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REVIEW

Molluscan antimicrobial peptides, a review from activity-based evidences to computer-assisted sequences

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Abstract

Antimicrobial peptides (AMPs) represent the most universal immune effectors. Molluscs constitute the second largest animal phylum, after Arthropods, in term of number of species. Only a negligible number has been investigated regarding AMPs. The choice of the species to be studied relied on their economical importance and availability. First studies on molluscan AMPs dated from 1996 and were based on biological activities of biochemical-purified fractions. Such approach released all the original structures we know, with biological activity sometimes different from one isoform to another. Then, molecular biology techniques were applied to molluscan AMPs starting in 1999. Complete screening of genome expression in various situations became available, as well as some exotic Molluscs, the ones collected from deep-sea hydrothermal vent, for instance. Full sequences of active peptides and precursors, and gene organizations were established. A breakthrough consisted in the discovery of numerous AMP variants, even within the same animal. In addition, computer homology revealed the existence of already known AMPs in new studied molluscan species.

Key Words: antimicrobial peptide; AMP; defensin; mussel; oyster; Bivalves; Gastropods

Introduction

Thousands of bioactive compounds identified in marine organisms reveal that sea creatures constitute a large reservoir for pharmacologically active drugs. Recently reviewed by (Mayer *et al.*, 2011), most of these active molecules are enzymatically synthesized. Exceptions are rare and consist on venoms and toxins, as regarding the Gastropod genus *Conus* with an estimated 50,000 different conotoxins (12-24 aa, 4-6 cysteines) reported for their neurotoxic effects, for instance (Han *et al.*, 2006). The first report on antibacterial activity observed in Molluscs was from mucus of the giant snail *Achatina fulica*. Named achacin, the protein of 150 kDa is composed of 2 subunits (Iguchi *et al.*, 1982; Kubota *et al.*, 1985). Due to its large MW, such molecule cannot be assimilated to a peptide. Also from Gastropod, the sea hare, *Aplysia kurodai,* were the bacteriostatic glycoproteins, aplysianin P, A and E, of 60-320 kDa (Kamiya *et al.*, 1984; Kamiya *et al.*, 1986; Kisugi *et al.*, 1987; Yamazaki *et al.*, 1990). Dolabellanin A from albumen gland (Kisugi *et al.*, 1989), C from coelomic fluid (Kisugi *et al.*, 1989), E from egg mass (Kamiya *et al.*, 1984), P from purple fluid (Yamazaki *et al.*, 1989), and B2 from skin and mucus (Iijima *et al.*, 2003) have been reported in another sea hare, *Dolabella auricularia*. It was only in 1996, that true antimicrobial peptides (*i.e.*, cationic, 4-6 kDa) have been isolated simultaneously from the Mediterranean

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List of abbreviations:
aa, amino acids; AMP(s), antimicrobial peptide(s); cds, coding sequence; EST, expressed sequence tags; ISH, *in situ* hybridization; PCR, polymerase chain reaction; SSH, suppression subtractive hybridization

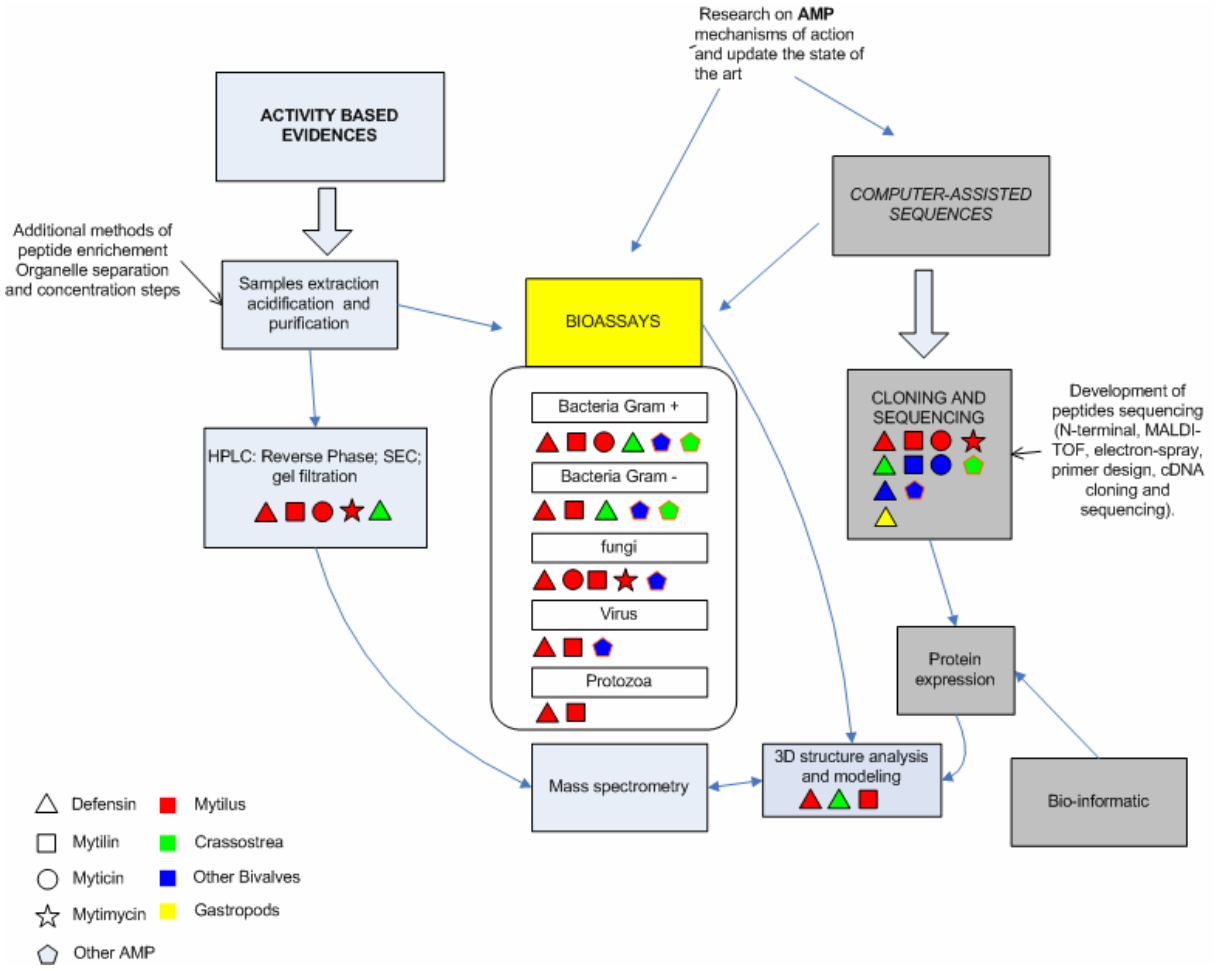

Fig. 1 Summary of the pathways regarding the discovery of Mollusc AMPs, linking species and molecules to the applied technologies.

mussel, *Mytilus galloprovincialis* (Hubert *et al.*, 1996a) and the blue mussel, *M. edulis* (Charlet *et al.*, 1996).

Previous mini-review related to AMPs from marine invertebrates dated from 2004 (Tincu and Taylor, 2004). At that time, only the biochemical approach was presented regarding the sole mussels with 4 defensins, 2 mytilins, 2 myticins and one partially characterized mytimycin. Since that time, knowledge on AMPs increased dramatically due to molecular biology techniques applied to invertebrates. More recent reviews included new discoveries, but were restricted to mussels, *Mytilus edulis, M. galloprovincialis* and oysters, *Crassostrea gigas, C. vir*ginica (Fleury *et al.*, 2008) or adding the Venerid clams, *Ruditapes* (Li *et al.*, 2009a). Since that time, 35 new AMPs isolated from 12 different molluscan species were released (Fig. 1). The present review will consider the same major mussel AMPs, now reported also from other molluscan species, and extended to some exotic AMPs grouped in as miscellaneous, organized chronologically moving from traditional antimicrobial activities assayed in biochemically-purified fractions, to recently applied computer-assisted nucleotide sequence investigations and data mining (Table 1).

Activity-based evidences

Defensin is probably the best known and appropriate term related to an immune effector molecule. It was introduced in 1985, regarding human neutrophils (Ganz *et al.*, 1985) following the discovery of antimicrobial activity and gene organization of the so-called arginine-rich cationic peptides reported in rabbit and guinea-pig leukocyte lysates in the 60's. Later, reviewers regarding anti-Gram positive peptides isolated from the flesh fly, *Phormia terranovae*, forced the term "insect defensin" (Lambert *et al.*, 1989; Cornet *et al.*, 1995). Mammalian and insect defensins resemble as 3 - 4 kDa peptides with 6 cysteines engaged in three intra molecular disulphide bonds. Meanwhile, knowledge of their 3-dimensional structures revealed crucial differences: 3 domains for insect defensins -one N-terminal loop, one amphipathic α-

Table 1 Chronology of AMP discovery in Molluscs

Species	AMP name	Nature	Technology used	Date	Reference
Mytilus galloprovincialis	defensin (MGD-1)	aa	HPLC	1996	Hubert *et al.*
Mytilus edulis	2 defensins (peptide A, B)	aa partial	HPLC	1996	Charlet *et al*.
Mytilus edulis	2 mytilins (A, B)	aa	HPLC	1996	Charlet *et al.*
Mytilus edulis	mytimycin	aa partial	HPLC	1996	Charlet *et al.*
Mytilus galloprovincialis	defensin (MGD-2)	nucleotides	cDNA	1999	Mitta *et al.*
Mytilus galloprovincialis	2 myticins (A, B)	aa partial	HPLC	1999	Mitta *et al.*
Mytilus galloprovincialis	3 mytilins (C, D, G1)	aa	HPLC	2000	Mitta *et al.*
Crassostrea virginica	big-defensin	nucleotides	cDNA	2001	database
Ruditapes philippinarum	big-defensin RPD-1	aa partial	HPLC	2003	Wei *et al.*
Mytilus trossolus	mytilin-C	nucleotides	mRNA	2004	database
Crassostrea virginica	defensin (AOD)	aa	HPLC	2005	Seo *et al.*
Biomphalaria glabrata	2 AMPs	nucleotides	EST	2005	Mitta *et al.*
Argopecten irradians irradians	2 defensins	nucleotides	EST	2006	Song *et al.*
Crassostrea gigas	defensin (*Cg*-Def)	nucleotides	EST	2006	Gueguen *et al.*
Crassostrea virginica	2 defensins	nucleotides	BAC	2006	Cunningham *et al.*
Argopecten irradians	big-defensin *Ai*BD	nucleotides	cDNA	2007	Zhao *et al.*
Crassostrea gigas	2 defensins (*Cg*-defh1, -2)	nucleotides	mRNA	2007	Gonzalez *et al.*
Bathymodiolus azoricus	mytilin	nucleotides	cDNA	2007	Bettencourt *et al.*
Ruditapes decussatus	mytilin	nucleotides	SSH	2007	Gestal *et al.*
Ruditapes decussatus	3 myticins (1-3)	nucleotides	SSH	2007	Gestal *et al.*
Chlamis farreri	histone H2A	nucleotides	cDNA	2007	Li *et al.*
Mytilus galloprovincialis	myticin-C	nucleotides	SSH	2008	Pallavicini *et al.*
Haliotis discus hannai	defensin (hd-def)	nucleotides	cDNA	2008	Hong *et al.*
Mercenaria mercenaria	big-defensin, defensin	nucleotides	SSH & cDNA	2009	Parrigault *et al.*
Haliotis discus discus	abhisin	nucleotides	cDNA	2009	De Zoysa *et al.*
Bathymodiolus thermophilus	defensin	nucleotides	SSH	2009	Boutet *et al.*
Littorina littorea	littorein	activity	HPLC	2009	Defer *et al.*
Crassostrea gigas	prolin-rich (*Cg*-Prp)	nucleotides	EST	2009	Gueguen *et al.*
Argopecten purpuratus	proline-rich AMP	aa	HPLC	2009	Arenas *et al.*
Hyriopsis cumingii	2 defensins	nucleotides	EST	2009	Bai *et al.*
Haliotis discus discus	defensin	nucleotides	cDNA	2010	De Zoysa *et al.*
Venerupis philippinarum	big-defensin *Vp*BD	nucleotides	EST	2010	Zhao *et al.*
Mytilus coruscus	2 mytilins (A, B)	nucleotides	cDNA	2010	database
Mytilus coruscus	6 mytilins (3-8)	nucleotides	cDNA	2010	database
Mytilus coruscus	9 myticins (1-9)	nucleotides	cDNA	2010	database
Crassostrea virginica	histone cv-H2B-1	aa partial	HPLC	2010	Seo *et al.*
Crassostrea virginica	histone cv-H2B-2, -3, -4	aa partial	HPLC	2011	Seo *et al.*
Haliotis laevigata	2 antiviral molecules	activity	none	2011	Dang *et al.*
Mytilus galloprovincialis	2 mytimycins (P, V)	nucleotides	cDNA	2011	Sonthi *et al.*

Gastropod species are grey boxed; Bivalve species are open boxed. Abbreviations: aa: amino acids; BAC: bacterial chromosome; cDNA: complementary DNA; EST: expressed sequence tags; HPLC: high pressure liquid chromatography; mRNA: messenger RNA; SSH: suppression subtractive hybridization; Database: information deposited in GenBank and never reported in publication.
Note the numerous discoveries and the diversity of investigated species in the last years.

helix and 2 C-terminal anti-parallel β-sheets (Bonmatin *et al.*, 1992), in contrast to the absence of α-helix in mammalian defensin. The term "plant defensins" introduced in 1995 regarding both mono- and dicotyledon species (Terras *et al.*, 1995) added confusion, although related to insect defensins due to a cystine-stabilized α-helix motif.

Defensin

The first molluscan defensin has been reported in 1996 from the Mediterranean mussels *M. galloprovincialis* following purification using several steps of HPLC applied to acidified plasma supernatant (Hubert *et al.*, 1996a). The first stepwise elution on C_{18} cartridge with 40 % acetonitrile resulted in several fractions displaying inhibitory growth activity against both *Escherichia coli* and *Micrococcus luteus (lysodeikticus).* The subsequent separations of the pooled active fractions on C_8 then on C_{18} columns resulted in a pure 4 kDa peptide named MGD-1. MGD-1 showed antibacterial activity also towards several *Vibrio*

species. rimary-structure analysis revealed 39 aa including 8 cysteines and a modified undetermined aa residue in position 28. The presence of 2 extra cysteines and of one modified aa suggested that MGD-1 represented a new member of the arthropod defensin family.

The same year, several cysteine-rich AMPs were isolated from the blood of immune-challenged mussels, *M. edulis* (Charlet *et al.*, 1996). The general strategy included solid phase extraction performed on acidified plasma supernatant, followed by HPLC on Aquapore OD 300 column, and gel permeation HPLC on Ultra-Spherogel SEC 3000 and SEC 2000 columns. Final HPLC purification step was on Aquapore OD 300 column. Each fraction was assayed for antibacterial (*E. coli* and *M. lysodeikticus*) and antifungal (*Neurospora crassa*) activities resulting in the partial characterization of 2 arthropod-like defensins, Peptide A and B, containing 6 cysteines.

Labeled by mouse antiserum directed against synthetic defensin MGD-1 observed with confocal and electron microscopy, defensins were located in nearly half the circulating hemocytes: 16 % contained defensin alone and 32 % contained both defensin and mytilin (Mitta *et al.*, 2000c). Such original and complex repartition reinforced the hypothesis on the existence of specialized hemocyte sub populations, never solved.

Despite long efforts regarding oysters only suggesting the presence of AMPs (Hubert *et al.*, 1996b), it was in 2005 that the first defensin-like, named American oyster defensin (AOD), has been purified from boiled acidified gill extract supernatant of *C. virginica* (Seo *et al.*, 2005). The supernatant was initially purified by preparative continuous acid-urea-polyacrylamide gel electrophoresis (CAU–PAGE). Anti-*E. coli* activity was monitored during the 12.5 h of elution. Final step of purification was by reverse phase HPLC through C_4 column. AOD had 38 aa, including 6 cysteines, possessed a strong activity against Gram-positive (*Lactococcus lactis* and *Staphylococcus aureus*) and Gram-negative (*E. coli* D31 and *V. parahemolyticus*) bacteria, and high sequence homology to arthropod and mussel defensins.

Three-dimensional solution structure of mussel and oyster defensins was established analyzing purified native defensins, completed by synthetic or recombinant defensins, with a combination of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), electro-spray ionization mass spectroscopy and nuclear magnetic resonance in spectroscopy with respect to hydrogen-1 nuclei (^{1}H-NMR). MGD-1 showed an arthropod defensins common cystine-stabilized alpha-beta motif (CS-$\alpha\beta$), but present an additional disulfide bond. The structure is characterized by an NH_2-terminal α-helix followed by 2 anti-parallel □-strands connected by distorted loops and constrained by 4 disulfide bonds (Yang *et al.*, 2000). Both oyster *C. gigas* and mussel defensins appeared with similar global fold but differed by the size of their loops and by the presence of 2 aspartic residues in Cg-Def (Gueguen *et al.*, 2006). Interesting, the nonapeptide corresponding to loop 3 connecting the 2 β-sheets of MGD-1 retained antibacterial and antifungal activities as demonstrated using synthetic peptides modified to maintain the 3-dimensional structure by creating a non-native disulfide bond (Romestand *et al*., 2003). The same nonapeptide was also found to be anti protozoa (*Trypanosoma brucei* and *Leishmania major*) and preventing the *in vitro* infection by virus HIV-1, but non-cytotoxic for human MAGIC-5B cells (Roch *et al.*, 2004). Increasing the net positive charge of the nonapeptides by selected aa replacements resulted in higher activities, whatever the target. On the opposite, unfolded linear sequences were not active revealing that the 3-dimensional hairpin structure is essential for activity.

Mytilin

During the HPLC process applied to *M. edulis* plasma and leading to defensin purification (see above), 2 isoforms of a novel peptide of 34 aa including 8 cysteines, with potent bactericidal activity named mytilins (isoform A and B) have been isolated (Charlet *et al.*, 1996). Using similar HPLC purification applied to *M. galloprovincialis* hemocyte granules, mytilin-B was recovered along with new isoforms C, D and G1 (Mitta *et al.*, 2000b). Although these isoforms share a high degree of homology in their primary structure, including the canonical cysteine array, they exhibited complementary antimicrobial properties against distinct pathogens, including Gram-positive and Gram-negative bacteria, fungi and protozoa. Also the kinetics of bacteriolytic effect was different according to the isoform, ranged from less than 3 min to 6 h. Existence of multiple mytilin isoforms, engaged in different biological activities, suggested that mussels possess an extended arsenal of molecules to counter microbial infestation.

Confocal microscopy analysis using rabbit anti-mytilin-B antiserum demonstrated that mytilins are stored in 69 % of *M. galloprovincialis* circulating hemocytes, whatever alone (37 %) or in addition to defensin in different granules (11 %) or in the same granules (21 %). Mytilin expressing hemocytes were particularly abundant in sinuses of adductor muscle, epithelia of gills, digestive gland and intestine (Mitta *et al.*, 2000c). They 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.*, 2000d).

White spot syndrome virus (WSSV), incubated with mytilin-B prior to injection into the shrimp, *Palaemon serratus,* was no longer able to kill the shrimps (Dupuy *et al.*, 2004). In the absence of 3-dimensional structure, one 10 aa fragment derived from the hypothesized loop 3 of mytilin-B, has been synthesized. It included 2 cysteines and its folding was supposed to form a stable β-hairpin structure. As obtained with complete mytilin-B, such synthetic peptide was able to reduce the shrimp mortality due WSSV when incubated *in vitro* with the virus prior injection. The 3-dimensional structure of *M. galloprovincialis* mytilin-B has been established. It resembles the CS-$\alpha\beta$ motif of defensin MGD-1, involving the same pattern of cysteine bonding (Roch *et al.*, 2008). One linear and 3 cyclic synthetic fragments corresponding to loop 3 sequence of mytilin-B have been synthesized. Only the fragment

of 10 aa constrained by 2 disulfide bonds in a stable β-hairpin structure was able to prevent the shrimp mortality due to WSSV. It was confirmed that the interaction occurred at the virus membrane level, and this in less than a min. In addition, such fragment inhibited the growth of several Vibrios, *Micrococcus lysodeikticus* and *E. coli*. On the opposite of what it has been reported with defensin fragments, increasing the positive net charge did not reinforce the antibacterial activity. Moreover, it completely suppressed the antiviral one (Roch *et al.*, 2008). In a study involving several AMPs, synthetic mytilin-A has been assayed for anti protozoan parasite activity. No parasiticidal effect was observed, as well as any inhibition of *Trypanosoma cruzi* growth. In contrast, *Leishmania braziliensis* growth was inhibited by mytilin-A, since the first cell cycle and even after 3 cell cycles (Lofgren *et al.*, 2008).

Native mytilin-A purified from *M. edulis*, presented some activity against several human virus when added simultaneously with the cell target (Vero, HEp-2 and MA104 cells). However, replication of the *herpes simplex* virus (HSV-1) was completely inhibited only when the virus was pre-incubated with mytilin-A (Carriel-Gomes *et al.*, 2007) suggesting direct interaction of the peptide at the virus membrane, as reported for mytilin-B and synthetic fragment (Roch *et al.*, 2008).

Myticin

In 1999, a novel AMP has been isolated from unchallenged *M. galloprovincialis.* Named myticin, such AMP was purified using 3 consecutive steps of HPLC on C_{18} - C_8 - C_{18} columns using linear gradients of acetonitrile, followed by Edman degradation by electro spray ionization on mass spectrometer. Two isoforms were obtained: myticin-A from hemocyte granules and cell-free plasma, and myticin-B only from hemocyte granules (Mitta *et al.*, 1999a). A partial sequence of 36 aa of myticin-A revealed the presence of 7 cysteines suggesting relationships with previously reported mussel AMPs. Only 7 NH_2-terminal aa sequence of myticin-B has been obtained and complete sequences were deduced from molecular biology data (see below). Myticins showed antimicrobial activity against several Gram-positive bacteria (*M. lysodeikticus, Bacillus megaterium, Enterococcus viridans*), but not against marine (Vibrios, Salmonellas) or non-marine (*Brucella suis*) Gram-negative bacteria or protozoa (*Perkinsus marinus*). Only myticin-B inhibited the growth of the fungus, *Fusarium oxysporum* (Mitta *et al.*, 1999a).

Mytimycin

A cysteine-rich peptide, named mytimycin, has been isolated from the plasma of immune-challenged and untreated mussels *M. edulis* using reversed phase HPLC purification. Of an estimated molecular mass of 6.2 kDa, mytimycin was strictly antifungal, inhibiting the growth of *Neurospora crassa* and *Fusarium culmorum* (Charlet *et al.*, 1996). Only 32 aa from NH_2-terminal sequence, corresponding roughly to half of the sequence, was released. Reduction and alkylation indicated the possible presence of 12 cysteines, a unique situation regarding AMPs.

Miscellaneous

A potent antibacterial histone H2B protein of 13.9 kDa (123 aa) has been isolated from boiled acidified gill extract supernatant from *C. virginica*, and named cvH2B-1 (Seo *et al.*, 2010). Only partial internal aa sequences have been released, confirming the similarity with *C. gigas*, *M. edulis* and *M. galloprovincialis* histone H2B counterparts. Recently, 3 isoforms named cvH2B-2, -3 and -4 have been reported (Seo *et al.*, 2011). Although active against Gram positive, *L. lactis*, cvH2B-2 is inactive against *S aureus*. Both cvH2B-1 and -2 had potent activity against the Gram-negative oyster pathogen *V. parahemolyticus* as well as against the human pathogen *V. vulnificus*. In addition, cvH2B-3 and -4 had similarly strong activity against *V. vulnificus*. Estimated concentration of histone H2B isoforms found normally within the oyster, were exceeding the *in vitro* inhibitory concentrations for Vibrios, revealing their potent protective function.

The big-defensin RPD-1 has been purified by a combination of precipitation, gel-exclusion and cation-exchange chromatography from the plasma of the Manila clam *Ruditapes philippinarum* (Wei *et al.*, 2003). Of 24.8 kDa, 11 NH_2-terminal aa sequence has been published with no known homology at that time. Activity was on Gram-positive and Gram-negative bacteria.

A proline-rich AMP of 5.1 kDa has been purified by several HPLC runs on C_{18} columns performed on hemocyte organelles collected from the Chilean scallop *Argopecten purpuratus*. The sequence of the 42 NH_2-terminal aa did not contain any cysteine, but presented some homology with *Drosophila melanogaster* ceratotoxin and human defensin hDB-1 (Arenas *et al.*, 2009). One synthetic peptide of 31 aa has been designed in order to enhance hydrophobicity and to increase cationicity. Both natural and synthetic peptides were clearly antifungal (*F. oxysporum, Saprolegnia parasitica, N. crassa*) with weak effect against bacteria and no cytotoxicity toward fish cell line. Increasing the positive net charge resulted in strong increase of antifungal activity confirming the importance of electrostatic interactions to develop the activity.

Littorein, an amphipathic cationic peptide of about 3.5 kDa, has been observed in the plasma of the marine Gastropod, *Littorina littorea* (Defer *et al.*, 2009a). The peptide was prepared by HPLC on C_{18} column, but not isolated in pure form. Bactericidal activity against *M. lysodeikticus* and anti viral activity against HSV-1, were reported. The same authors investigated the same activities in the hemolymph of commercially important Bivalves and Gastropods. The broadest antibacterial activity was found in the Bivalve, *Cerastoderma edule*, whereas the highest activity was found in the oyster, *Ostrea edulis* (Defer *et al.*, 2009b). Also antiviral activity, but not cytotoxicity, was detected in *C. edule*. Acid extracts from the other examined species were cytotoxic and presented less antiviral capacity then *C. edule.* As no pure fractions have been obtained, the molecular supports of activities remain unknown.

Antiviral activity of the abalone *Haliotis laevigata* was assessed against HSV-1 using a plaque assay technique (Dang *et al.*, 2011). Hemolymph and lipophilic extract of the digestive gland substantially decreased the number and size of plaques. Antiviral activity of hemolymph was rapid, in contrast to antiviral activity of lipophilic extract, which was greater when added 1 h after infection, suggesting that abalone have at least two antiviral compounds with different modes of action.

Computer-based evidences

In the last decade, biochemical approaches were rapidly submerged by molecular biology-driven investigations, i.e. addressing mRNA and genomic DNA sequences. Not only were the origins of RNA diverse, from whole animal to dissected tissues or hemocytes, but also the techniques used: EST, SAGE, SSH, DGGE, 454 pyrosequencing, Illumina, high-density microarrays, etc. Regarding small proteins, frequently present in small quantities, including multiple isoforms with different biochemical properties, a real breakthrough was introduced by the molecular biology techniques, revealing AMPs in all the invertebrates analyzed so far.

Defensin

Using back-translation of the aa sequence from *M. galloprovincialis* MGD-1, a new isoform, MGD-2, has been obtained from a cDNA library (Mitta *et al.*, 1999b). The signal peptide of 21 aa is followed by 39 aa of the mature defensin and 21 aa of the C-terminal extension. The presence of 8 cysteines engaged in 4 intra chain disulfide bonds did not match with the 6 cysteines reported for *M. edulis* defensins peptide-A and -B (Charlet *et al.*, 1996). MGD-1 and -2 precursors were predominantly expressed in hemocytes. Mature defensins were located in vesicles of hemocytes containing small granules, in large clear granules of another subclass of hemocytes and in some enterocytes. Challenge with heat-killed *Vibrio alginolyticus* triggered the plasmatic increase of MGD-1 concentration. The gene coding MGD-2 has been cloned and sequenced: one intron interrupted the signal peptide and another intron is located within the C-terminal extension (Mitta *et al.*, 2000a). Remarkable was the fact that the sequence coding for the mature defensin is not interrupted. Analyzed in Southern blot, the defensin gene appeared present as a single copy in the genome.

Recent studies performed in real-time PCR shown that MGD-1 gene expression, the less expressed gene in untreated *M. galloprovincialis* compared to mytilin and myticin, was highly dependent on the season with higher expression in summer (Li *et al.*, 2009b). In addition, its expression was up regulated only by injection of *V. splendidus* (1 to 24 h) (Cellura *et al*., 2007).

Using EST libraries, the first oyster defensin was isolated from the mantle of the Pacific oyster *C gigas* and named *Cg*-Def (Gueguen *et al.*, 2006). The deduced aa sequence starts with a 22 aa signal peptide followed by the 43 aa of the mature peptide. On the opposite of mussel defensins, *Cg*-Def precursor does not contain C-terminal extension. Meanwhile, its genomic organization is similar to that of the mussel defensin genes. The *in vitro* antimicrobial effect of recombinant *Cg*-Def was tested in conditions mimicking the seawater environments. Activity was directed against Gram-positive bacteria but no or limited activity was observed against Gram-negative bacteria and fungi. *Cg*-Def in solution shares the cystine-stabilized alpha-beta (CS-$\alpha\beta$) motif with arthropod defensins, with an exception of the presence of an additional disulfide bond. This structure was similar to that of mussel defensin MGD-1 (Romestand *et al.*, 2003). However, *Cg*-Def and MGD-1 structures could be distinguished by the size of their respective loops and by the presence of two aspartic residues in *Cg*-Def. Expression of *Cg*-Def gene was mainly in mantle edge as revealed by mass spectrometry analyses. *Cg*-Def mRNA concentration was unchanged after bacterial challenge, which could be explained by its high level of continuous expression (Gueguen *et al.*, 2006). One year later, two new defensin isoforms, *Cg*-defh1 and -2, were identified by cDNA cloning technology from circulating hemocytes of *C. gigas*. After *in silico* translations, the precursor was of 73 aa, 43 of which representing the mature peptide. *Cg*-Defh1 and *Cg*-Defh2 aa sequences shared 86 % of identity and they belong to the "arthropod-Molluscs defensin family". Similarly to the expression behavior of *Cg*-Def in mantle, *Cg*-defh2 is continuously expressed in hemocytes. In addition, the level of *Cg*-defh2 transcripts decreases dramatically in circulating hemocytes following bacterial challenge. Correlation with an increase in gills and mantle suggested migration of hemocytes expressing *Cg*-defh2 towards tissues considered as representing the first defense barrier (Gonzalez *et al.*, 2007). Sequence alignment of *Cg*-defh1 and -2 suggested they aroused from a multigenic family displaying variety of gene structures (Schmitt *et al.*, 2010). Consequently, different antimicrobial peptides from Molluscs appeared to have been subject to distinct patterns of diversification.

Another economically important oyster species, *C. virginica*, was used to construct large-insert genomic bacterial artificial chromosome (BAC) library. The library of 55,296 clones was screened with probes derived from selected oyster genes, including defensins, using high-density filter arrays. Seven defensin positive clones were identified in this BAC library which can be grouped in 2 clusters, probably resulting from only one gene copy (Cunningham *et al.*, 2006).

Some information recently emerged regarding the hydrothermal-vent mussel, *Bathymodiolus thermophilus*. Suppression subtractive hybridization (SSH) libraries were used to reveal the presence of defensin which expression is regulated by temperature (Boutet *et al.*, 2009).

Triangle snail mussel, *Hyriopsis cumingii*, is the most important mussel species producing freshwater pearls in China. Two defensins analogous to the *C. gigas* ones were identified among 1,165 singletons generated by EST technology (Bai *et al.*, 2009).

Among the genes newly identified from an EST library constructed from the whole body except

digestive tract and intestine of the bay scallop, *Argopecten irradians irradians*, 10 ESTs out of 2,853 sequences were related to defensin (Song *et al.*, 2006). They were organized into 2 groups: one of 3 ESTs with a size of 518 nucleotides, the other of 7 ESTs with a size of 824 nucleotides. No more details were released.

Several genes involved in the defense against the parasite QPX (Quahog Parasite Unknown) were identified in the hard clam, *Mercenaria mercenaria,* using SSH libraries constructed from hemocytes (Perrigault *et al.*, 2009). One big-defensin and one hemocyte defensin homologous to the one of *C. gigas* were discovered. Quantitative PCR revealed that the gene from hemocyte defensin was up-regulated 14 to 48 days in gills after experimental infestation.

Defensins were also identified in Gastropods. Regarding the abalone, *Haliotis discus hannai*, the major cultured species in China, sequence homology analysis of the 42 defensin-related sequences identified from an EST library constructed from liver and kidney, revealed high similarity with arthropod defensins, including the 6 cysteines. Non-expressed in untreated abalone, the defensin gene is triggered only in hepatopancreas after injection of *V. anguillarum* or *S. aureus* (Hong *et al.*, 2008). Complete study of defensin has been performed in the disk abalone, *Haliotis discus discus*, starting from cDNA library constructed from whole tissues to tissue specific expression and regulation after bacterial challenge (De Zoysa *et al.*, 2010). The pro-defensin comprised a putative signal peptide of 18 aa followed by the mature defensin of 48 aa. The presence of 6 cysteines, the putative three disulfide bonds and 3-dimensional structure, joined to phylogenic relationship, suggested disk abalone defensin is related to arthropod defensins. Constitutively expressed mainly in mantle and hepatopancreas, expression was up regulated principally in hemocytes 3 - 6 h and 24 - 48 h post-challenge with a mixture of bacteria

Mytilin

The nucleotide sequence of mytilin-B precursor from *M. galloprovincialis*, one of the 5 mytilins purified by HPLC, was obtained from a hemocyte cDNA library (Mitta *et al.*, 2000b). The precursor starts by a signal peptide of 22 aa followed by the mature mytilin-B of 34 aa including 8 cysteines, and ended with 48 aa from the C-terminal extension. The gene coding for mytilin-B has been cloned and sequenced. Its structure is similar to the MGD-2 one: one intron interrupted the signal peptide and another intron is located within the C-terminal extension (Mitta *et al.*, 2000a). Analysis in Southern blot suggested the presence of one copy of mytilin-B gene per genome, with possibly another gene displaying homology. Such last observation is in agreement with the evidence of numerous variants of mytilin (see below).

The extended variability of mytilin has been investigated from circulating hemocytes focusing on mytilin-B (Parisi *et al.*, 2009). One mussel expressed simultaneously 2 to 10 different mytilin-B mRNAs as observed in denaturing gradient gel electrophoresis (DGGE), defining 10 individual DGGE patterns. Nucleotide substitutions were observed at specific locations, mainly within C-terminus and 3'UTR, leading to 36 nucleotide sequence variants and 21 different coding sequences segregating in 2 different clusters. Most of the nucleotide substitutions were silent, and the number of pro-peptide variants was restricted to 4. Finally, as the 2 aa replacements occurred within the C-terminus, mytilin-B mature peptide appeared unique. Multiple sequencing of partial mytilin-B gene from one mussel revealed that 1 to 4 randomly distributed nucleotide substitution points occurred within intron-3. Only one sequence out of the 91 analyzed contained 16 nucleotide substitution points. In addition, this sequence was the only one containing 4 out of the 6 nucleotide substitution points occurring within exon-4, that codes for most of the C-terminal domain, including the unique aa replacement. In addition, mytilin-B locus did not appear to be under directed selection (Boon *et al.*, 2009), a situation contradictory to myticin-C.

Mytilin-B gene expression slightly varied in *M. galloprovincialis* according to the season (Li *et al.*, 2009b). However, bacterial challenges modified its expression in hemocytes. Strongly up regulated by *V. anguillarum* (24 - 48 h), expression was down regulated by *V. splendidus* and *M. lysodeikticus* (Cellura *et al.*, 2007), a situation confirmed by Li *et al.* (2010), except regarding *V. anguillarum*.

In a study considering also myticin, a mytilin of 34 aa, similar to the mussel counterpart, has been characterized in the clam, *Ruditapes decussates,* using SSH technique (Gestal *et al.*, 2007). The clam mytilin differs by only 2 aa from mussel mytilin-C. Expression level of clam mytilin increased 24 h and 48 h post-injection with the mixture of dead bacteria (*M. lysodeikticus, V. splendidus*, *V. anguillarum*) and on those injected with live *V. anguillarum*, as measured by real-time PCR. Expression patterns were similar and confirmed the higher expression level at 48 h post-injection, as suggested by SSH.

One mytilin-C complete mRNA sequence isolated from the bay mussel *M. trossulus*, hemocytes has been deposited in GenBank in 2004 (accession number AY730626). Similarly, mytilin-A and -B complete mRNA sequences from *M. coruscus* have been deposited in GenBank in 2010 (accession numbers FJ973154 and 5), together with mytilin-3 to -8 complete mRNA sequences (accession numbers GU324718 to 23).

Regarding the deep-sea hydrothermal vent mussel, *Bathymodiolus azoricus*, degenerated primers have been used in RT-PCR to evidence mytilin transcript in gills (Bettencourt *et al.*, 2007). Mytilin mRNA has been located in hemocytes underlying gill epithelium and its expression was induced by exposure to *V. parahemolyticus* (Bettencourt et al., 2009)

Myticin

Myticin precursor nucleotide sequences of *M. galloprovincialis* had been established by cloning based on partial aa sequences. They consist of 96 aa with a signal peptide of 20 aa, followed by the mature myticin of 40 aa including 8 cysteines arranged in an original array, and a 36 aa C-terminal extension (Mitta *et al.*, 1999a). Southern-

blot analysis suggested myticin gene is present as a single copy in the mussel genome. Hemocytes are the main site for precursor production of myticins. Nevertheless, mantle, labial palps, gills and digestive glands showed a faint band with the same mobility as that of hemocytes in Northern blotting. Myticin-B transcript has been observed by using *in situ* hybridization (ISH) on 16 % of circulating hemocytes from untreated mussels, identified as small granulocytes. Such expression pattern was not modified by bacterial challenges (Li *et al.*, 2010). Myticin-B gene is subject to variation of expression according to season with lower expression in winter as observed during 3 consecutive years (Li *et al.*, 2009b). Expression was also down regulated by injection of *V. splendidus* (1 to 12 h), and *M. lysodeikticus* (1 to 72 h), and to a less extent of *V. anguillarum* (1 to 9 h) (Cellura *et al.*, 2007). In a comparative study involving *M. galloprovincialis* from different geographical areas, myticin-B gene expression was also found down regulated, but with different kinetics following bacterial challenges and origin (Li *et al.*, 2010).

First *in silico* data on myticin was obtained by production and sequencing of 3'end-specific EST from multiple tissues of unchallenged, *M. galloprovincialis* (Venier *et al.*, 2003). A variety of genes potentially involved in stress responses, including the cysteine-rich peptide precursor myticin-A, were identified. Later, primary and SSH libraries were prepared from hemolymph of *M. galloprovincialis* after heat-inactivated bacteria or poly I:C challenge. The 232 ESTs matching with myticin-A and -B displayed considerable sequence variability and revealed a third cluster proposed as myticin-C. Analysis of the translated myticin-C yielded 74 and 25 variants of the precursor and active peptide, respectively (Pallavicini *et al.*, 2008). The individual sequences of myticin-C are unique for each mussel, independently of the geographic origin, age, sex or gonad maturation. Comparative analysis of genomic and cDNA sequences from the same individual showed that all detected variants shared a very high homology with the most frequent genomic isoforms, suggesting that variations were generated from the most common sequences (Costa *et al.*, 2009). Meanwhile, the genetic mechanism(s) that maintains such high diversity is poorly understood.

It has been suggested that the high sequence variability of myticin transcripts in *M. galloprovincialis* hemocytes reflected ancient host-pathogen interactions (Pallavicini *et al.*, 2008). Using phylogeny-based models of sequence evolution and several statistical tests for neutrality, the role of natural selection process in the evolution of multiple variants of myticin-C has been confirmed. Statistical tests rejected the hypothesis that all polymorphism within myticin-C loci is neutral (Padhi and Verghese, 2008), a result on the opposite to the analysis of mytilin-B polymorphism (Parisi *et al.*, 2009).

Analysis of differentially expressed genes in response to bacterial challenge in hemocytes of the carpet-shell clam, *Ruditapes decussatus*, using SSH technique resulted in the identification of clam myticin-1, -2 and -3 (Gestal *et al.*, 2007). Clam myticins share the cysteine array defining the 4 disulfide bonds which are the signature of the mussel myticin family. However, they constitute a separated sister taxon. The challenge with dead bacteria mixture or with alive *V. anguillarum* resulted in maximum 2xfold up-regulation of the clam myticins 48 h post challenge. Here also it was suggested that the variations in aa sequences might be a response to the recognition of different pathogens.

Nine myticin complete mRNA sequences, named myticin-1 to -9, isolated from *Mytilus coruscus* have been deposited in GenBank in 2010 (accession numbers GU324724 to 32).

Mytimycin

Such strictly antifungal peptide from *M. edulis* was reported in 1996 as partial NH_2-terminus 33 aa sequence (Charlet *et al.*, 1996). MytiBase, the first species-specific catalogue of *M. galloprovincialis* ESTs publicly available at http://mussel.cribi.unipd.it, has been constructed from different *M. galloprovincialis* tissues, including gills, mantles and hemocytes (Venier *et al.*, 2009). Using bioinformatics, several hits related to mytimycin were identified. Primers designed from a *consensus* sequence have been used to obtain the complete cds of 456 nucleotides. Mytimycin precursor is constituted by a signal peptide of 23 aa, followed by the mature mytimycin of 54 aa and a C-terminal extension of 75 aa (Sonthi *et al.*, 2011). Only 2 major aa precursor sequences emerged according to the geographical origin of the mussels: one shared by *M. galloprovincialis* from Venice-Italy and Vigo-Spain, and by *M. edulis*, the other belonging to *M. galloprovincialis* from Montpellier-France, with 9 aa difference between the two mature mytimycins. The suspected presence of 12 cysteines has been confirmed and predicted disulfide bonds suggested the presence of 2 constrained domains joined by a 4 aa tract. Mature mytimycin appeared more closely related to mytilin than to any other antibacterial-antifungal peptide, possibly due to the C-terminal domain. Intriguing was the presence of conserved canonical EF-hand-motif located in the C-terminal extension. Mytimycin gene of *M. galloprovincialis* is interrupted by 2 introns. Intron 2 presented 2 forms, a long one and a short one resulting from the removal of the central part of the long one, both being simultaneously present in each mussel (Sonthi *et al.*, 2011).

Miscellaneous

Two clusters from EST library constructed from hemocytes of the Gastropod, *Biomphalaria glabrata*, maintained in non-axenic conditions showed sequence similarity to theromacin, the AMP of the Annelid leech, *Theromyzon tessulatum*, particularly the cysteine array (Mitta *et al.*, 2005).

The first proline-rich AMP from Mollusc, *Cg*-Prp, was identified from the oyster *C. gigas*. The cDNA sequence codes for a 61 aa precursor with homologies to proline-rich AMP, and was composed of a hydrophobic signal peptide of 37 aa followed by mature *Cg*-Prp, composed of an acidic region and a cationic proline-rich region. Expression of *Cg-prp* is restricted to hemocytes and induced following

bacterial challenge. As *Cg*-Prp was located by immune cytochemistry in some hemocytes together with defensins, antimicrobial activity was suspected to be through synergy with defensins (Gueguen *et al.*, 2009).

Histone H2A was reported to participate in host defense response through producing novel AMP from its N-terminus in the scallop, *Chlamys farreri*. Homology cloning and genomic DNA walking have been used to identify the open reading frame of 375 bp coding for a polypeptide of 125 aa (Li *et al.*, 2007). As expression in hemocytes was not modified by bacterial challenge, it was concluded that H2A did not participate in immune response directly. Partial recombinant H2A, restricted to 39 N-terminal aa, has been produced. Its antibacterial activity was 2.5 times higher toward Gram-positive bacteria (*M. lysodeikticus*) than toward Gram-negative bacteria (*V. splendidus, V. anguillarum, V. vulnificus*).

Histone H2A full-length cDNA was cloned from disk abalone, *Haliotis discus discus*, and a 40 aa AMP, named abhisin, was identified from the N-terminus of H2A sequence (De Zoysa et al., 2009). Abhisin shares 80 % aa identity with the buforin I from Asian toad histone H2A. Antimicrobial activity was investigated on synthetic abhisin, revealing growth inhibition of Gram-positive (*Listeria monocytogenes*), Gram-negative (*Vibrio ichthyoenteri*) bacteria, and fungi (yeast *Pityrosporum ovale*). Additionally, abhisin has cytotoxic activity against cancer cells but not against normal cells. H2A gene expression was significantly induced at 3 h post-infection with bacteria in abalone gills and digestive tract.

So-called big-defensins have been identified using various techniques in several bivalves. They are proteins composed of an N-terminal hydrophobic sequence followed by the C-terminal cationic defensin portion, similar to those of mammalian defensins, particularly the disulfide array, but different from the «Arthropod-Mollusc defensins» (see above). The first one was deposited in 2001 as cDNA from the oyster, *C. virginica* (GenBank accession number BG624524). The second, named *Ai*BD, was released from the bay scallop, *A. irradians,* also as a cDNA coding for a signal peptide of 28 aa followed by the mature peptide of 94 aa sharing homologies with big-defensins from Arthropods and amphioxus (Zhao *et al.*, 2007). Constitutive expression in hemocytes was up regulated in the first 8h after injection of *V. anguillarum*, followed by a drastic increase at 32 h post-injection. In addition, recombinant *Ai*BD inhibits the growth of Gram-positive and Gram-negative bacteria, and possesses strong fungicidal activity. Existence of a third big-defensin has been reported in the hard clam, *Mercenaria mercenaria* after experimental challenge with the protistan parasite QPX (Quahog Parasite Unknown) (Perrigault *et al.*, 2009). Its expression was significantly induced in gills at 48 days post-infection. The fourth big-defensin, *Vp*BD, belongs to hemocytes of the Manila clam, *Venerupis philippinarum*. The precursor is composed of a signal peptide of 20 aa followed by the active molecule of 74 aa with a positive net charge of +5 and 36 % of hydrophobic residues. As others defensins, *Vp*BD possesses remarkable microbicidal activity against Gram-positive and Gram-negative bacteria, and showed strong fungicidal activity towards yeast (Zhao *et al.*, 2010). Its expression was up regulated during the first 24 h after *V. anguillarum* challenge and its activity was directed toward a broad spectrum of bacteria species.

Two other AMPs from the oyster, *C. gigas*, gigasin-2 and -3, have been deposited in GenBank in June 2003 under the accession numbers AJ582629 and AJ582628 respectively. Alignments of the deduced aa sequences revealed that gigasin-2 is identical to defensin Cg-Defh2 and gigasin-3 closely related to *Cg*-Defh1 (Gonzalez *et al.*, 2007).

Surprisingly, some authors did not report the presence of AMP-related sequences from cDNA libraries constructed from different Bivalves, such the ones from *C. gigas* and *C. virginica* (Jenny *et al.*, 2007), *C. gigas*, *M. edulis*, *R. decussatus* and *Bathymodiolus azoricus* (Tanguy *et al.*, 2008), *Laternula elliptica* (Park *et al.*, 2008), *C. gigas* (Roberts *et al.*, 2009) and *Chlamys farreri* (Wang *et al.*, 2009). Such apparent absence of existing AMPs highlights the limits of EST technology, which is obviously non-exhaustive.

Concluding remarks

It is remarkable that, on the opposite to the variety of structures characterizing Arthropods AMPs, Mollusc AMPs reported so far belong to a unique molecular family, *i.e.*, the CS-$\alpha\beta$, with the exception of proline-rich AMP from the Chilean scallop, *A. purpuratus,* which did not contain cysteine.

The 70's have been the era of biological activity-driven technology completed by biochemical identification of the active molecules. Such approach lead to the discovery of new aa sequences displaying antimicrobial activities and revealed unknown peptide frameworks with antibiotic capabilities. Multiple molecules were discovered revealing the importance and universality of AMPs and establishing the basis of unsuspected innate immune mechanisms. Since the mid-90's, the field of AMP exploded due to molecular biology techniques, confirming their existence in all the living creatures, including plants and bacteria. Regarding only Molluscs, several databases were made available, in addition to number of EST libraries constructed for various purposes. Nowadays, bio-informatic data are so numerous that data mining is applied also to Molluscs sequences (Yu and Li, 2008; Wang *et al.*, 2008). Meanwhile, nucleotide sequences from genomes or ESTs in themselves cannot demonstrate biological pathways, and functional assays must be performed to confirm, species by species, challenge by challenge, the real involvement of AMPs in anti infectious defense.

Only few Mollusc families were investigated for AMPs, mainly the Bivalve *Mytilidae*, *Ostreidae* and *Pectinidae* (Table 2). Some information came from Gastropods (*Haliotidae*) but no one from Cephalopods or Polyplacophors. In addition, routinely applied molecular biology techniques

Table 2 Number of reported AMPs within the major classes of the Mollusc phylum

Class	Estimated number of species	Number of reported AMPs
Polyplacophors	900 - 1,000	0
Gastropods	70,000 – 103,000	8
Cephalopods	730 - 900	0
Bivalves	12,000 – 20,000	61
Scaphopods	400 - 500	0

Note that only 2 classes have been investigated and the discrepancy between the number of species and the number of reported AMPs in Gastropods versus Bivalves.

allowed reporting AMPs only if closely-related sequences were already deposited in databases and mined by computer. In other words, molecular biology techniques alone cannot reveal new AMP structures, probably underestimating the diversity of such universal immune effectors. Biochemical purification linked to biological activity of isolated fraction represents the main approach capable to reveal new structures. Meanwhile, sequence mining might represent an alternative approach which, using rational prediction models (Torrent *et al.*, 2011) and various algorithms like Random Forests (RF), Support Vector Machines (SVM) and Discriminant Analysis (DA) (Thomas *et al.*, 2010), is already used to develop theoretical new antibiotic generation(s).

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