Molluscan Research 29(1): 17–26
http://www.mapress.com/mr/

ISSN 1323-5818
Magnolia Press

A review of advances in research on marine molluscan antimicrobial peptides and their potential application in aquaculture

LI CHENG-HUA[1*], ZHAO JIAN-MIN[2*] & SONG LIN-SHENG[2]

[1]Yantai Institute of Coastal Zone Research for Sustainable Development, Chinese Academy of Sciences, Yantai, 264003, China. Email: chli@yic.ac.cn

[2]Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, China. Email: lshsong@ms.qdio.ac.cn

Abstract

Antimicrobial peptides (AMPs) are important components of the host innate immune response against microbial invasion. They are usually characterized by their small-size, heat-stability and broad range of antimicrobial activity. This review covers research advances on marine mollusc AMPs, specifically those isolated from mussels, scallops, oysters, venerid clams and abalone, which mainly include MGD, mytilin, myticin, mytimycin, big defensin, and RPD-1. Their structural characteristics, antibacterial activity, and expression pattern as well as peptide distribution and their release following microbial challenge are also discussed. In addition, the prospect of the application of AMPs as food additives or their use in immunostimulation to prevent diseases of aquatic animals, as well as their potential hazards, are also discussed.

Key words: Innate immunity, immunostimulation, oysters, scallops, mussels, abalone

Introduction

The gene-encoded cationic antimicrobial peptides (AMPs), widely distributed in nature, are important mediators in the primary host defense system against bacterial and fungal pathogens to ensure either a systemic or a local protection of the host. Since the discovery of cecropin from the insect *Hyaophora cecropia* (Linnaeus, 1758) (Steiner *et al.* 1981), reports of the occurrence and characterisation of low-molecular-mass AMPs from a wide variety of organisms has accumulated rapidly. They are notable for their biochemical diversity, broad specificity against bacteria or fungi (Terras *et al*. 1993; Mor and Nicolas 1994; Casteels-Josson *et al*. 1994; Storici *et al*. 1996; Sitaram and Nagaraj 2002; Zasloff 2002), and also because some of them have anti-viral, (Murakami *et al*. 1991; Zhang *et al.* 1992; Frechet *et al*. 1994; Wachinger *et al*. 1998; Rozek *et al*. 2000; Sitaram and Nagaraj 2002; Roch *et al*. 2004), anti-tumoural (Gesell *et al*. 1997; Winder *et al*. 1998; Rozek *et al*. 2000) or wound-healing effects (Murphy *et al*. 1993; Fernandes *et al*. 2002). To date, over 1 000 AMPs have been identified from numerous phylogenetically diverse organisms (for detailed information see the Antimicrobial Sequences Database (http://www.bbcm.units.it/~tossi/amsdb.html).

In this review we first outline the classification and mode of action of AMPs in general and briefly discuss some issues in aquaculture and their potential utilisation as substitutes for antibiotics in aquaculture. We then review the state of knowledge of molluscan AMPs and some of their applications.

Classification of AMPs

Although all characterized AMPs have widely diverse sequences, they are tentatively classified into four distinct groups based on amino acid sequences, secondary structures, and functional similarities (Table 1): (1) the linear basic peptides forming amphipathic α-helices conformation and deprived of cysteine residues; (2) peptides containing cysteine residues with one to six intra-molecular disulphide bonds; (3) peptides with an over-representation of proline, arginine, glycine residues or tryptophan-rich peptide, such as apidaecins, drosocin, attacins and diptericins (Bachère *et al*. 2000; Lee *et al*. 2003); and (4) the peptides produced by the hydrolysis of large inactive or proteins with little activity, such as oncorhyncin I, oncorhyncin II and oncorhyncin III from histone H1 and H6 of fish *Oncorhynchus mykiss* (Walbaum, 1792) (Fernandes *et al*. 2002, 2003, 2004). Some researchers have also divided AMPs according to their original host, with five different types of AMPs usually recognised: insect, mammalian, plant, microbial and amphibian (Ma *et al*. 2008). Accordingly, AMPs from marine molluscs will be designated as molluscan AMPs.

Mode of action of AMPs

Despite their variations in structure and size, AMPs are usually characterised by their cationic and hydrophobic nature, which was considered crucial for the initial interaction between the peptide and bacterial membrane (Handcock and Chapple 1999). According to previous research, their mode of action was similar to a pore-forming action or by a detergent effect regardless of the actual targeted action (Destoumieux *et al*. 2000). Three models have been proposed to interpret the action mechanisms; the Barrel-stave model, the Toroidal model and the Carpet-like model (Figure 1). The three models are all established on the presumption that AMPs could initially interact with the bacterial cytoplasmic membrane by electrostatic bonding between the cationic peptide and the negatively charged components present on the outer bacterial envelope, such as phosphate groups within the Lipopolysaccharide (LPS) of gram-negative bacteria or lipoteichoic acids present on the surfaces of gram-positive bacteria.

TABLE 1. Summary of the AMPs identified in marine molluscs.

AMP Classification	AMP Characteristics	Molluscan Examples	References
Class 1	α-helices AMP without cysteine residues like magainin and cecropin	Not known	
Class 2	AMP with intra-molecular disulphide bonds, such as defensin, mylitin and myticin	MGD, mytilin, myticin, mytimycin from mussel (*Mytilus*, Mytilidae).	Charlet *et al*. 1996,Mitta *et al*. 1999, Mitta *et al*. 2000c, Mitta *et al*. 2000d
Class 3	AMP rich with a specific amino acids as apidaecin and attacins	Big defensin from scallops (Argopecten, Pectinidae)	Zhao *et al*. 2007 Wei *et al*. 2003,Gestal *et al*. 2007
Class 4	AMP from large inactive or little active proteins like Oncorhyncin I, II and III from *Oncorhynchus mykiss* (Walbaum, 1792)	RPD-1,mytilin and myticin from clams (*Ruditapes*, Veneridae)	Seo *et al*. 2005,Gueguen *et al*. 2006, Gonzalez *et al*. 2007
		Defensin from oysters (Crassostrea, Ostraeidae) and abalone (*Haliotis*, Haliotidae)	
		Not known	
		Scallops (Chlamys, Pectinidae) H2A derived AMP	Li *et al*. 2007

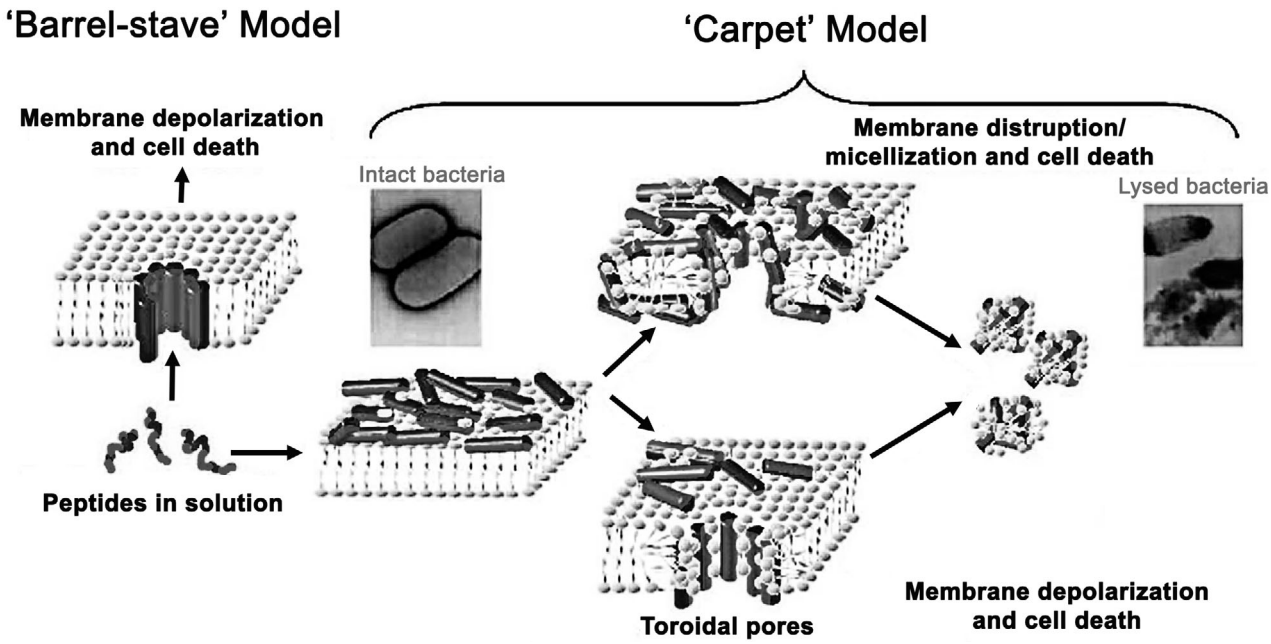

FIGURE 1. Three proposed bactericidal mechanisms of cationic antimicrobial peptides (modified from Shai *et al*. 2002, reproduced with permission). After binding to the bacterial cytoplasmic membrane by electrostatic bonding, the membrane is rendered permeable by the formation of transmembrane pores composed of a bundle of amphipathic helices as in the Barrel-stave model. The toroidal model differs from the barrel-stave model in that the peptides are always associated with the lipid headgroups. The lipid monolayer bends continuously from the top to the bottom in the fashion of a toroidal hole, so that the pore is lined by both the peptides and the lipid headgroups. In the 'carpet-like' mechanism, peptides act as a detergent and disrupt the membrane via, eventually, the formation of transitory pores.

Barrel-stave model: In this model, the attached peptides aggregate and insert into the membrane bilayer so that the hydrophobic peptide regions align with the lipid core region while the hydrophilic peptide regions form the interior region of the pore. The membrane is then permeabilised by the transmembrane pores composed of a bundle of amphipathic helices as observed in alamethicin (Baumann and Mueller 2004).

Toroidal model: In this model, lipids are inserted between helices to form a mixed pore as described in magainin (Ludtke *et al*. 1996). Lipids interpose magainin helices oriented perpendicular to the membrane surface such that the polar faces of the amphiphilic helices and the polar heads of the lipids constitute the pore lining. As a result, both membrane leaflets form a continuous surface, which allows for free diffusion of lipids between the outer and inner membrane layers (Figure 1).

Carpet-like model: A carpet-like mechanism was put forward to explain some AMPs without insertion into the hydrophobic core of the membrane to form transmembrane channels (Figure 1). After binding to the bacterial surface, the peptides reorient themselves so that their hydrophobic face is toward the lipids and the hydrophilic face toward the phospholipid head groups. When the peptides reach a threshold concentration, they are in contact with the phospholipid head group throughout the formation of transitory pores, eventually leading to membrane disintegration (Shai 1999).

Practical applications in aquaculture

In the last century, the discovery of antibiotics revolutionised the treatment of infectious diseases, leading to a dramatic reduction in morbidity and mortality, and also contributing significantly to the success of aquaculture. However, due to the overuse of antibiotics, the resistance of bacteria to antibiotics has drastically increased. There are also environmental risks with these substances being distributed through the environment. The question is whether AMPs are a suitable substitute for conventional antibiotics.

Due to their specific actions, AMPs have been shown to exert little or no toxic effect on healthy eukaryote cells, their real target being prokaryotes and abnormal eukaryote cells. Thus AMPs are considered to be promising candidates for screening as new antibiotics. More importantly, certain AMPs have shown the ability to inhibit viral infection (Murakami *et al*. 1991; Zhang *et al*. 1992; Frechet *et al*. 1994; Wachinger *et al*. 1998; Rozek *et al*. 2000; Sitaram and Nagaraj 2002; Roch *et al*. 2004). However, it appears to be impossible to predict antiviral activity based primarily on the secondary structures of peptides. The potential mechanism for antiviral activity would appear to be: (1) blocking of viral entry by heparan sulfate interaction; (2) blocking of viral attachment or entry into the host cell by interaction with specific cellular receptors; (3) blocking viral entry into the host cell by interaction with viral glycoproteins; (4) influencing host cell antiviral response or blocking viral gene/protein expression (Jenssen *et al*. 2006).

The aquaculture of molluscs is of considerable importance, as it contributes greatly to the economic development of many countries. Nearly 60% of shellfish (mainly molluscs) consumed by humans was produced by aquaculture (Qian *et al*. 2001), with abalone, scallop, oyster and mussel farming an important part of aquaculture worldwide, and they represent more than 50% of the shellfish consumed by humans (Qian *et al*. 2001). With the rapid development of mollusc aquaculture, many infectious and non-infectious diseases have been identified (Zhang *et al*. 1999) and have become the main limitations for development of this industry (Table 2). This situation is not helped by the limited knowledge of the physiology of these animals, particularly regarding their immune defence systems (Bachère *et al*. 2004). Characterisation of immune effectors would provide a better understanding of the immune defence mechanisms of molluscs and provide new insights into health management and disease control in mollusc aquaculture. AMPs, as an important immune effector in innate mollusc immunity, need to be investigated urgently. The remainder of this review presents recent findings regarding the antimicrobial properties of AMPs isolated from molluscs and the potential applications of AMPs in preventing diseases of aquatic animals.

TABLE 2. Major cultured mollusc species and their pathogens in China (modified from Zhang *et al*. 1999).

Species	Year of deaths	Death rate	Pathogen type
Ruditapes philippinarum (Adams & Reeve, 1850)	1987	100% in local place	*Vibrio*
Chlamys nobilis (Reeve, 1852)	1988	95%	*Vibrio*
Pinctada maxima (Jameson, 1901)	1992	97%	Rickettsia
Pinctada martensii (Dunker, 1872)	1992	52%	Rickettsia
Ostrea rivularis Tchang et Lou, 1956 (in part) (non Gould, 1861)	1992	80–90%	Rickettsia
Argopecten irradians (Lamarck, 1819)	1992–1993	70–80%	*Vibrio* and spherical virus
Haliotis discus (Reeve, 1846)	1993	90% (Larval stage)	*Vibrio* and spherical virus
Patinopecten yessoensis (Jay， 1857）	1994	50–60%	Not determined
Scapharca broughtonii (Schrenck, 1867)	1995	70%	Not determined
Mytilus edulis Linnaeus, 1758	1995	70%	Spherical virus
Bullacta exarata (Philippi, 1848)	1997	Large-scale mortality	*Vibrio*
Chlamys farreri (Jones & Preston， 1904)	1997	80–90%	*Vibrio* and virus
Haliotis diversicolor (Reeve, 1846)	1999	70%	*Vibrio*

MPs in molluscs

Molluscs rely predominantly on cellular responses mediated by haemocytes and humoral immune responses that employ AMPs to lyse invading microorganisms (Hooper *et al*. 2007). The presence of lower molecular mass AMPs started to be investigated in the last decade. The first AMP discovered in bivalve molluscs was in the 1990s through reverse genomics,

i.e. from biochemical purification of active peptides, to cDNA and gene sequencing (Hubert *et al*. 1996). To date, there are approximately 20 AMPs known that have been isolated or cloned from molluscs, mainly bivalves (Table 1). Mussels (Mytilidae).

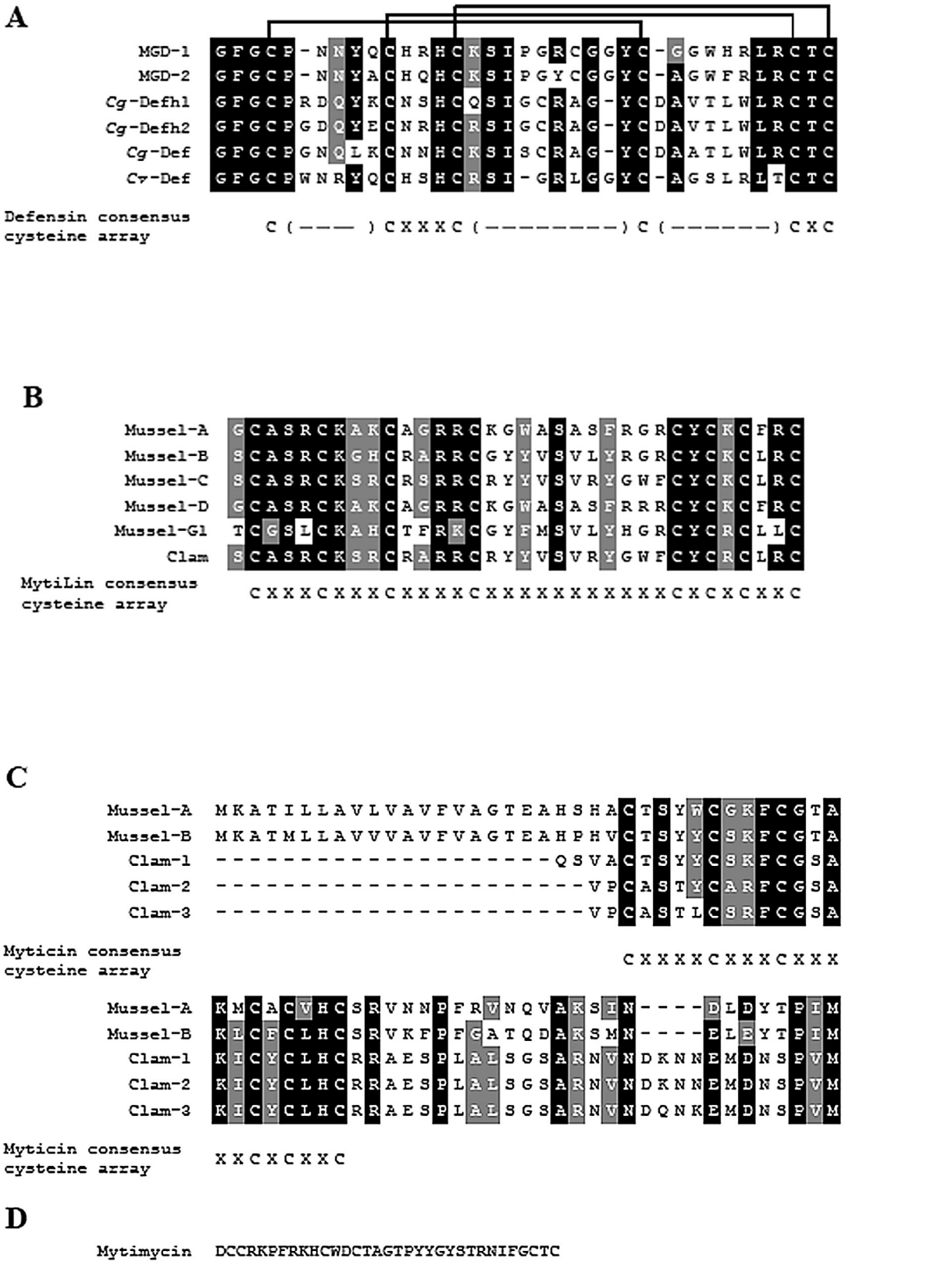

FIGURE 2. Diversity of the mussel antimicrobial peptide families: **A**. MGD. MGD1 and MGD2 were defensin cloned from *Mytilus*; Cg-Defh1, Cg-Defh2 and Cg-Def from *Crassostrea gigas* (Thunberg, 1793); Cv-Def was purified from *Crassostrea virginica* (Gmelin, 1791). **B**. mytilin. Mussel-A, B, C, D, and G1 are five isoform mytilins from *Mytilus edulis* Linnaeus, 1758 or *Mytilus galloprovincialis* Lamarck, 1819; clam indicated mytilin was cloned from *Ruditapes philippinesis* (Adams & Reeve, 1850). **C**.Myticin. Mussel-A and Mussel-B were two myticin from *M. galloprovincialis*; Clam-1, 2 and 3 indicated myticin identified from *Ruditapes decussatus* (Linné, 1785). **D**. Mytimycin from *M. edulis*.

Since the discovery of the first molluscan AMP from *Mytilus galloprovincialis* Lamarck, 1819 (Hubert *et al*. 1996), a total of 10 peptides have been identified from *Mytilus edulis* Linnaeus, 1758 and *M. galloprovincialis*. These AMPs were characterized by their hydrophobic and cationic properties, and they had an amphipathic structure (α-helix, β-hairpin-like β-sheet, β-sheet, or α-helix/β-sheet mixed structures) that was believed to be essential for their anti-microbial action (Bulet *et al*. 2004). These molecules were classified into four groups according to common features of their primary structure: MGD (defensins), mytilins, myticins and mytimycin.

MGD (Defensin): MGD is a 4 kDa antimicrobial peptide with two isoforms MGD1 and MGD2. MGD1 was purified from the plasma of *M. galloprovincialis*, while MGD2 was cloned from the haemocyte cDNA library (Mitta *et al*. 1999a). Sequence analysis indicated that MGD1 and MGD2 shared significant homology. The cysteine, hydrophilic and hydrophobic residues were highly conserved between them (Figure 2A). *In vivo*, MGDs were synthesized as precursors consisting of a putative signal peptide of 21 residues, the active peptide of 39 amino acids and a 21 residue carboxyl-terminal extension rich in acidic amino acids (Mitta *et al*. 1999a). These molecules had an unambiguous signature of arthropod defensins, but the presence of two extra cysteines and one modified amino acid suggested that it was a new member of that family (Hubert *et al*. 1996).

The three-dimensional structure of MGD1 was established using NMR analysis (Yang *et al*. 2000). A helical part (Asn7-Ser16) and two anti-parallel α-strands (Arg20-Cys25 and Cys33-Arg37) gave rise to the common cysteine-stabilized R-α motif, which was stabilized by four disulfide bonds (Cys4-Cys25, Cys10-Cys33, Cys14-Cys35, and Cys21-Cys38), instead of the three disulfide bonds commonly found in arthropod defensins. The fourth disulfide bond of MGD1 was not essential for its biological activity as it exerted similar bactericidal anti-Gram-positive activity to insect defensin A (Yang *et al*. 2000). The active domain was located by synthesizing a series of peptides corresponding to the main known secondary structures of MGD1 (Romestand *et al*. 2003). The region corresponding to residues 25–33 of MGD1 (CGGWHRLRC) was demonstrated to play central roles on the antimicrobial activity of MGD1, and the activity of the sequence 25–33 was strictly dependent on the bridging of Cys25 and Cys33. Modelling studies showed that positively charged and hydrophobic residues of MGD1 were organized in two layered clusters, which might have a functional significance in the docking of MGD1 to the bacterial membrane (Romestand *et al*. 2003).

Tissue location analysis at both the optical and ultra-structural levels showed MGD were predominantly located in vesicles of a granulocyte subclass of haemocytes containing small granules, and also in large clear granules of another granulocyte subclass. Immune reactivity of MGD existed in granular structures of enterocytes (Mitta *et al*. 1999a).

The expression pattern of the MGD2 had been analyzed in the haemocytes of animals subjected to various stress factors, as well as during larval development. Results suggested that in adult mussels, the MGD2 gene could be over-expressed with physical and temperature stress, but that reduced expression occurred with bacterial challenge (Mitta *et al*. 2000b). Moreover, the expression of MGD2 transcript was developmentally regulated, and was not detected until the completion of larval settlement and metamorphosis (Mitta *et al*. 2000b).

Mytilin: The second group of effector molecules in mussels, the mytilins family, consisted of five isoforms (A, B, C, D and G1) (Figure 2B). Isoforms A and B were isolated from *M. edulis* plasma (Charlet *et al*. 1996), and isoforms B, C, D and G1 from *M. galloprovincialis* haemocytes (Mitta *et al*. 2000d), which exhibited complementary antimicrobial properties to increase the antimicrobial capabilities of mussels. The cDNA sequence encoding mytilin B was obtained from a haemocyte cDNA library and the corresponding genome sequence was amplified from the genomic library (Mitta *et al*. 2000b). Compared with the purified peptide, it was demonstrated that mytilin B was synthesized as a precursor. The precursor contained a putative signal peptide of 22 residues, a processing peptide sequence of 34 amino acids, and a C-terminal extension of 48 residues rich in acidic residues (Mitta *et al*. 2000d).

Mytilins were found to be partially located in different sub-types of circulating haemocytes by confocal microscopy analysis, and exerted their microbicidal effect within the cells through the process of phagosome-mytilin granule fusion leading to the co-location of ingested bacteria and mytilins (Mitta *et al*. 2000a; d). As for their antimicrobial spectrum, mytilin A showed marked activities against many gram-positive strains: *Aerococcus viridans* Williams *et al*., 1953, *Bacillus megaterium* de Bary, 1884, *Micrococcus luteus* (Schroeter, 1872) (MIC: 0.6–1.2 mM), *Enterococcus faecalis* (Andrewes and Horder, 1906) (MIC: 2.5–5) and *Staphylococcus aureus* Rosenbach, 1884 (MIC: 5–10 mM), and had similar effects on the two gram-negative strains *Escherichia coli* (Migula, 1895) D31 (MIC: 2.5–5 mM) and *E. coli* D22 (MIC: 5–10 mM), and also affected the marine species *Alteromonas carrageenovora* Akagawa-Matsushita et al., 1992, *Pseudomonas alginovora*, and *Cytophaga drobachiensis* (MIC: 2.5–5 mM) (Charler *et al*,. 1996). A 10 amino acid derived fragment of mytilin located in two disulfide bonds in a stable β-hairpin structure was able to inhibit the mortality of the shrimp *Palaemon serratus* (Pennant, 1777) injected with white spot syndrome virus (Roch *et al*. 2008).

The expression pattern of the mytilin B indicated that it had a similar trend to that of MGD2 under various stress factors, as well as during larval development (Mitta *et al*. 2000b).

Myticin: Myticin was identified as a novel cysteine-rich peptide characterized from haemocytes (isoform A of 4.438 kDa and B of 4.562 kDa) and plasma (isoform A) of *M. galloprovincialis* (Figure 2, C). The two molecules displayed antimicrobial activity against gram-positive bacteria,

whereas only isoform B was active against the fungus *Fusarium oxysporum* and a gram-negative bacterium *E. coli* D31. The mature molecules comprise 40 residues with four intra-molecular disulfide bridges and a cysteine array in the primary structure different to that of the previously characterized cysteine-rich antimicrobial peptides. Sequence analysis of the cloned cDNAs revealed that myticin precursors consist of 96 amino acids with a putative signal peptide of 20 amino acids and a 36-residue C-terminal extension (Mitta *et al*. 2000c, d).

Mytimycin: This novel cysteine-rich antifungal peptide was isolated from the plasma of *M. edulis* with the molecular weight of 6233.5 Da. Twelve cysteines were engaged in the formation of six intra-molecular disulfide bridges (Figure 2D). A search in the peptide sequence data bases did not yield any homology with known peptides (Mitta *et al.* 2000d).

Scallops (Pectinidae)

An important AMP, Big defensin, was firstly isolated from horseshoe crabs. It includes a cationic cysteine-rich C-terminal defensin domain and a highly hydrophobic N-terminus. Its N-terminal region shows potent activity against gram-positive bacteria, while the C terminal defensin domain is more potent against gram-negative bacteria (Saito *et al*. 1995). In our laboratory, a mollusc counterpart of Big defensin (designated AiBD) was identified from the haemocyte cDNA library of the bay scallop *Argopecten irradians* (Lamarck, 1819). AiBD contained a typical signal peptide at the N-terminus, a transmembrane domain ranging from position 39 to position 58 and a defensin domain in the C-terminus. Multiple alignment of AiBD revealed that the hydrophobic region GAAAVT(A)AA at N-terminus and the consensus pattern C-X6-C-X3-C-X13(14)- C-X4-C-C in the defensin domain were highly reserved (Figure 3). The AiBD was a constitutive protein and the relative expression level of AiBD transcript was up-regulated evenly in the first 8 h, followed by a drastic increase, and increased 131.1-fold at 32 h after *Vibrio anguillarum* Bergeman, 1909 challenge (Figure 4). Biological activity assay with the recombinant protein showed its activity spectrum included both gram-positive and gram-negative bacteria, and also certain fungi (Zhao *et al*. 2007).

FIGURE 3. Multiple alignment of scallop *Argopecten irradians* (Lamarck, 1819)AiBD with big defensin from *Tachypleus tridentatus* (Leach, 1819) and defensin from *Branchiostoma belcheri singtaunese* (Gray, 1847) (Zhao *et al.* 2007). The black colour indicates positions where all three sequences share the same amino acid residue. Dashes indicate gaps inserted to improve the alignment.

Additionally, a potential AMP derived from the Zhikong scallop *Chlamys farreri* (Jones & Preston,1904), histone H2A, was also investigated in our group (Li *et al*. 2007). The N-terminal 39aa of H2A, homology to buforin I in vertebrates, was demonstrated to be a potential AMP with significant antibacterial activity. Currently, we are conducting experiments to confirm whether H2A is really involved in scallop immune response, with emphasis on the dynamic change of this peptide before and after microbial challenge.

Venerid clams (Veneridae)

The Big defensin in clams named RPD-1 was purified from the plasma of *Ruditapes philippinesis* (Adams & Reeve, 1850) (Wei *et al*. 2003), and no homologous peptides could be identified by databank mining using its N-terminal amino acid sequence. RPD-1 strongly inhibited the growth of Gram-negative and Gram-positive bacteria, and the minimal inhibitory concentration against *Staphylococcus aureus*, *Bacillus subtilis* (Ehrenberg, 1835), *Micrococcus tetragenus*, *Escherichia coli*, *Vibrio parahaemolyticus* (Fujinno *et al*., 1951), *V. anguillarum* was 9.6 mg/L, 76.8 mg/L, 38.4 mg/L, 76.8 mg/L, 19.2 mg/L, 19.2 mg/L respectively (Wei *et al*. 2003). Recently, clam myticin isoforms 1, 2 and 3, and clam mytilin, similar to myticins and mytilins previously identified in *M. galloprovincialis* (Figure 2B, C), were identified and characterized in *Ruditapes decussatus* (Linnaeus, 1758) (Gestal *et al*. 2007). Quantitative real time PCR analysis revealed that the expression levels of clam myticin and mytilin in haemocytes were increased after dead or alive *V. anguillarum* challenge. The highest expression levels were observed 48 h post-infection in both challenges (Gestal *et al*. 2007).

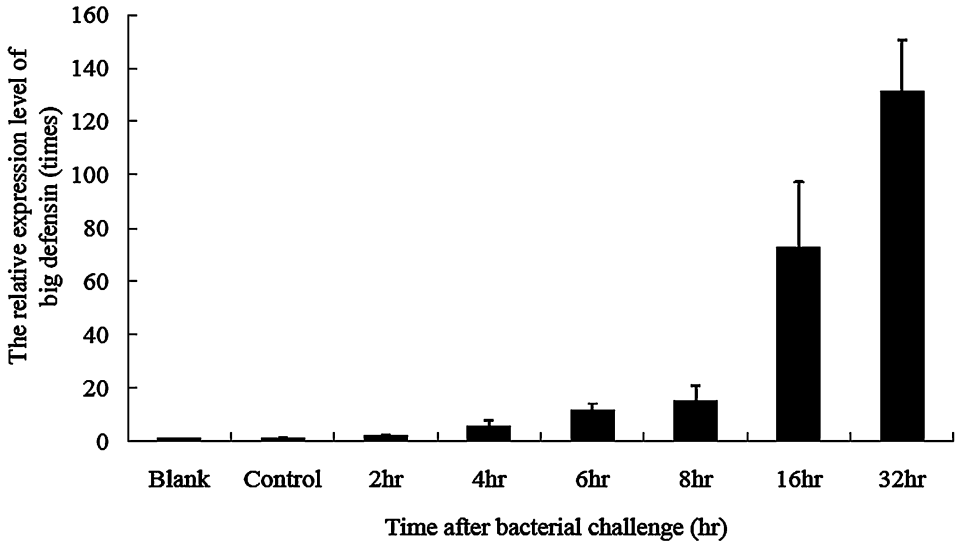

FIGURE 4. Temporal expression of the scallop *Argopecten irradians* (Lamarck, 1819) AiBD transcript in haemolymph after *Vibrio anguillarum* challenge (Zhao *et al.* 2007). All data were given in terms of relative mRNA expression as means with the error bars representing the standard deviation.

Oysters (Ostraeidae)

Defensin was the only antimicrobial peptide family having been identified and characterized in oysters. The first defensin was purified from acidified gill extract of the American oyster *Crassostrea virginica* (Gmelin, 1791) by preparative acid-urea-polyacrylamide gel electrophoresis and reversed-phase high performance liquid chromatography (Seo *et al*. 2005). The peptide (denoted as *Cv-Def*) had 38 amino acids with 6 cysteines and the molecular mass was 4265.0 Da. The primary structure of the peptide had a highly conserved consensus sequence pattern seen in most arthropod defensins: Cys1-[- - -]-Cys2-Xaa-Xaa-Xaa-Cys3-[- - -]-Gly-Xaa-Cys4-[- - -]-Cys5-Xaa-Cys6, indicating it was a member of the arthropod defensin family (Figure 2A). Activity spectrum assay showed *Cv-Def* had antimicrobial activity against both gram-positive bacteria and gram-negative bacteria, including some pathogenic microorganisms for *C. virginica* (Seo *et al*. 2005). Three defensins were also identified and characterized from the mantle (denoted as *Cg-Def*) or haemocytes (designated as *Cg-defh*1 and *Cg-defh*2) of the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) (Gueguen *et al*. 2006; Gonzalez *et al*. 2007), which shared the so-called cystine-stabilized alpha-beta motif (CS-αβ) with arthropod defensins, but were characterized by the presence of an additional disulfide bond, as previously observed in the mussel defensin (MGD-1) (Figure 2A).

Cg-Def was continuously expressed in the mantle, and the *Cg-Def* messenger concentration was unchanged after bacterial challenge in *Crassostrea gigas*. Furthermore, the recombinant peptide was active *in vitro* against gram-positive bacteria but showed no or limited activities against gram-negative bacteria and fungi. Regarding *Cg-defh*1 and *Cg-defh*2, their deduced amino acid sequences revealed two peptides of 73 amino acid residues with a mature portion consisting of 43 amino acid residues. *Cg*-Defh1 and *Cg*-Defh2 shared 86% amino acid identity and belong to the 'arthropod-mollusc defensin' family. qRT-PCR analysis indicated that *Cg-defh*2 was continuously expressed in the haemocytes of *C. gigas*. In addition, after a bacterial challenge, the level of *Cg-defh*2 transcripts decreased drastically in the circulating haemocyte population and this decrease could be correlated with an increase of *Cg-defh*2 transcripts in the gill and the mantle tissue, suggesting a possible migration of the haemocytes expressing *Cg-defh*2 towards the tissues are implicated as the first defence barrier of the oyster (Gonzalez *et al*. 2007).

Abalone (Haliotidae)

To date, there are no reports of abalone AMPs apart from several papers on antimicrobial or antiviral activity in the haemolymph (Hooper *et al*. 2007). The only sequence information was from random sequencing of the abalone cDNA library. Blast analysis revealed that one abalone EST was homologous to the defensin family, and was speculated to be an antimicrobial peptide. This work is under investigation by Professor Xiuqin Sun at the First Institute of Oceanography, State Oceanic Administration, People's Republic of China.

Discussion

Prospects for antimicrobial peptides as antibiotics in molluscan aquaculture

In view of the increasing microbial antibiotic resistance to available agents, there is a broad consensus that there needs to be much more effort to develop new anti-infection

drugs (Handcock and Chapple 1999; Bachère 2003; Jenssen *et al*. 2006). AMPs were considered as novel therapeutic candidates for their innate advantages of less bacterial resistance and very specific targets (Jenssen *et al*. 2006). To date, several antimicrobial peptides have been developed and entered into clinical trials, and there are also peptides that are currently in the preclinical development stage (Jenssen *et al*. 2006). However, because resistance is determined by the bacterial genome and its plasmid, there remains a possibility that bacteria might evolve resistance to AMPs if they are administered on a large scale in aquaculture over long periods (Serrano 2005). On the other hand, AMPs are likely to initiate antibody-based specific immune responses on their potential therapeutic use in humans, which also could limit their practical utility.

Another prospective application for AMPs is to develop disease-resistant strains of the cultured organisms. This has been very successful in crops through trans-genetic technology to integrate AMP genes into the plant chromosomes.(e.g., Liu 2007; Zhou *et al*. 2007). In molluscs, AMPs have mainly been adopted as molecular markers for molecular assisted selection breeding. The general approach is to infect molluscs in experimental conditions with a causative agent, and use the survival information in genetic evaluation to find the molecular marker. Selective breeding of oysters significantly increased growth as well as the resistance to MSX and QX disease (Zhang and Liu 2006). A frame chart of genetic improvement of molluscs had been derived from the breeding application of abalone and scallop (Zhang and Liu 2006). To our knowledge, no trans-genetic strains are currently available.

In other invertebrates, some AMPs were successfully utilised in viral control (Dupy *et al*. 2004). The synthetic AMP from *M. galloprovincialis* has been successfully utilised and can significantly reduce mortality of white spot syndrome virus in palaemonid shrimps (Dupy *et al*. 2004). This inspired us to adopt a new strategy to utilise these important effectors. We are currently engaged in unicellular eukaryotic expression systems of diatoms to produce scallop antimicrobial peptides, and to explore the feasibility of utilisation of trans-genetic algae as feed to enhance the innate immunity of abalone. Because of pathogen diversity (Table 2) and specific activity spectrum for most of AMPs, there was little chance of success with only one AMP, 'combination therapy' (utilisation of more than one AMP synchronously) seemed like an effective alternative means in practice. 'Combination therapy' could create an additive or synergistic effect analogous to a multidrug target approach – as for example in reducing the resistance to HIV therapies in humans (Jenssen *et al*. 2006). Many questions must be resolved before the practical implementation of AMPs, such as their destiny after they have entered the body of aquatic animals, and exactly how they exert their function. No matter what strategies are used in future, simultaneous assessment of their efficiency and their environmental safety is required. Taking into account the safety issues of transgenic aquaculture species that express AMPs, another alternative appears to be the simplest and most effective approach. This involves the use of immunomodulators to enhance *in vivo* expression, synthesis and release of AMPs. Chinese traditional medicine, β-glucans and polysaccharides were successfully implicated in aquatic disease-control as immunostimulants to some extent, and some of them could have increased expression of some of the AMPs (Bachère 2003; Costa *et al*. 2008). Other mechanisms have also been proposed to explain the mode of action of immunostimulants. In the crayfish *Pacifastacus leniusculus* (Dana, 1852), it has been demonstrated that, upon stimulation by β-glucans and polysaccharides, haemocyte exocytosis is activated leading to the release of effectors belonging to the proPO system, which can contribute to the elimination of microorganisms (Sritunyalucksana *et al*. 2000). In mussels, β-glucans increased the release of free oxygen radicals and also were able to enhance the phorbol 12-myristate 13-acetate (PMA) mediated effect on this haemocyte activity (Costa *et al*. 2008). The intrinsic relationship between the effective component of immunostimulants and prevention of mortality probably also varies from species to species (Costa *et al*. 2008). On the other hand, the dosage of immunostimulants was another key factor (Tseng *et al*. 2008). The optimal level for the administration of immunostimulants to improved disease resistance and enhanced immune activity should be carefully determined by trials before general use.

Prospects for the future use of mollusc AMPs

Antimicrobial peptides are critical in the immune defence reactions developed by living organisms to fight infection by microorganisms. The recent discovery of antimicrobial peptides in molluscs provides new clues for a fundamental understanding of molluscan immunity. Moreover, knowledge of the function, expression and regulation of AMPs will be particularly important for further establishment of disease control in mollusc aquaculture. More importantly, the expression profile of molluscan AMPs has been shown in oysters and scallops (Bachère *et al*. 2004; Gonzalez *et al*. 2007; Zhao *et al*. 2007) to be precisely regulated as they are in fruit flies (Leclerc and Rrichhart 2004). This indicates that similar signal transduction pathways like Toll and Imd exist in molluscs. Moreover, several molecules involved in the Toll pathway, such as Toll, Myd88, NF-κB, IκB, have already been identified by our group in scallops (unpublished data). The advancement in this area will greatly enrich our knowledge of the regulation of AMPs and assist in promoting the development of a new generation of therapeutic agents that will find potential applications in aquaculture or agronomy.

Acknowledgements

We thank the editor and reviewers for their valuable comments on earlier versions of our manuscript. This research was supported by Chinese Academy of Sciences Innovation Program (HK0810BX-049).

References

Bachère, E. (2003) Anti-infectious immune effectors in marine invertebrates: potential tools for disease control in larviculture. *Aquaculture* 227, 427–438.

Bachère, E., Destoumieux, D. & Bulet, P. (2000) Penaeidins, antimicrobial peptides of shrimp: a comparison with other effectors of innate immunity. *Aquaculture* 191, 71–88.

Bachère, E., Gueguen, Y., Gonzalez, M., Lorgeril, J., Garnier, J. & Romestand, B. (2004) Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. *Immunological Reviews* 198,149–168.

Baumann, G. & Mueller, P. (2004) A molecular model of membrane excitability. *Journal of Supeamolecular Structure* 2, 538–557.

Bulet, P., Stocklin, R. & Menin, L. (2004) Antimicrobial peptide: from invertebrate to vertebrate. *Immunological Reviews* 198, 169–184,

Casteels-Josson, K., Zhang, W., Capaci, T., Casteels, P. & Tempst P. (1994) Acute transcriptional response of the honeybee peptide-antibiotics gene repertoire and required post-translational conversion of the precursor structures. *Journal of Biological Chemistry* 269, 28569–28575.

Charlet, M., Chernysh, S., Philippe, H. & Hoffmann, J. (1996) Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. *Journal of Biological Chemistry* 271, 21808–21813.

Costa, M. M., Novoa, B. & Figueras, A. (2008) Influence of β-glucans on the immune responses of carpet shell clam (*Ruditapes decussatus*) and Mediterranean mussel (*Mytilus galloprovincialis*). *Fish and Shellfish Immunology* 24, 498–505.

Destoumieux, D., Munoz, M., Bulet, P. & Bachère, E. (2000) Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). *Cellular and Molecular Life Sciences* 57, 1260–1271.

Dupuy, J.W., Bonami, J.R. & Roch, P. (2004) A synthetic antibacterial peptide from *Mytilus galloprovincialis* reduces mortality due to white spot syndrome virus in palaemonid shrimp. *Journal of Fish Diseases* 27, 57–64.

Fernandes, J., Molle, G., Kemp, G. & Smith, V. (2003) Oncorhyncin III: a potent antimicrobial peptide derived from the non-histone chromosomal protein H6 of rainbow trout, *Oncorhynchus mykiss*. *Biochemical Journal* 373, 621–628.

Fernandes, J. M., Kemp, G. D., Molle, M. G. & Smith, V. J. (2002) Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Biochemical Journal* 368, 611–620.

Fernandes, J. M., Kemp, G. D. & Smith, V. J. (2004) Isolation and characterization of oncorhyncin II, a histone H1-derived antimicrobial peptide from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Developmental & Comparative Immunology* 28, 127–138.

Frechet, D., Guitton, J. D., Herman, F., Faucher, D., Helynck, G.., Monegier du, S., Ridoux, J. P., James-Surcouf, E. & Vuilhorgne, M. (1994) Solution structure of RP 71955, a new 21 amino acid tricyclic peptide active against HIV-1 virus. *Biochemistry* 33, 42–50.

Gesell, J., Zasloff, M. & Opella, S. J. (1997) Two-dimensional 1H NMR experiments show that the 23-residue magainin antibiotic peptide is an alpha-helix in dodecylphosphocholine micelles, sodium dodecylsulfate micelles, and trifluoroethanol/water solution. *Journal of Biomolecular NMR* 9, 127–135.

Gestal, C., Costa, M., Figueras, A. & Novoa, B. (2007) Analysis of differentially expressed genes in response to bacterial stimulation in hemocytes of the carpet-shell clam *Ruditapes decussatus*: Identification of new antimicrobial peptides. *Gene* 406, 134–143.

Gonzalez, M., Gueguen, Y., Desserre, G., Lorgeril, J., Romestand, B. & Bachère, E. (2007) Molecular characterization of two isoforms of defensin from hemocytes of the oyster *Crassostrea gigas*. *Developmental & Comparative Immunology* 31, 332–339.

Gueguen, Y., Herpin, A., Aumelas, A., Garnier, J., Fievet, J., Escoubas, J.M., Bulet, P., Gonzalez, M., Lelong, C., Favrel, P. & Bachère E. (2006) Characterization of a defensin from the oyster *Crassostrea gigas*. Recombinant production, folding, solution structure, antimicrobial activities, and gene expression. *Journal of Biological Chemistry* 281, 313–323.

Handcock, R. E. W. & Chapple, D. S. (1999) Peptide antibiotics. *Antimicrobial Agents and Chemotherapy* 43, 1317–1323.

Hooper, C., Day, R., Slocombe, R., Handlinger J. & Benkendorff K. (2007) Stress and immune responses in abalone: Limitations in current knowledge and investigative methods based on other models. *Fish and Shellfish Immunology* 22, 363–379.

Hubert, F., Noel, T. & Roch, P. (1996) A member of the arthropod defensin family from edible Mediterranean mussels (*Mytilus galloprovincialis*). *European Journal of Biochemistry* 240, 302–306.

Jenssen, H., Hamill, P. & Hancock, R. E. W. (2006) Peptide antimicrobial agents. *Clinical Microbiology Reviews* 19, 491–511.

Leclerc, V. & Reichhart J.M. (2004) The immune response of *Drosophila melanogaster. Immunological Reviews* 198, 59–71

Lee, S., Lee, B. & Soderhall, K. (2003) Processing of an antibacterial peptide from hemocyanin of the freshwater crayfish *pacifastacus leniusculus. Journal of Biological Chemistry* 278, 7927–7933.

Li, C., Song, L., Zhao, J., Zhu, L., Zou, H., Zhang, H., Wang H. & Cai, Z. (2007) The preliminary study on a potential antibacterial peptide derived from histone H2A in hemocytes of scallop *Chlamys farreri*. *Fish and Shellfish Immunology* 22, 663–672.

Liu, G. (2007) Five trans-genetic foods authorized and entered into the trial phase in China. *Beijing Agriculture* 12, 44–44.

Ludtke, S. J., He, K., Heller, W. T., Harroun, T. A., Yang L. & Huang, H.W. (1996) Membrane pores induced by magainin. *Biochemistry* 35, 13723–13728.

Ma, W., Liao F., Fan X. & Zhang W. (2008) Research and implication of bacterium's antibacterial peptide. *Biotechnology Bulletin* 3, 39–49.

Mitta, G., Vandenbulcke, F., Hubert, F. & Roch, P. (1999a) Mussel defensins are synthesized and processed in granulocytes then released into the plasma after bacterial challenge. *Journal of Cell Science* 112, 4233–4242.

Mitta, G., Vandenbulcke, F., Hubert, F. & Roch, P. (2000a) Involvement of mytilins in mussel antimicrobial defense. *Journal of Biological Chemistry* 275, 12954–12962.

Mitta, G., Hubert, F., Noel, T. & Roch P. (1999b) Myticin, a novel cysteine-rich antimicrobial peptide isolated from haemocytes and plasma of the mussel *Mytilus galloprovincialis*. *European Journal of Biochemistry* 265, 71–78.

Mitta, G., Vandenbulcke, F., Noel, T. & Roch, P. (2000b) Differential distribution and defense involvement of antimicrobial peptides in mussel. *Journal of Cell Science* 113, 2759–2769.

Mitta, G., Hubert, F. & Roch, P. (2000c) Mytilin B and MGD2, two antimicrobial peptides of marine mussels: gene structure and expression analysis. *Developmental & Comparative Immunology* 24, 381–393.

Mitta, G., Vandenbulcke, F. & Roch, P. (2000d) Original involvement of antimicrobial peptides in mussel innate immunity. *FEBS Letters* 486, 185–190.

Mitta, G., Vandenbulcke, F., Hubert, F., Salzet, M. & Roch, P. (2000e) Involvement of Mytilins in Mussel Antimicrobial Defense *Journal of Biological Chemistry* 275, 12954–12962.

Mor, A. & Nicolas, P. (1994) Isolation and structure of novel defensive peptides from frog skin. *European Journal of Biochemistry* 219, 145–154.

Murakami, T., Niwa, M., Tokunaga, F., Miyata, T. & Iwanaga, S. (1991) Direct virus inactivation of tachyplesin I and its isopeptides from horseshoe crab hemocytes. *Chemotherapy* 37, 327–334.

Murphy, G. J., Foster, B. A., Mannis, M. J., Selsted, M. E. & Reid, T. W. (1993) Defensins are mitogenic for epithelial cells and fibroblasts. *Journal of Cellular Physiology* 155, 408–413.

Qian, P., Wu, M. C. S. & Ni, I. (2001) Comparison of nutrients release among some maricultured animals. *Aquaculture* 200 305–316.

Roch, P., Beschin, A. & Bernard E. (2004) Antiprotozoan and antiviral activities of non-cytotoxic truncated and variant analogues of mussel defensin. *Evidence-based Complementary and Alternative Medicine* 1, 167–174.

Roch, P., Yang, Y., Toubiana, M. & Aumelas, A. (2008) NMR structure of mussel mytilin, and antiviral-antibacterial activities of derived synthetic peptides. Developmental *& Comparative Immunology* 32, 227–238.

Romestand, B., Molina, F., Richard, V. & Roch, P. (2003) Key role of the loop connecting the beta strands of mussel defensin in its antimicrobial activity. *European Journal of Biochemistry* 270, 2805–2813.

Rozek, A., Friedrich, C. L. & Hancock, R. E. W. (2000) Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles. *Biochemistry* 39, 15765–15774.

Rozek, T., Wegener, K. L., Bowie, J.H., Olver, I. N., Carver, J. A., Wallace, J. C. & Tyler, M. J. (2000) The antibiotic and anticancer active aurein peptides from the Australian bell frogs *Litoria aurea* and *Litoria raniformis*—the solution structure of aurein 1.2. *European Journal of Biochemistry* 267, 5330–5341.

Saito, T., Kawabata, S., Shigenaga, T., Takayenoki, Y., Cho, J., Nakajima, H., Hirata, M. & Iwanaga S. (1995) A novel big defensin identified in horseshoe crab hemocytes: isolation, amino acid sequence and antibacterial activity. *Journal of Biochemistry* 117, 1131–1137.

Seo, J. K., Crawford, J. M., Stone, K. L. & Noga, E. J. (2005) Purification of a novel arthropod defensin from the American oyster, *Crassostrea virginica*. *Biochemical and Biophysical Research Communications* 338, 1998–2004.

Serrano, P. H. (2005) Responsible use of antibiotics in aquaculture. *FAO Fisheries Technical Paper.* No. 469. Rome, FAO. 2005. 97p.

Shai, Y. (1999) Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochimica et Biophysica Acta (BBA). Biomembranes* 1462, 55–70.

Shai, Y. (2002) Mode of action of membrane active antimicrobial peptides. *Biopolymers* 66, 236–248.

Sitaram, N. & Nagaraj, R. (2002) Host-defense antimicrobial peptides: importance of structure for activity. *Current Pharmaceutical Design* 8, 727–742.

Steiner, H., Hultmark, D. & Engstorm, A. (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 292, 246–248.

Storici, P., Tossi, A., Lenarcic, B. & Romeo, D. (1996) Purification and structural characterization of bovine cathelicidins, precursors of antimicrobial peptides. *European Journal of Biochemistry* 238, 769–776.

Sritunyalucksana, K. & Söderhäll, K. (2000) The proPO and clotting systems in crustaceans. *Aquaculture* 191, 53– 69.

Terras, F. R. G., Torrekens, S., van Leuven, F., Osborn, R. W., Vanderleyden, J., Cammue, B. P. & Broekaert, W. F. (1993) A new family of basic cysteine-rich plant antifungal proteins from Brassicaceae species. *FEBS Letters* 316**,** 233–240.

Tseng, D., Ho, P., Huang, S., Cheng, S., Shiu, Y., Chiu, C. & Liu, C. (2008) Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20. *Fish and Shellfish Immunology* 26, 339–344.

Wachinger, M., Kleinschmidt, A., Winder, D., von Pechmann N., Ludvigsen, A., Neumann M., Holle R., Salmons B., Erfle V. & Brack-Werner R. (1998) Antimicrobial peptides melittin and cercropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. *Journal of General Virology* 79, 731–740.

Wei, Y., Guo, D., Li, R., Chen, H. & Chen, P. (2003) Purification of a big defensin from *Ruditapes philippinesis* and its antibacterial activity. *Acta Biochimica et Biophysica Sinica* 35, 1145–1148.

Winder, D., Gunzburg, W. H., Erfle, V., & Salmons, B. (1998) Expression of antimicrobial peptides has an antitumor effect in human cells. *Biochemical and Biophysical Research Communications* 242, 608–612.

Yang, Y., Mitta, G., Chavanieu, A., Calas, B., Sanchez, J. & Roch, P. (2000) Solution structure and activity of the synthetic four-disulfide bond Mediterranean Mussel defensin (MGD-1). *Biochemistry* 39, 14436–14447.

Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415, 389–395.

Zhang, G. & Liu, X. (2006) Theory and method of genetic improvement in mariculture mollusks: a review. *Journal of Fisheries of China* 30, 130–137.

Zhang, G., Li, X. & Xue, Z. (1999) Potential reasons and controlling strategies of mollusk dramatic death in China. *Chinese fishery* 9, 34–39.

Zhang, X. L., Selsted, M. E. & Pardi, A. (1992) NMR studies of defensin antimicrobial peptides. 1. Resonance assignment and secondary structure determination of rabbit NP-2 and human HNP-1. *Biochemistry* 31, 11348–11356.

Zhao, J., Song, L., Li, C., Ni, D., Wu, L., Zhu, L., Wang H. & Xu W. (2007) Molecular cloning, expression of a big defensin gene from bay scallop *Argopecten irradians* and the antimicrobial activity of its recombinant protein. *Molecular Immunology* 44, 360–368.

Zhou, S., Chen, X., Xue, X., Zhang, X. & Li, Y. (2007) Physiological and growth responses of tomato progenies harboring the betaine alhyde dehydrogenase gene to salt stress. *Journal of Integrative Plant Biology* 49, 628–637.