificity and memory (71). Marine invertebrates rely solely on innate immune mechanisms that include both humoral and cellular responses. Humoral immunity in marine invertebrates is characterized by antimicrobial agents present in the blood cells and plasma (92), along with reactions such as hemolymph coagulation or melanization (79, 85). Cellular immunity in marine invertebrates is based on cell defense reactions, including encapsulation, nodule formation, and phagocytosis (92). The cellular component of marine invertebrate immunity is mediated by hemocytes, motile cells that phagocytize microbes and secrete soluble antimicrobial and cytotoxic substances into the hemolymph (53). This differs from insects, especially *Drosophila melanogaster*, which rely largely on the challenge-induced synthesis of antimicrobial peptides by the fat body (30, 88) and use exclusion, via a tough exoskeleton, as their major antimicrobial defense. The circulating hemolymph in marine invertebrates contains biologically active substances such as complement, lectins, clotting factors, and antimicrobial peptides (57). All of these factors contribute to a self-defense system in marine invertebrates against invading microorganisms, which can number up to 10^6 bacteria/ml and 10^9 virus/ml of seawater (2). The survival of marine invertebrates in this environment suggests that their innate immune system is effective and robust (52).

Antimicrobial peptides are a major component of the innate immune defense system in marine invertebrates. They are defined as molecules less than 10 kDa in mass which show antimicrobial properties (12) and provide an immediate and rapid response to invading microorganisms (8). The major classes of antimicrobial peptides include (i) α-helices, (ii) β-sheet and small proteins, (iii) peptides with thio-ether rings, (iv) peptides with an overrepresentation of one or two amino acids, (v) lipopeptides, and (vi) macrocyclic cystine knot peptides (24). There is evidence that antimicrobial peptides are widespread in invertebrates (15), especially in tissues such as the gut and respiratory organs in marine invertebrates, where exposure to pathogenic microorganisms is likely. In spite of variations in structure and size, the majority of antimicrobial peptides are amphiphilic, displaying both hydrophilic and hydrophobic surfaces. These peptides generally act by forming pores in microbial membranes or otherwise disrupting membrane integrity (82), which is facilitated by their amphiphilic structure. This mode of action is unlikely to lead to the development of resistance (9, 58), although it must be noted that this presumption is debatable (10). Recently, cationic antimicrobial peptides have been reported to be involved in many aspects of innate host defenses, associated with processes such as acute inflammation (25). The value of antimicrobial peptides in innate immunity lies in their ability to function without either high specificity or memory, and their small size makes them easy to synthesize (72). In addition, many antibacterial peptides show remarkable specificity for prokaryotes with low toxicity for eukaryotic cells (97). This is a characteristic that has favored their investigation and exploitation as potential new antibiotics (97).

The recent appearance of a growing number of bacteria resistant to conventional antibiotics has become a serious medical problem. To overcome this resistance, the development of antibiotics with novel mechanisms of action is a pressing issue (48). Endogenous antimicrobial peptides are exciting candidates as new antibacterial agents due to their broad antimicrobial spectra, highly selective toxicities, and the difficulty for bacteria to develop resistance to these peptides (11, 26, 47). The ocean covers 71% of the surface of the earth and contains approximately half of the total global biodiversity, with estimates ranging between 3 and 500×10^6 different species (28). Marine macrofauna alone comprise 0.5 to 10×10^6 species (23). Therefore, the marine environment, especially marine invertebrates that rely solely on innate immune mechanisms for host defense, is a spectacular resource for the development of new antimicrobial compounds. This minireview will encompass what is known about gene-encoded antimicrobial peptides from marine invertebrates, covering the phyla Arthropoda, Chordata, and Mollusca (Table 1).

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ARTHROPODA

Chelicerata. In 1982, a cationic protein that inhibits the *Limulus* hemolymph coagulation system was isolated in hemocyte lysate from the Japanese and American horseshoe crabs

TABLE 1. Antimicrobial peptides from marine invertebrates

Group	Species	Peptide	Class[a]	Reference
Tunicates	*Styela clava*	Styelin	α	46
		Clavanin	α	44
		Clavaspirin	α	45
	Styela plicata	Plicatamide	ND	86
	Halocynthia aurantium	Dicynthaurin	α (dimer)	43
		Halocidin	ND	34
	Halocynthia roretzi	Halocyamines	ND	4
Crustaceans	*Penaeus vannemi*	Penaeidins 1 to 3	β-3	18
		2.7, 7.9, and 8.3 kDa	ND	22
	Litopenaeus vannamei	*Lv*1 to 6	ND	8
	Litopenaeus setiferus	*Ls*1 to 3	ND	8
		Penaeidin 4	ND	17
	Carcinus maenas	6.5 kDa	ND	75
		Crustin Cm-1	ND	72
	Callinectes sapidus	Callinectin	ND	37
Chelicerates	*Tachyplesus tridentatus*	Tachyplesin I	β-2	68
		Big defensin	β-3	74
	Limulus polyphemus	Tachyplesin II	β-2	57
		Polyphemusin I and II	β-2	57
	Tachyplesus gigas	Tachyplesin III	β-2	66
Mollusks	*Mytilis edulis*	Defensins A and B	β-3	14
		Mytilin A and B	β-4	14
		Mytimicin	β-6	14
	Mytilis galloprovincialis	Myticin A and B	β-4	54
		Defensins 1 and 2	β-4	55
		Mytilin B, C, D, and G1	β-4	56

[a] Class assignments are as follows: α; amphipathic alpha helix; β; beta sheet (the number refers to the number of disulfide bonds). ND, not determined.

Tachypleus tridentatus and *Limulus polyphemus* and named anti-lipopolysaccharide (anti-LPS) factor (1, 61, 67, 83). Anti-LPS factor has strong antimicrobial effect on the growth of gram-negative R-type bacteria and shows hemolytic activity on red blood cells sensitized with LPS (69). Nakamura et al. (68) then isolated a new cationic peptide, tachyplesin, from acid extracts of large hemocyte granules of *Tachypleus tridentatus* (78) that are constitutively expressed and released after contact with microbial endotoxins by regulated exocytosis (33). Tachyplesin consists of 17 residues with a C-terminal arginine α-amide and four cysteine residues comprising two disulfide bridges (Fig. 1.).

Tachyplesin inhibits growth of gram-negative and -positive bacteria, the marine bivalve pathogens *Bonamia ostreae*, *Perkinsus marinus*, and *Vibrio* P1 (62), and forms a complex with bacterial LPS (29). Tachyplesin significantly inhibits the LPS-mediated activation of factor C in a manner similar to that of anti-LPS factor by binding to bacterial LPS to neutralize the factor C-activating activity of LPS (68). In addition, tachyplesin causes a rapid K^+ efflux from *Escherichia coli* cells concurrent with a reduced cell viability (50) by permeabilizing both bacterial and artificial lipid membranes (41, 51). Tachyplesin suppresses the development of cytopathic effects of human immunodeficiency virus by 70% when added during the adsorption period of the virus and has been shown to inactivate vesicular stomatitis virus and slightly inactivate influenza A virus (60, 65). The stability of tachyplesin at low pH and high temperatures apparently is due to the rigid anti-parallel β-hairpin structure connected by a β-turn that tachyplesin forms in aqueous solution (36, 41) (Fig. 2.). However, the disulfide bonded β-sheet structure may not be essential for antimicrobial activity (70), although it seems to play a role in maintaining antimicrobial activity in high salt concentrations (82). The three tandem repeats of a tetrapeptide sequence, hydrophobic amino

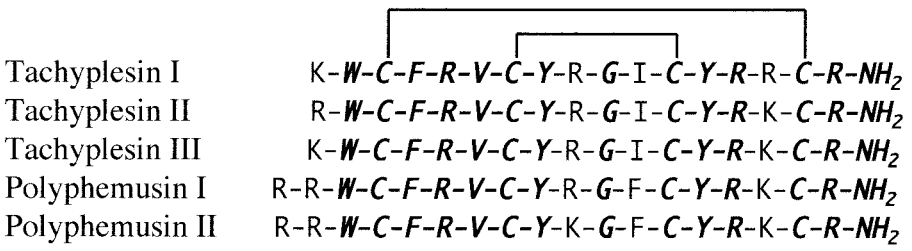

FIG. 1. Amino acid sequences of tachyplesins and polyphemusins isolated from horseshoe crabs. Identical amino acids are shown in bold and italics. The disulfide linkages between Cys-3 and Cys-16 and between Cys-7 and Cys-12 are shown by the heavy solid lines connecting the respective cysteine residues. (Adapted with permission from Muta et al. [66].)

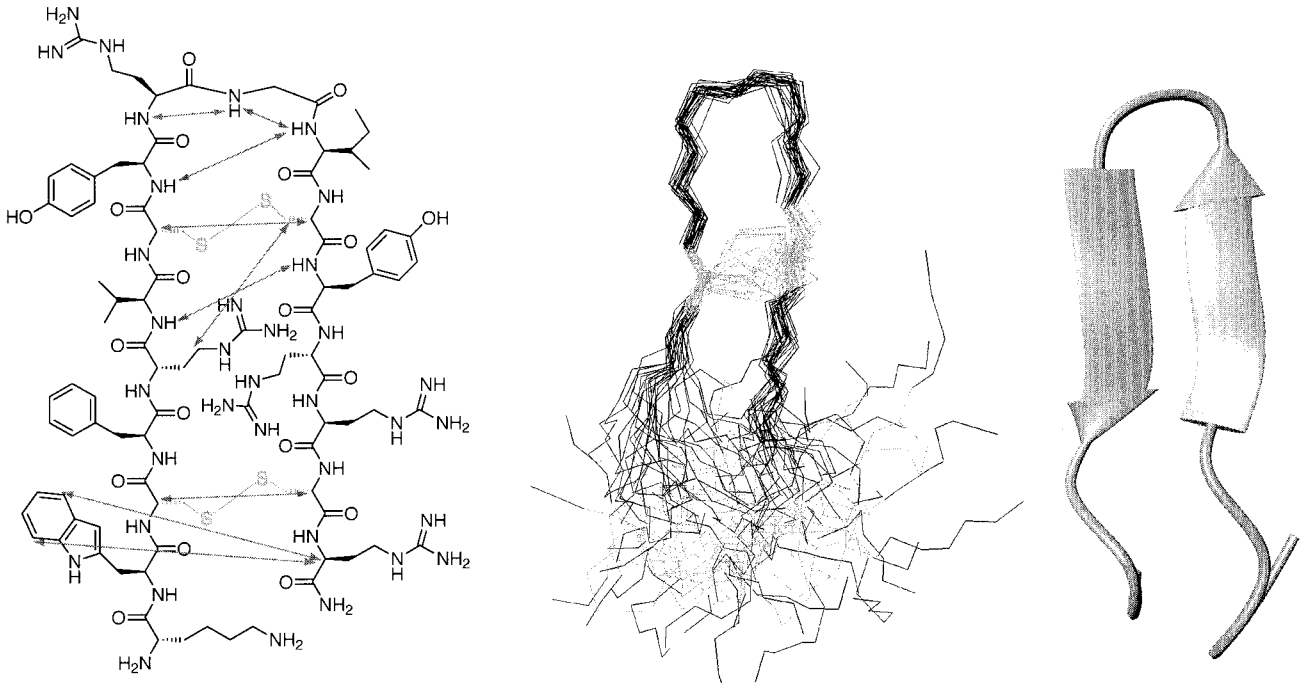

FIG. 2. Solution structure of tachyplesin I. (Left panel) Schematic representation showing selected nonsequential NOEs as gray arrows. Disulfide bonds and cysteine residues are shown in yellow. (Center panel) Composite of 30 solution structures. (Right panel) Minimized average structure in an aqueous environment. (Reprinted from reference 41 with the permission of the publisher.)

acid-Cys-hydrophobic amino acid-Arg, within the peptide indicate that its amphipathic nature, as confirmed by nuclear magnetic resonance structural investigations (36), is probably associated with biological activity (68). In view of the amphipathic cationic structure of tachyplesin and antiparallel β-sheets as a general DNA-binding motif, DNA binding using footprinting analysis of the peptide was examined and indicates that tachyplesin interacts with the minor groove of DNA duplex (96).

Additional studies on tachyplesin found isopeptides, tachyplesin II and tachyplesin III, and also polyphemusins I and II in hemocytes of the American horseshoe crab *Limulus polyphemus* and the southeast Asian horseshoe crab *Tachypleus gigas* (Fig. 1) (57, 66). Tachyplesin II differs from tachyplesin I in an Arg substitution in position 1, and both tachyplesin II and III differ from tachyplesin I by a Lys substitution in position 15. Polyphemusins I and II were found to contain 18 residues due to an additional Arg residue at the N terminus and differ from tachyplesin I by a Arg substitution in position 2, a Lys substitution in position 10 for polyphemusin II, a Phe substitution in position 12, and a Lys substitution in position 16. Tachyplesin II and polyphemusins I and II inhibited the growth of not only gram-negative and -positive bacteria but also fungi, including *Candida albicans* M9 (57). In addition, complex formation between these peptides and bacterial LPSs was observed (57), and polyphemusin I demonstrated an ability to translocate across lipid bilayers (98).

Sequence analysis of cloned cDNAs encoding tachyplesin precursors revealed that the precursors consist of 77 amino acids with (i) 23 residues in a presegment containing a putative signal peptide, (ii) a mature peptide with a peptide processing sequence and a C-terminal amidation signal (Gly-Lys-Arg), and (iii) an additional C-terminal sequence of 34 residues including an acidic amino acid cluster that may help balance the cationic portion of the tachyplesin peptide prior to processing (77). Interestingly, a tachyplesin peptide derivative with a C-terminal extension of glycine-lysine was found in the hemocytes of *Carcinoscorpius rotundicauda*, which appeared to be an intermediate derived from a tachyplesin precursor during processing to the mature form (66). Naturally occurring peptides containing amidated C termini have been reported in antimicrobial peptides, polypeptide toxins, and sarcotoxins previously and may impart proteolytic resistance, as well as contribute to an increased overall positive charge (39, 49, 87). Like tachyplesin, the porcine neutrophil peptides, protegrins, are composed of 16 to 18 amino acid residues and contain two intramolecular cysteine disulfide bonds (38).

In addition to the tachyplesin family of arthropod antimicrobial peptides, Saito et al. (74) described a novel defensin-like substance present in the hemocytes of *Tachypleus tridentatus* that inhibits the growth of gram-negative and -positive bacteria, as well as fungi. "Big defensin" consists of 79 amino acid residues, of which the C-terminal 37 residues have a sequence that is related to mammalian neutrophil derived defensins. The disulfide array of big defensin is identical to that of β-defensin from bovine neutrophils; however, big defensin has an extension of the N-terminal hydrophobic sequence with 35 amino acid residues, followed by the C-terminal cationic defensin portion (74). Again, the amphipathic nature of big defensin is presumably associated with the potent antimicrobial activity but, like the tachyplesins, data to support this hypothesis are not available.

FIG. 3. Sequence comparisons of penaeidins. (A) Mature penaeidin primary sequences. Proline and cysteine residues are in boldface, and identical amino acids are shaded gray. pE stands for pyroglutamic acid, and asterisks indicate C-terminal amidation. Assumed posttranslational modifications are in parentheses. Gaps were introduced to optimize the alignment. (B) N-terminal sequences of penaeidin precursors. Underlined amino acids indicate sequences absent from the mature molecules. Arrows indicate predicted cleavage sites. The "1" and "2" indicate preferential and secondary cleavage sites, respectively. Identical residues are shaded gray, and the dipeptide Glu-Ala found only in Pen-2 is in boldface. (Reprinted from reference 20 with the permission of the publisher.)

Crustacea. Antimicrobial peptide defense in Crustacea has long been suspected. In 1972, bactericidal activities were observed in the lobster *Homarus americanus* plasma (81) and hepatopancreas (59) but were absent from the plasma of the crab *Carcinus maenas* (20). This led White et al. (91) to speculate that elimination of pathogenic microorganisms from the bloodstream of crustaceans is likely to be mediated by hemocytes, a hypothesis that over the past decade has been supported by the isolation of several peptide factors displaying antimicrobial activities from crabs and shrimp. Prominent among crustacean antimicrobial peptides are the penaeidins, which display antifungal and antibacterial properties and were isolated from the hemolymph of the shrimp *Penaeus vannamei* (18). To determine the activity spectrum in more detail, large quantities of penaeidins were produced by using recombinant technologies. Penaeidins have broad-spectrum fungicidal activity against filamentous fungi but are inactive against yeasts such as *Candida albicans* or *Saccharomyces cerevisiae* (19). Interestingly, penaeidins are active against the shrimp pathogen *Fusarium oxysporum* where such infections remain unreported in *Penaeus vannamei*. Penaeidin antibacterial activity was found to be rather specific and is directed against gram-positive bacteria via a strain-specific inhibition mechanism that includes a rapid killing activity or bacteriostatic properties (19). Activity against gram-negative strains, including the *Vibrio* spp., which are responsible for many crustacean bacterial infections (73, 80), was minimal.

Penaeidins are synthesized as precursor molecules consisting of a 19- to 21-amino-acid signal peptide immediately preceding the bioactive compound (18). The penaeidins were purified in their active form (5.48 to 6.62 kDa) and fully characterized at the amino acid (Pen-1, -2, and -3a) and nucleic acid (Pen-2, -3a, -3b, and -3c) levels by using biochemistry and cDNA cloning (Fig. 3). Penaeidins are highly cationic and are composed of an N-terminal proline-rich region followed by a C-terminal domain stabilized by three intramolecular disulfide cross-links (20) (Fig. 3A) which initially attributes these peptides to the defensin class of antimicrobial peptides. However, by the placement of their cysteine residues, four of which are arranged in doublets, the penaeidins differ from all of the known defensin peptides (7, 94). The overall biological activity of the penaeidins may be associated with distinct properties of these two regions (7, 20). Pen-2 and -3a are C terminally amidated, and Pen-3a is blocked at the N terminus by a pyroglutamic acid that has been reported in some bovine β-defensins (76) and in the insect hymenoptaecin (13). These posttranslational modifications were found to have little effect on penaeidin antimicrobial properties (19) but may contribute to the stability of penaeidins that are highly resistant to proteolysis (18). Pen-2 has two additional amino acids (Glu-Ala) at

the C-terminal position of the precursor region so maturation must involve an additional processing step compared to Pen-3A (Fig. 3B). Cuthbertson et al. (17) reported the existence of a fourth class of penaeidins in the hemocyte cDNA library of *Litopenaeus vannamei* that shared an identical leader sequence with the previously described penaeidins while differing dramatically in the remainder of the peptide.

Hemocytes are the main site for penaeidin synthesis and storage (63) with penaeidins representing the bulk of proteinaceous material observed by reversed-phase high-pressure liquid chromatography in acid extracts from hemocytes (21). Granulocytes and hyaline hemocytes are the two major distinct groups or lineages of shrimp blood cells and are responsible for coagulation processes, phagocytosis, and encapsulation (21). Immunogold experiments show that penaeidins are contained in the large cytoplasmic granules of granular hemocytes but are absent from the hyaline cells (21). Penaeidin transcription is not upregulated in shrimp hemocytes after microbial challenge; however, challenge induces an increase in the penaeidin concentration in plasma. Thus, it has been speculated that penaeidins are released from hemocytes after intracellular degranulation and cell lysis in response to microbial stimulation (63). In addition, microbially challenged shrimp show increased immunoreactivity for penaeidins on the gill cuticle surface, where penaeidins can attach through their chitin-binding properties and ensure optimal protection of the whole animal (21). Munoz et al. (64) highlighted the potential involvement of penaeidins during larval development by using reverse transcription-PCR analysis to show that penaeidin mRNAs are present in the first early larval stage of Nauplius V in *Penaeus vannamei*. Destoumieux et al. (22) subsequently isolated antifungal peptides from the plasma of the shrimp *Penaeus vannamei* and *Penaeus stylirostris*. Three molecules with molecular masses of 2.7, 7.9, and 8.3 kDa were purified and displayed 95 to 100% sequence identity with a C-terminal sequence of hemocyanin, indicating that they are cleaved fragments of the shrimp respiratory protein. Thus, the production of antifungal peptides by proteolysis of hemocyanin may be relevant to shrimp immune reactions and would impart a new function to the respiratory pigment of crustaceans (22).

The hemocytes of the shore crab *Carcinus maenas* have been shown to contain broad-spectrum antibacterial activity, and similar activity is displayed by the hemocytes of several other crustacean species (15, 16). Schnapp et al. (75) reported the presence of several constitutive broad-spectrum antibacterial proteins in the hemocytes of *Carcinus maenas*. Partial N-terminal sequence analysis indicates that the smallest of this group (6.5 kDa) is proline-rich and 60% identical in a 28-amino-acid overlap with mature bactenecin 7, an antimicrobial peptide from bovine neutrophils that is a member of the cathelicidin family of mammalian antimicrobial peptides (75). Relf et al. (72) partially characterized a cysteine-rich 11.5-kDa gram-positive specific antibacterial peptide from *Carcinus maenas*, which is biochemically and functionally different from the 6.5-kDa protein but contains a disulfide domain signature, indicating that it might be a member of the four-disulfide core proteins. Expressed sequence tag analysis of hemocyte cDNA libraries from the shrimp *Litopenaeus vannamei* and *Litopenaeus setiferus* revealed transcripts with strong sequence similarity to the 11.5-kDa (crustin *Cm*-1) peptide isolated from *Carcinus maenas* (8). Analysis of these cDNA libraries gave six isoforms of this peptide in *Litopenaeus vannamei* (crustin *Lv*1 to *Lv*6) and three isoforms in *Litopenaeus setiferus* (crustin *Ls*1 to *Ls*3) (8). Callinectin is a cationic antimicrobial peptide of 3.7 kDa that represents the major antibiotic activity from the blue crab *Callinectes sapidus* (37). In addition, antimicrobial activity has been found in the hemolymph and hemocytes of the Northern shrimp *Pandalus borealis*, the hermit crab *Pagurus bernhardus*, the spider crab *Hyas araneus*, and the king crab *Paralithodes camtschatica*, although no primary sequence information has been reported for these compounds (27).

CHORDATA

Urochordata. Much of the work on antimicrobial peptides from the Urochordata has been performed on hemocytes of ascidians of the family *Styelidae*, which tend to be mildly acidic in nature. The clavanins, a family of four α-helical, amphipathic, histidine-rich antimicrobial peptides that contain 23 amino acids and exhibit C-terminal amidation were purified from the hemocytes of the ascidian *Styela clava* (44) (Fig. 4). Clavanins A to D resembled the magainins, well-characterized antimicrobial peptides from the skin of the amphibian *Xenopus laevis*, in size, primary sequence, and antimicrobial activity. Synthetic clavanin A displayed antimicrobial activity comparable to that of magainins and cecropins (44). The activity of clavanin A against *Escherichia coli*, *Listeria monocytogenes*, and *Candida albicans* was substantially greater at pH 5.5 than at pH 7.4, and clavanin A permeabilized the outer and inner membranes of *Escherichia coli* very effectively at pH 5.5 but not at pH 7.4. This is likely a function of the high net positive charge of clavanins at pH 5.5 due to the histidine component, which has a pK_a of ca. 6.5 (42). Clavanin A efficiently inserts into different phospholipid monolayers via hydrophobic interactions, suggesting that the membrane is the target for this molecule (90), probably through interactions with membrane proteins that generate transmembrane ion gradients (89). In addition, clavanins were broadly effective against gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*. cDNA sequence analysis indicates that clavanins are synthesized as 9.2-kDa prepropeptides. These prepropeptides contain a 19-residue signal peptide succeeded by a highly polar "pro" region with five glutamic acid residues, the mature clavanin peptide consisting of 23 residues, a glycine necessary for C-terminal amidation, and a C-terminal extension of 27 residues that is removed during processing (99). Using a polyclonal antibody and light and electron microscopy, Menzel et al. (52) showed that clavanins were present in the cytoplasm and/or cytoplasmic granules of five different types of granulocytes and that they occurred everywhere in the cytoplasm of macrophages. Degenerate PCR with primers corresponding to amino acids 10 to 16 of the clavanins identified a histidine-rich, amidated 23-residue antimicrobial peptide named clavaspirin from *Styela clava* (45). A synthetic clavaspirin was prepared that killed gram-positive and –negative bacteria, permeabilized the outer and inner membranes of *Escherichia coli*, lysed phosphatidylglycerol liposomes and was hemolytic toward human and bovine erythrocytes, all more effectively at an acidic pH (45).

Subsequent work on *Styela clava* generated two partially

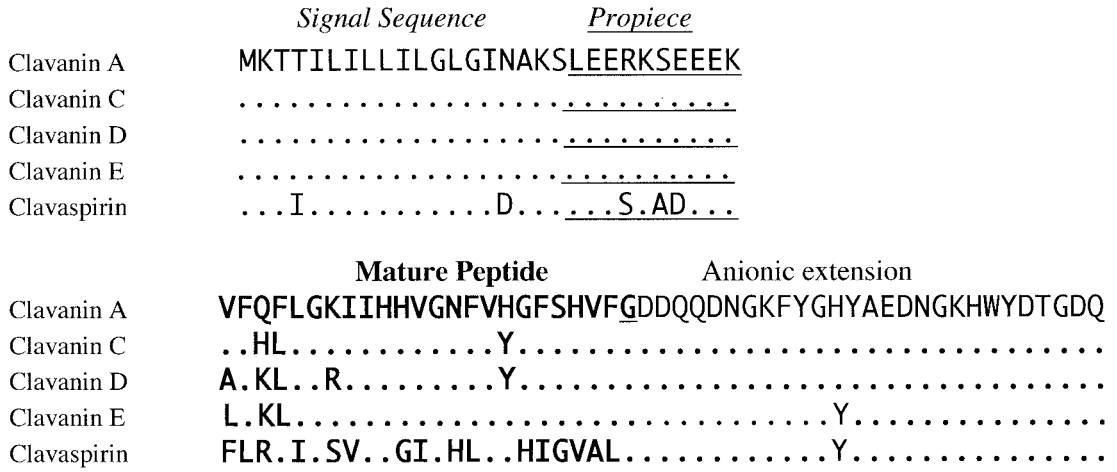

FIG. 4. Amino acid sequences of clavanins A, C, D, and E and clavaspirin. The full sequence of preproclavanin A is shown, and residues identical to clavanin A are indicated by a dot. Differing residues are shown. The mature peptides are in boldface type, and the anionic propieces are underlined. The C-terminal glycine (underlined) is converted to an amide in mature clavanins. (Reprinted from reference 45 with the permission of the publisher.)

characterized phenyalanine-rich antimicrobial peptides, styelin A and B, that are effective against gram-negative and -positive bacterial pathogens of humans, with low MICs even in the presence of high salt concentrations (46). Styelins also killed marine bacteria *Psychrobacter immobilis* and *Planococcus citreus* in 0.4 M NaCl which approximates seawater salt concentrations (46). Zhao et al. (100) cloned precursors of styelins C, D, and E from a pharyngeal cDNA library representing the hemopoietic tissue. The amino acid sequences resembled those of dipteran and lepidoteran preprocecropins, well-characterized antimicrobial peptides found in flies, moths, and butterflies, while the mature domain of styelin C resembled cecropin P1, an antimicrobial peptide isolated from porcine intestine. Styelins D and E differed significantly from styelins A to C but closely resembled each other. The styelins are highly basic polypeptides, encoded as prepropeptides, with a signal sequence and with cationic sequences in the mature protein counterbalanced by a polyanionic C-terminal extension in its precursor. Taylor et al. (84) characterized the remarkably extensive posttranslational modifications of styelin D, including two novel amino acids, dihydroxyarginine and dihydroxylysine, and two unusual ones, 6-bromotryptophan and 3,4-dihydroxyphenylalanine, as well as a C-terminal amidation. In addition, styelin D exhibits microheterogeneity due to differential hydroxylation of several lysine residues. Styelin D displayed activity against gram-negative and -positive bacteria in 200 mM NaCl, the role of the extensive posttranslational modifications possibly being to preserve activity under low-pH or high-salt conditions in which an unmodified synthetic analogue was considerably less active (84).

Plicatamide (Phe-Phe-His-Leu-His-Phe-His-dcΔDOPA), in which dcΔDOPA is decarboxy-(*E*)-α,β-dehydro-3,4-dihydroxyphenylalanine, is a potently antimicrobial octapeptide from the blood cells of the ascidian *Styela plicata* (86, 87). Wild-type and methicillin-resistant *Staphylococcus aureus* respond to plicatamide exposure with a massive potassium efflux that begins within seconds. Soon thereafter, treated bacteria largely cease consuming oxygen, and most become nonviable. Plicatamide forms cation-selective channels in model lipid bilayers composed of bacterial lipids. Methicillin-resistant *Staphylococcus aureus* treated with plicatamide contains prominent mesosomes, as well as multiple, small dome-shaped surface protrusions that suggest the involvement of osmotic forces in its antimicrobial effects. Plicatamide is potently hemolytic for human red blood cells but does not lyse ovine erythrocytes. Plicatamide is an interesting peptide because it violates conventional notions about antimicrobial peptides. Plicatamide contains only eight residues and is modestly cationic at pH 7.4, at which its activity is greatest. Typically, one expects such peptides to be cationic and amphipathic molecules with 16 to 40 residues (87), although two bovine peptides, dodecapeptide and indolicidin, are 12 to 13 amino acids long. In addition, plicatamide and other ascidian peptides (4, 85–87), including the halocyamines (below), are characterized by an oxidatively decarboxylated aromatic C-terminal residue. This structural feature may represent an alternative to C-terminal amidation for conferring proteolytic resistance and removal of the carboxylate's negative charge.

Halocyamine A and B are two antimicrobial tetrapeptides isolated from the hemocytes of the ascidian *Halocynthia roretzi* (4). Halocyamines, along with 5-S-GAD from hemocytes of the injured flesh fly *Sarcophaga peregrina*, are the only two antimicrobial peptides smaller than plicatamide that have been found in animals (4). The structures of halocyamine A and B were determined to be L-histidyl-L-6,7-dihydroxyphenylalanyl-glycl-6-bromo-8,9-didehydrotryptamine and L-threonyl-L-6,7-dihydroxyphenylalanyl-L-histidyl-6-bromo-8,9-didehydrotryptamine by spectroscopic analysis and degradation studies. Halocyamine A was reported to inhibit the growth of yeast, *Escherichia coli* (4) and the marine bacteria *Achromobacter aquamarinus* and *Pseudomonas perfectomarinus* (5, 6). In addition to antimicrobial activity, both halocyamine A and B showed cytotoxic activities against rat fetal brain neuronal cells, mouse neuroblastoma cells, and human hepatoma cells. Although the small, secondary metabolite-like halocyamines may be the cleavage product of larger gene encoded polypeptide precursors and not the product of

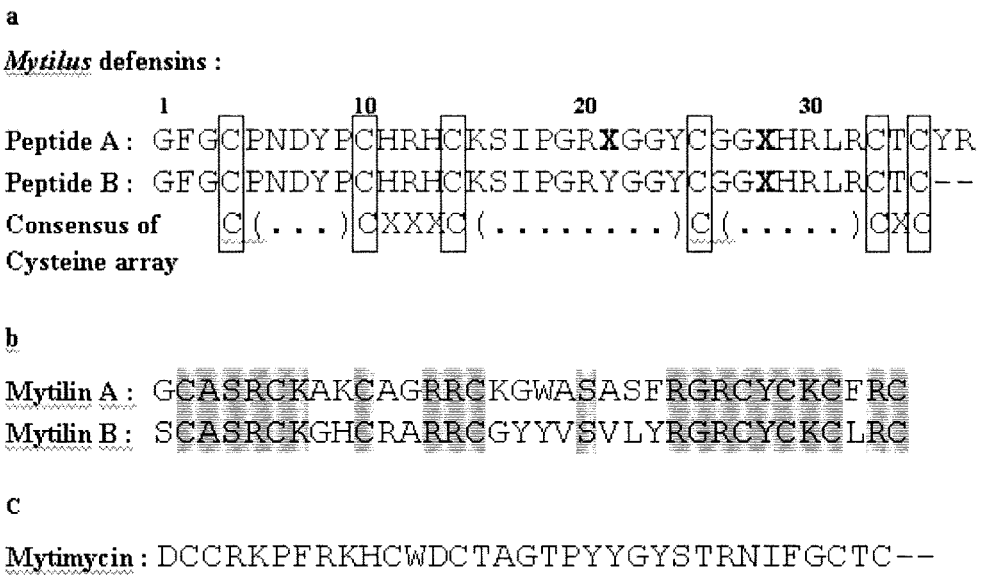

FIG. 5. Amino acid sequences of antimicrobial peptides isolated from *Mytilus edulis.* (a) *Mytilus* defensins A and B. Cysteines are boxed following the consensus cysteine array deduced from arthropod defensins. Unidentified residues are indicated by boldface X's. (b) Mytilins A and B. Identical residues are shaded. (c) Partial N-terminal sequence of *Mytilus* antifungal peptide mytimycin. (Reprinted from reference 14 with the permission of the publisher.)

nonribosomal synthesis (5, 85), it is important to note that there is no direct evidence to support this hypothesis. In addition to the halocyamines, antimicrobial peptides of 6.2 and 3.4 kDa have been isolated from *Halocynthia roretzi*. The first, dicynthaurin, is composed of two 30-residue monomers without any sequence homology to previously identified peptides. Most dicynthaurin molecules are C terminally amidated and are linked covalently with a single cysteine disulfide bond. In membrane-mimetic environments dicynthaurin displayed largely α-helical conformations (43). Dicynthaurin's broad-spectrum activity included *Micrococcus luteus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa* but not *Candida albicans* (43). The second, halocidin, has a mass of 3,443 Da and is composed of two subunits containing 18 and 15 amino acid residues that are linked by a single disulfide bond (34). In antimicrobial assays performed with synthetic congeners of halocidin, congeners of the 18-residue monomer were more active than those of the heterodimer or the 15-residue monomer against methicillin-resistant *Staphylococcus aureus* and multidrug-resistant *Pseudomonas aeruginosa* (34).

MOLLUSCA

Bivalvia. Mollusks rely predominantly on cellular defense reactions in which invading microorganisms are encapsulated by blood cells or phagocytosed (14). The presence of antimicrobial activity in Mollusca has been reported from the mucus of the giant snail *Achatina fulica* (31, 40), from the egg mass and purple fluid of the sea hare *Aplysia kurodai* (35, 93), and from the body wall of the sea hare *Dolabella auricularia* (32). Work on marine mollusks has focused on the mussels *Mytilus edulis* and *Mytilus galloprovincialis*. Charlet et al. (14) isolated from the blood of immune-challenged and untreated *Mytilus edulis* antibacterial and antifungal peptides. Two antimicrobial peptides, defensins A and B, were purified that were close in sequence and show a high degree of similarity with arthropod defensins, a large family of cysteine-rich cationic peptides (Fig. 5). The positions of the cysteines in arthropod defensins are highly conserved, and this array is identical to that of defensins A and B from *Mytilus edulis* (14). A defensin-like peptide,

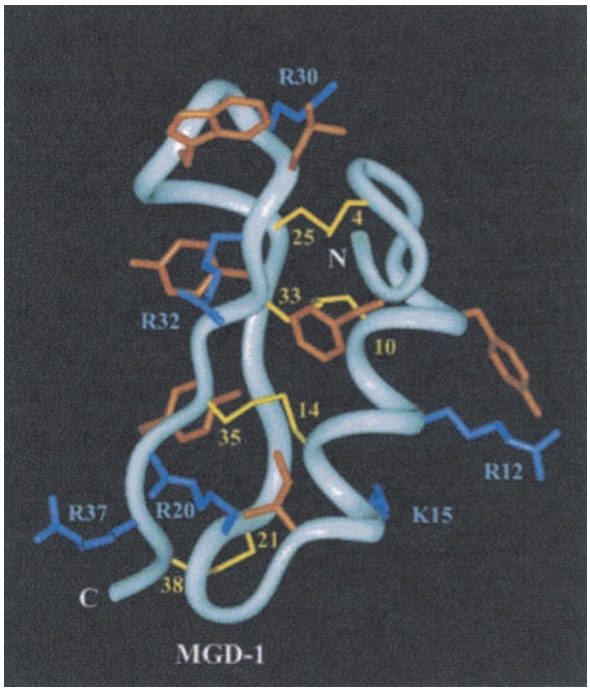

FIG. 6. MGD-1 solution structure. The location of the hydrophobic (orange) and positively charged (blue) side chains are clearly shown. Disulfide bonds are drawn as yellow lines. Only Lys and Arg side chains are labeled for the sake of clarity. (Reprinted from reference 95 with the permission of the publisher.)

MGD-1, and a second isoform, MGD-2, containing eight cysteines were isolated from the plasma and mRNA of the mussel *Mytilus galloprovincialis* (55) (Fig. 6.). MGDs are synthesized as precursors consisting of a signal peptide of 21 residues, the active peptide of 39 amino acids, and a 21-residue C-terminal extension that is rich in acidic amino acids. Bacterial challenge triggered an increase of MGD-1 in *Mytilus galloprovincialis* plasma and stimulated the release of MGD-1 and MGD-2 from hemocytes (55).

Mytilins A and B, cationic cysteine-rich antimicrobial peptides, were isolated and fully characterized from *Mytilus edulis* and showed no homology with known peptides in the peptide sequence database (14). The mytilin isoforms C, D, and G1 were isolated from *Mytilus galloprovincialis* and exhibited complementary antimicrobial properties (56). The mytilins are notably rich in cysteine residues with respect to their small size, indicating that their three-dimensional structure is highly compact (14), but the connectivity of their disulfide bonds has yet to be determined. In addition, a novel antifungal peptide that delays the growth of *Neurospora crassa* and *Fusarium culmorum*, mytimycin, has been isolated and partially characterized in conjunction with the defensins and mytilins from *Mytilus edulis* (14). Mytimycin shows no homology with reported peptide sequences in protein databases.

Myticins A and B, isolated from the hemocytes (A and B) and plasma (A) of the mussel *Mytilus galloprovincialis*, comprise 40 residues with four intramolecular disulfide bridges and a cysteine array different from that of previously characterized cysteine-rich antimicrobial peptides (54). Sequence analysis of the cloned cDNAs reveal that myticin precursors comprise 96 amino acids, including a signal peptide of 20 amino acids, the antimicrobial peptide sequence, and a C-terminal extension of 36 amino acids. This arrangement suggests that myticins are synthesized as preproproteins and processed before storage in hemocytes. Myticin A and B display antibacterial activity against gram-positive bacteria, and myticin B is active against the fungus *Fusarium oxysporum* and gram-negative bacteria *Escherichia coli* D31. In addition to *Mytilus edulis* and *Mytilus galloprovincialis*, antibacterial activity has been measured in unfractionated plasma from the mussel *Geukensia demissa* and from the oyster *Crassostrea virginica* (3).

CONCLUSION

Marine invertebrates have developed an effective use of their innate immune system to defend against pathogenic attack by microorganisms. Excellent examples of small, cationic, amphipathic peptides from the Arthropoda, Mollusca, and Urochordata are presented here to review what is known about innate immunity in marine invertebrates. Obviously, the field of marine invertebrate antimicrobial peptides is underdeveloped and provides the opportunity for a breadth of research on antimicrobial peptides. As pathogenic microorganisms continue to evolve resistance to conventional antibiotics, the development of novel antimicrobial agents will become a pressing issue. One may need to look no further than the immune system of the common marine invertebrate.

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