ISJ 14: 95-111, 2017 ISSN 1824-307X

REVIEW

Immune-related genes in gastropods and bivalves: a comparative overview

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Accepted March 29, 2017

Abstract

The biological diversity of molluscs and their adaptation to highly diverse environments offer a unique opportunity for studying the evolution of the innate immune system in invertebrates. This review provides an updated account about the progresses made over the past few years in the study of the molecular players involved in the recognition of pathogen associated molecular patters (PAMPs), in the transduction of immune signaling and in the elimination of potentially pathogenic microbes in gastropod and bivalve molluscs. A major focus will be put on the differences and peculiarities of the molecular immune system of the two major molluscan classes, which have developed specific adaptations to cope with diverse living environments, pathogenic and non-pathogenic microbes over the course of several hundred million years of independent evolution. Intriguing but still poorly understood aspects, such as antiviral response and immune priming, will be also explored, highlighting the present challenges and opportunities connected to the application of modern genomics techniques to the study of the immune system in these fascinating metazoans.

Key Words: mollusca; bivalves; gastropods; innate immunity; lectins; antimicrobial peptides; antiviral response

Introduction

The phylum Mollusca is one of the largest and most diverse group of metazoans, only second to Arthropoda in number of described species. Molluscs are highly heterogeneous in terms of size, morphology, adaptations to diverse habitats (terrestrial, freshwater and marine) and feeding and behavior, reflecting the massive radiation this phylum underwent during the Cambrian period.

The phylogeny of molluscs is still a matter of debate, as many different but equally plausible hypotheses have been proposed over the years, either based on morphological features, molecular data, or both. The reasons behind the apparently unsolvable topology of the molluscan tree of life are multiple and mainly need to be sought in narrow taxonomical sampling, focus on specific target clades and lack of appropriate outgroups (Sigwart and Lindberg, 2015). While molecular studies have long been expected to disentangle the complex evolutionary relationships among molluscs, this task still appears far to be accomplished and poses a great challenge for the interpretation of the results of comparative studies.

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Despite these uncertainties, most of the over 100,000 estimated extant species are currently classified within two main classes, Gastropoda and Bivalvia. The former is thought to comprise more than 80 % living molluscs, including species adapted both to terrestrial and aquatic life, while the latter strictly includes aquatic organisms, altogether accounting for ~14 % of the described species (Nicol, 1969). The remaining six classes (Scaphopoda, Cephalopoda, Aplacophora, Monoplacophora and Polyplacophora), despite their unique peculiarities and key position for evolutionary studies, overall only account for less than 2 % of all extant mollusc species.

The two major molluscan classes are likely to have diverged from each other during the Cambrian explosion and are therefore separated by over 450 million years of independent evolution. Although a broad range of species, from chitons, to tusk shells, land snails, squids, mussels and oysters are all classified under the same taxonomical umbrella as molluscs, such a large time of divergence permitted the development of highly specialized, novel genetic and morphological adaptations.

All molluscs rely on a similar cellular and molecular framework of immune cells and molecules for surviving in challenging environments, often rich of potentially pathogenic microorganisms. In particular, free-living bacteria and viruses in seawater can reach considerable concentrations, in

the range of 10^{-6} and 10^{-7} ml^{-1}, respectively (Bezdek and Carlucci, 1972; Drake *et al*., 1998), posing a great challenge for filter-feeding animals, including bivalves. The powerful innate immune system of molluscs, which comprises a broad array of soluble and membrane-bound Pattern Recognition Receptors (PRRs) for the recognition of pathogens, a finely regulated cytosolic signaling machinery and a battery of effector molecules aimed at eliminating invading microbes and recruiting specialized immune cells at the site of infection, is partially shared by other large invertebrate phyla which, unlike vertebrates, are not equipped with an adaptive immune system.

Far from being simple and less developed than in vertebrates, the invertebrate immune system has followed slightly divergent evolutionary routes in different taxa over the past few million years. This complex evolutionary process, driven by environmental selective pressure, has led the development of remarkable and diverse immune capabilities, which are still poorly understood (Loker *et al*., 2004).

Similarly, the ancient split between gastropods and bivalves, as well as their adaptation to different habitats and lifestyles, determined the evolution of unique features in their immune system. While extensive works have been carried out to document the genetic and molecular background of both gastropod (Loker 2010, Coustau *et al*., 2015) and bivalve (Gerdol and Venier, 2015; Song *et al*., 2015; Zhang *et al.,* 2015) innate immune system, little attention has been paid so far at providing a comparative account of the differences two major molluscan classes.

Indeed, important observations provided more than a decade ago concerning differences in the agglutinating and opsonizing activity of bivalve and gastropod plasma have not been followed by extensive molecular comparative research. Yakovleva and colleagues (2001) described these two alternative defense strategies as “low-promiscuous” and “high-promiscuous” for gastropods and bivalves, respectively. It was hypothesized that, while the former comprised a set of plasma lectins with a broad spectrum of carbohydrate recognition, the latter mainly relied on plasma lectins with narrow binding specificity. It was also observed that the rate of phagocytosis in gastropods was highly dependent on the concentration of plasma lectins, as opposed to bivalves, where opsonization was likely triggered by membrane-bound lectins. As it will be pointed out below, these definitions still appear to be valid, and closely mirror the situation which is starting to emerge from the analysis of the large-scale molecular data collected over the past 15 years.

This review will focus on this particular comparative aspect, exploring the massive amount of literature recently produced on this topic and presenting molecular data in a genomic context, whenever possible. Unless differently stated, data concerning the size and the sequence diversity of immune-related gene families will be inferred from the available molluscan genomes as of March, 2017. Namely, the reference genomes taken into account in this study are: *Crassostrea gigas* v.9 and *Pinctada fucata* v.2.0 for bivalve molluscs; *Lottia gigantea* v.Helro1, *Aplysia californica* v.3.0 and *Biomphalaria glabrata* v.ASM45736v1 (unpublished) for gastropod molluscs.

Molluscan lectins: a molecular repertoire characterized by lineage-specific expansions and extreme diversification

Ever since the first molecular studies carried out in the early ‘80s it became evident that the immune response of molluscs largely relied on the production of a large number of soluble proteins with marked carbohydrate binding properties, acting as PRRs (Miller *et al*., 1982; Renwrantz, 1983; Pipe, 1990; Fisher and DiNuzzo, 1991).

While most efforts have been put into their isolation of these molecules from hemocytes, the central players in molluscan cellular immunity, considerable results have been also attained from the mucus of land snails, which covers their entire bodily surface, acting as a barrier from a potentially pathogen-rich environment. Like gastropod skin, bivalve gills and mantle represent main tissues of interface with the external environment, they are covered by mucus and constantly exposed to microbes present in the water column and conveyed to the intervalvar space by filter-feeding. While the majority of lectin-like proteins of bivalves have been described and isolated from hemolymph, the important role of bivalve pallial tissues in immune recognition is starting to emerge, as evidenced by the gills- or mantle-specificity of several PRRs (Gourdine and Smith-Ravin, 2007; Jing *et al*., 2011).

It is now clear from genome and transcriptome data mining that molluscan lectin-like molecules are extremely variable, both in terms of their carbohydrate recognition domain (CRD), sugar-binding properties, and sequence diversity among members of the same lectin family (Gerdol and Venier, 2015; Gorbushin and Borisova, 2015; Zhang *et al*., 2015). The high molecular diversity and the remarkable plasticity of CRDs allow, even in absence of the genetic re-arrangements typical of the vertebrate adaptive immune system, a very broad spectrum of recognition for invertebrate lectins (Vasta *et al*., 2007).

Over the past few decades, scientific literature has evidenced some remarkable differences in the repertoires of these molecules in bivalve and gastropod molluscs. While some of these apparent discrepancies could be simply explained by the higher amount of efforts put into immunological research of economically important bivalves, whole-genome analyses fully confirm that all the main lectin-like families show a different size and degree of diversification between these two molluscan classes, as reported in the comparative summary in Figure 1. This divergent evolution might be linked to environmental factors (terrestrial vs freshwater vs marine) and adaptation to the associated microbiomes and pathobiomes, as well as to the development of alternative low- and high-promiscuity defense strategies, as previously proposed by other authors (Yakovleva *et al*., 2011).

The most evident case is certainly that of C1q

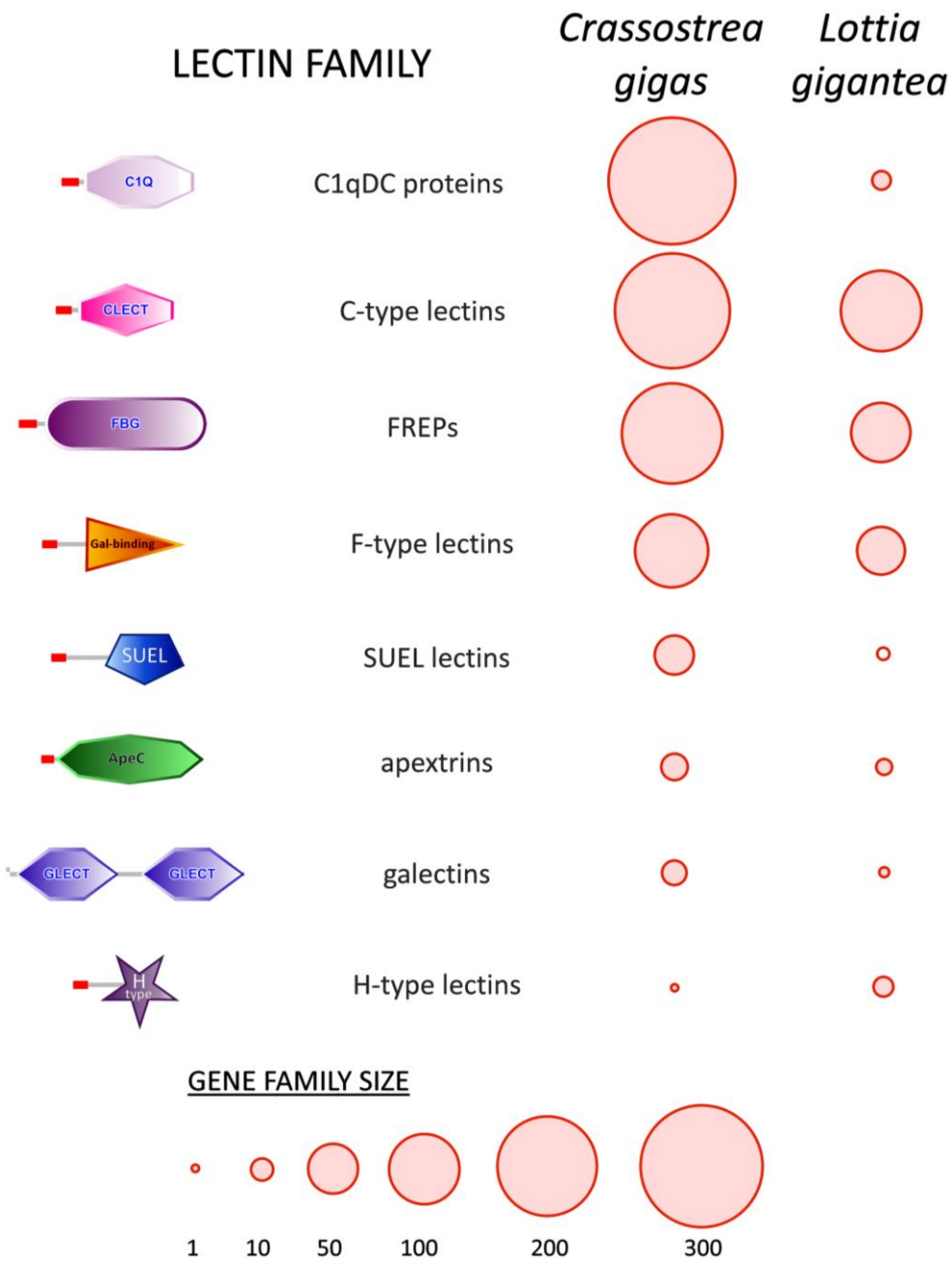


Fig. 1 Domain organization and size of the main lectin gene families in two representative species for Bivalvia (the Pacific oyster *Crassostrea gigas*) and Gastropoda (the owl limpet *Lottia gigantea*).

domain containing (C1qDC) proteins, which are present in hundreds different variants in bivalves, compared to the few (usually less than 20) gene copies found in gastropods. The C1q domain takes its name from the homonymous protein complex of the vertebrate complement system, whose three main components are characterized by the presence of the globular head domain C1q. The astounding plasticity and binding properties of this domain led to an extraordinary evolutionary success in metazoans (Carland and Gerwick, 2010), as highlighted by the nearly 6,500 C1qDC protein sequences deposited so far in public databases.

In protostomes, C1qDC proteins have been linked on multiple occasions to a lectin-like activity, such as in the snail *Cepaea hortensis* (Gerlach *et al*., 2004). However, since this first report in 2004, very scarce references have been made in literature to gastropod C1qDC proteins as PRRs, with the only exception of some abalone proteins linked to lipopolysaccharide (LPS) and peptidoglycan (PGN) response (Bathige *et al*., 2016). Comparatively,

C1qDC proteins have been the target of a much higher number studies in bivalves (Zhang *et al*., 2008; Gestal *et al*., 2010; Kong *et al.,* 2010; Xu *et al.,* 2012), where they have been often linked to hemocyte-specificity, upregulation in response to multiple PAMPs, bacterial agglutination and growth inhibition. This disparity concerning the reports in the two major mollusk classes is certainly correlated to very low number of C1qDC proteins found in gastropod genomes, as opposed to the a massive gene family expansion that occurred in bivalves (Fig. 1). This event, which is estimated to have brought to the development of almost 1,000 different paralogs in *Mytilus* spp., only involved Pteriomorphia and Imparidentia, but not freshwater unionoids and the basal branch of protobranch bivalves (Gerdol *et al.,* 2015).

Despite the low number of C1qDC proteins, gastropods produce several other lectin-like molecules, as evidenced by a large scale transcriptomic survey of the hemocyte transcriptome of the periwinkle *Littorina littorea* (Gorbushin and Borisova, 2015), even though, on average, their number at the whole genome level appears to be lower than in bivalves (Fig. 1).

C-type lectins are the second largest lectin family in the Pacific oyster. These molecules are characterized by a CLECT domain which can be often associated with other conserved modules, creating a broad range of domain combinations. C-type lectins can cover multiple biological functions, but one of the best documented is innate immune recognition, such as in the case mannose-binding lectin, a key component of the lectin pathway of the vertebrate complement system (Zelensky and Gready, 2005). The role of C-type lectins in the molluscan immune system has also been clearly established and thoroughly investigated (Wang *et al.,* 2011). In gastropods, the abalone HdhCTL is involved in the agglutination of Gram-negative bacteria (Zhang *et al* 2014) and incilarins have been shown to be major components of the mucus of the land snail *Meghimatium fruhstorferi* (Yuasa *et al.,* 1998). In bivalves, the immune role of C-type lectins has been best defined in scallops. Different proteins, characterized by the presence of one to four CLECT CRDs, function both as PRRs with a broad spectrum of recognition (LPS, PGN, mannose and galactose) and as opsonins capable of enhancing the phagocytic activity of hemocytes (Mu *et al.,* 2012; Huang *et al.,* 2013). Interestingly a C-type lectin of the Eastern oyster *Crassostrea virginica* has been implicated in mucosal immunity, due to its expression in the epithelial mucocytes of pallial organs (Jing *et al.,* 2011) and, similarly, codakines have been implicated in the recognition of pathogenic or symbiotic bacteria in the gills of *Codakia orbicularis* (Gourdine and Smith-Ravin, 2007).

Fibrinogen-related proteins (FREPs) constitute the third molluscan lectin family in terms of number of sequences per genome. Like C-type lectins, FREPs also find their counterpart in key molecules of the lectin pathway of the vertebrate complement system, ficolins. The primary role of FREPs in invertebrates has been demonstrated to be linked to immune defense (Hanington and Zhang, 2011), as it had been already suggested by their isolation in the mucus gland of land slugs in the late '90s (Kurachi *et al.,* 1998). The most remarkable differences between bivalve and gastropod FREPs involve the somatic mutations that occur in the latter, an aspect which will be discussed in detail in the next section.

Many other lectins have been implicated in the immune recognition system of molluscs and, despite pertaining to smaller gene families compared to C1qDC, C-type lectins and FREPs, they almost invariably appear to be more diversified in bivalves than in gastropods. Among these, the multifunctional F-type lectins are certainly worth of a mention as they have been linked to PAMP recognition in bivalves (Chen *et al.,* 2011). Despite its small size, the galectin gene family has been studied in detail (Fig. 1). Compelling evidence has been indeed produced linking the up-regulation of galectins to infection by *Perkinsus* spp. (Tasumi and Vasta, 2007; Kim *et al.,* 2008) and various types of bacteria (Zhang *et al.,* 2011; Maldonado-Aguayo *et al.,* 2014).

In stark contrast with the carbohydrate-binding proteins reported so far, H-type lectins have been only studied in gastropods, whereas no information has been provided so far in bivalves. These constituents of the perivittelin fluid protect the fertilized eggs of snails from bacterial infections (Gerlach *et al.,* 2005; Sanchez *et al.,* 2006). Although SUEL rhamnose/galactose-binding lectins cover a very similar role in sea urchin, they have been only marginally studied in molluscs so far, with the lone report of a Gram-negative bacteria agglutinating protein in the mantle of the pearl oyster *Pteria penguin* (Naganuma *et al.,* 2006).

The field of lectinomics is in constant expansion and the popularity of high throughput sequencing technologies is bringing a new impulse to the discovery of novel molecules with carbohydrate-binding potential in non-model species (Gorbushin and Borisova, 2015). For example, apextrin-like proteins, which have been recently described as novel PGN sensors in amphioxus (Huang *et al.,* 2014), have also been unveiled as PRRs in mussels (Estévez-Calvar *et al.,* 2011). In this case, the occasional association of the apextrin domain with a MACPF domain would combine PAMP-binding and pore-forming activities within the same protein product (Gerdol and Venier, 2015).

Interestingly, such a functional combination, reminiscent of the link between the lectin pathway and the terminal pathway of the complement system, has been reported in two other cases in molluscs. The first one involves a defense system from the eggs of the snail *Pomacea canaliculata*, which uses a complex between a MACPF pore-forming and a *Limulus* lectin L-6-like subunit for microbe recognition and killing (Dreon *et al.,* 2013). However, unlike mussel MACPF/apextrin proteins, in this case the pore-forming and PAMP-binding activities are provided by two distinct proteins encoded by two different genes.

The second case is that of mytilectins, a multigenic family identified in *Mytilus galloprovincialis*, which is characterized by a ricin-B-

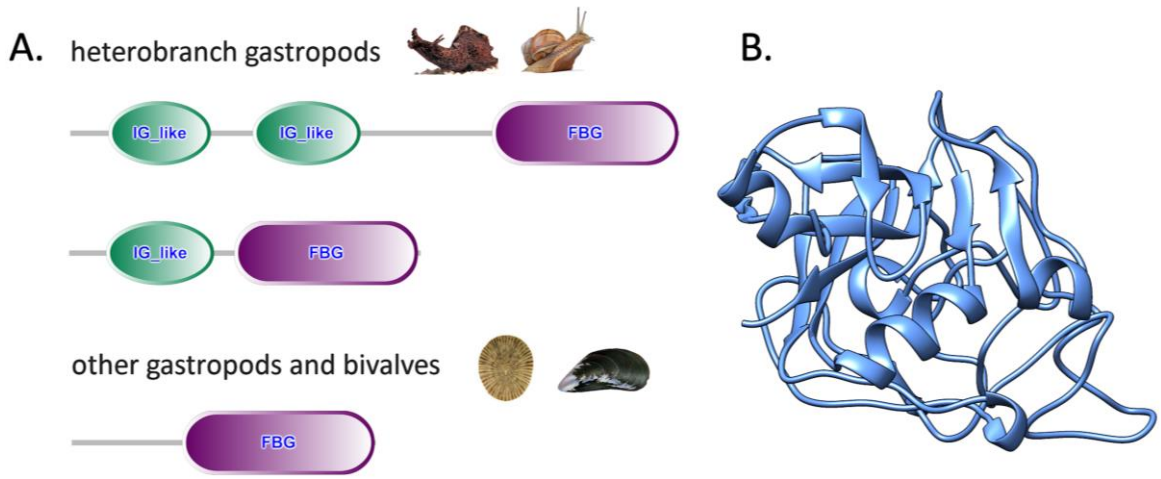


Fig. 2 Panel A: Schematic domain organization of FREPs in Mollusca. Panel B: Three-dimensional modeling of the fibrinogen-like domain of *Biomphalaria glabrata* FREP3. FBG: Fibrinogen; IG: immunoglobulin.

like fold. Although the structure of the sugar-binding domain of these molecules vaguely resembles that of an agglutinin described in gastropods (Arreguín-Espinosa *et al.,* 2001), mytilectins: (i) are taxonomically restricted to mytiloids and a few other bivalve groups and (ii) sometimes display a C-terminal aerolysin-like pore-forming domain (Hasan *et al.,* 2016). The very same structural pore-forming motif is also present in biomphalysins from land snails, although in this case there is no association with a lectin-like domain (Galinier *et al.,* 2013).

Clearly, additional studies will be needed to establish whether these hybrid proteins provide a functional equivalent to the terminal pathway of the complement system, which only appears to be present as a primitive version in protostomes (Nonaka and Kimura, 2006). At the same time, it is still largely unclear how the large arsenal of secreted molluscan PRRs can transmit signals of infection within the cell and coordinate the cellular immune response.

Somatic diversification: towards a definition of immune memory in invertebrates?

As briefly mentioned in the previous section, proteins containing a C-terminal fibrinogen-like domain (FREPs) are among the most important and best studied families of PRRs in molluscs. The structural similarity between the C-terminal domain of mammalian fibrinogen and the horseshoe crab tachylectin first revealed that this three-dimensional fold (Fig. 2) is shared by molecules involved in innate immunity and blood clotting, which therefore likely originated from a common ancestor.

So far FREPs have been mostly studied in gastropods, and specifically in the snail *B. glabrata*, where they play a fundamental role in conferring resistance to infections by the digenean trematode *Schistosoma mansoni* (Gordy *et al.,* 2015). This fascinating and complex host/pathogen relationship is characterized by patterns of resistance and susceptibility to infection which are governed by the somatic mutation of snail FREP sequences (Zhang *et al* 2004). Since somatic mutations had long been long thought to be confined to vertebrate immune systems, this phenomenon has attracted a considerable attention, making FREPs the best candidate molecules for immune memory in invertebrates (Milutinović and Kurtz, 2016).

Indeed, it has been demonstrated that somatic mutation in FREPs could permit not just to establish, but also to maintain resistance to infection in snail populations (Hanington *et al.,* 2012). This is supported not just by the different susceptibility to infection of snails expressing different FREP isoforms, but also by the acquisition of resistance towards secondary homologous infections through a mechanism of immune priming reminiscent of vertebrate adaptive immunity (Portela *et al.,* 2013). This idea was further reinforced by the observation of complexes between FREPs and highly variable mucin molecules produced by trematodes, in an interaction which could be seen as quite similar to that occurring between antigens and antibodies (Moné *et al.,* 2010).

So far, somatic diversification has only been demonstrated for a small subclass of FREPs called IgSF-FREPs which, in addition to the C-terminal Fibrinogen-like domain, also contain one or two N-terminal Immunoglobulin-like domains (Fig. 2) where the process of somatic mutation appears to take place (Zhang *et al.,* 2004). Curiously, bivalves lack IgSF-FREPs and, while similar proteins are present in *Aplysia*, they are absent in *Lottia*, revealing that they are likely to represent evolutionary innovations of heterobranch gastropods (Gorbushin *et al.,* 2010).

Although bivalve FREPs display a simpler domain organization (Fig. 2) and lack significant homology to the sequences of *B. glabrata*, they are also extremely diversified. While events of allelic recombination or somatic mutation have been invoked to explain this remarkable sequence diversity (Zhang *et al.,* 2012), the high number of FREP genes found in the oyster genome and in various transcriptomes of other species suggest that

the major driving force behind the hyervariability of bivalve FREPs is gene duplication, not somatic diversification (Gerdol and Venier, 2015; Zhang *et al.,* 2015).

Other authors have also revealed the presence of CREPs and GREPs, *i.e.,* molecules whose structure is similar to that of IgSF-FREPs, but whose N-terminal lectin-like region is either a C-type lectin or by a galectin domain (Dheilly *et al.,* 2015). While somatic diversification has not been observed yet for CREPs and GREPs, these molecules are absent in bivalves and therefore they probably also represent gastropod innovations.

In bivalves, somatic mutation has been suggested, but not demonstrated yet in myticin C (Vera *et al.,* 2011), an antimicrobial peptide characterized by extreme levels of polymorphism and probably evolving under positive selection. However, in absence of definitive evidence, it is reasonable to assume that the factors underlying this sequence hypervariability might be linked to the extreme rate of heterozygosity of the mussel genome (Murgarella *et al.,* 2016).

Overall, while convincing evidence has been collected concerning the existence of immune somatic diversification in *B. glabrata* and possibly also in other gastropods where IgSF-FREPs are present, the possibility that such mechanism of molecular diversification takes place also in bivalve mollusks still remains to be explored. However, as reported in the previous section, it is certainly noteworthy that lectin-like gene families underwent much more relevant expansion and diversification in bivalves, where somatic mutations have not been reported, compared to gastropods, where the lower sequence diversity at the whole genome level seems to be compensated by somatic diversification.

Immune memory in invertebrates is certainly a hot topic, which has been revolutionized by important discoveries in the past 20 years, which have introduced concepts such as immune priming and immune memory also in non-vertebrate metazoans. At the same time this aspect of invertebrate immunology is still poorly understood and applied research in this field is certainly complicated by the highly divergent strategies adopted by different phyla. As an example, the fascinating strategy adopted by arthropods to generate up to 10,000 different isoforms of the Down Syndrome Cell Adhesion Molecule (DSCAM) (Brites and Du Pasquier, 2015), is not used at all by other animals. As opposed to the presence of a hypervariable array of duplicated exons and complex alternative splicing patterns of insects, the genomic organization of DSCAM in molluscs is only consistent with the production of a single invariable protein product (Gorbushin and Iakovleva 2013).

The broad repertoire of molluscan Toll-like receptors converge in a highly conserved intracellular signaling pathway

Toll-like receptors (TLRs) have been recognized as central players in the innate immune response of vertebrates, where they have been implicated in the recognition of a broad range of bacterial, fungal and viral molecular patterns. In *Drosophila melanogaster*, Toll has a dual role in the determination of embryonic dorsal-ventral polarity and in the transduction of immune signaling in response to Gram-positive bacteria upon the binding with the proinflammatory cytokine Spätzle, which is in turn activated by an extracellular proteolytic cascade mediated by Peptidoglycan Recognition Proteins (PGRPs) and Gram-Negative Binding Proteins (GNBPs). In many invertebrate organisms, TLRs have been linked with pathogen detection and the subsequent production of immune effectors through the activation of Nf-κB signaling, even though no Spätzle-like cytokine has been identified yet in non-arthropod protostomes. Genomic studies have further revealed that some invertebrates possess a very large repertoire of TLRs, as perfectly exemplified by the several hundred members identified in the sea urchin *Strongylocentrotus purpuratus* (Hibino *et al.,* 2006). However, echinoderms are not an isolated case, as a similar number of these membrane-bound receptors have been found in other deuterostomes and protostomes, including the Pacific oyster (Zhang *et al.,* 2015).

Compared to bivalves, gastropod genomes appear to possess a significantly lower number of TLR genes, ranging from 10 to 20 in the genomes which have been sequenced so far. Despite this numerical difference, both bivalves and gastropods are characterized by the presence of two different sets of structurally divergent receptors which co-exist (Gerdol *et al.,* 2017), *i.e*., single cysteine cluster (scc) and multiple cysteine cluster (mcc) TLRs. While the former type resembles vertebrate receptors due to the presence of a single set of N-terminal and C-terminal-type Leucine Rich Repeats (LRRs), the latter are more similar to *Drosophila* Toll, with two consecutive sets of N- and C-terminal LRRs.

So far no comprehensive functional study has been carried out to assess whether the high sequence diversity of molluscan TLRs mirrors a functional specialization in immune recognition, in embryonic development, or in both processes. However, an increasing number of reports suggest that at least some TLRs likely play a crucial role in mediating immune response upon infection. For example, mccTLRs have been linked to antibacterial and antiviral response in abalone (Elvitigala *et al.,* 2013) and a TLR of the snail *B. glabrata* was found to be strictly associated with specimens resistant to infections by the trematode *S. mansoni* (Pila *et al.,* 2016). Convincing evidence concerning the immune function of TLRs and their role in regulating the production of AMPs in bivalves have been also produced by the observation of their up-regulation in response to experimental bacterial infections (Toubiana *et al.,* 2013; Ren *et al.,* 2014).

Surprisingly, in spite of the highly diversified repertoire of molluscan TLRs, a single molecule, named MyD88, has been so far determined as they lone cytosolic partner and positive regulator of downstream intracellular immune signaling. Although different MyD88 isoforms have been characterized in bivalve and gastropod molluscs

(Toubiana *et al.,* 2013; Ning *et al.,* 2015), it seems quite unlikely that such a small number of intracellular proteins are able to mediate signals originated from hundreds different receptors, as such a massive diversification would allow the recognition of a potentially very broad range of ligands, requiring the subsequent production of specialized immune effectors.

A regent genomic survey tried to fill this knowledge gap, pointing out that a very large amount of evolutionarily conserved cytosolic TIR-domain containing (ecTIR-DC) proteins are present in bivalves, and enabling the possible characterization of novel TLR intracellular partners in the near future (Gerdol *et al.,* 2017). Although the total number of ecTIR-DC proteins found in gastropods genomes is somewhat lower than that found in bivalves, some highly conserved gene families are present in both the two main Mollusca classes. These also include SARM, a probable negative regulator of Toll signaling. The other conserved TIR-DC families identified in both gastropods and bivalves were: ecTIR-DC 2, 3, 5, 6, 8, 9, 11, 14 and 15. While the precise role of these conserved proteins remains unknown, their remarkable conservation across metazoans suggests an important function in the regulation of intracellular immune signaling, which might warrant future research in the near future.

Notwithstanding the elusive nature of the cytosolic interactors of TLRs, the downstream machinery responsible of cytosolic immune signal transduction appears to be well conserved in molluscs, mirroring almost perfectly that of vertebrates and showing a less relevant overlap with the simpler pathway of *Drosophila* (Fig. 3).

In vertebrates, the first complex activated by MyD88 and by other related adaptors is composed by the serine/threonine kinases IRAK1, IRAK2 and IRAK4, which associate with TNF receptor associated factor (TRAF) 6. This evolutionarily conserved protein, despite having been identified in multiple bivalve species, has never been characterized so far in gastropods (He *et al.,* 2013; Toubiana *et al.,* 2014). Nevertheless, genome data clearly reveal that TRAF6 is present as a single copy gene in *L. gigantea*, *A. californica* and *B. glabrata*. IRAK kinases on the other hand represent one of the few cases of significant divergence between vertebrates and mollusks: in particular, while proteins similar to IRAK4 have been described in abalone (Ge *et al.,* 2011) and mussels (Toubiana *et al.,* 2014), no sequence displaying convincing homology with IRAK1 and IRAK2 is encoded by molluscan genomes, leaving the molecular partners of IRAK4 and TRAF6 (if any) presently unknown.

The next step of signaling involves the transforming growth factor-β activated kinase 1 (TAK1) which, together with the two associated proteins TAB1 and TAB2, is well conserved also across all metazoans. The activated TAK1 complex subsequently phosphorylates and activates the IκB kinase (IKK) complex, which is composed by IKKα and IKKβ, whose homologs have been identified in *M. galloprovincialis* and *P. fucata* (Xiong *et al.,* 2008; Toubiana *et al* 2014). Alternatively, TAK1 can also activate the MAPK pathway (not shown in Fig. 3), which ultimately leads to the activation of the AP-1 transcription factor complex in the nucleus, triggering the expression of various immunity and stress-related genes (Gerdol and Venier, 2015).

The inhibitor of NF-κB (IκB) is the key molecule in the entire intracellular immune signaling cascade, as is it binds and sequesters NF-κB in the cytosol. This inhibition can be reversed by phosphorylation by the IKK complex described above, which permits the dissociation between the transcription factor and its inhibitor. IκB is also perhaps the molecular component of the pathway which has so far been the object of the most studies, both in bivalves (Zhang *et al.,* 2009; Mu *et al*; 2010; Valenzuela-Muñoz and Gallardo-Escárate, 2014) and in gastropods (Kasthuri *et al.,* 2013; Zhang *et al.,* 2014).

Finally NF-κB, whose homologs have been reported in different molluscan species (Jiang and Wu, 2007; Huang *et al.,* 2012; Li *et al.,* 2015), can migrate to the nucleus, where it turns on the expression of pro-inflammatory genes and immune effectors, such as AMPs (Fig. 3).

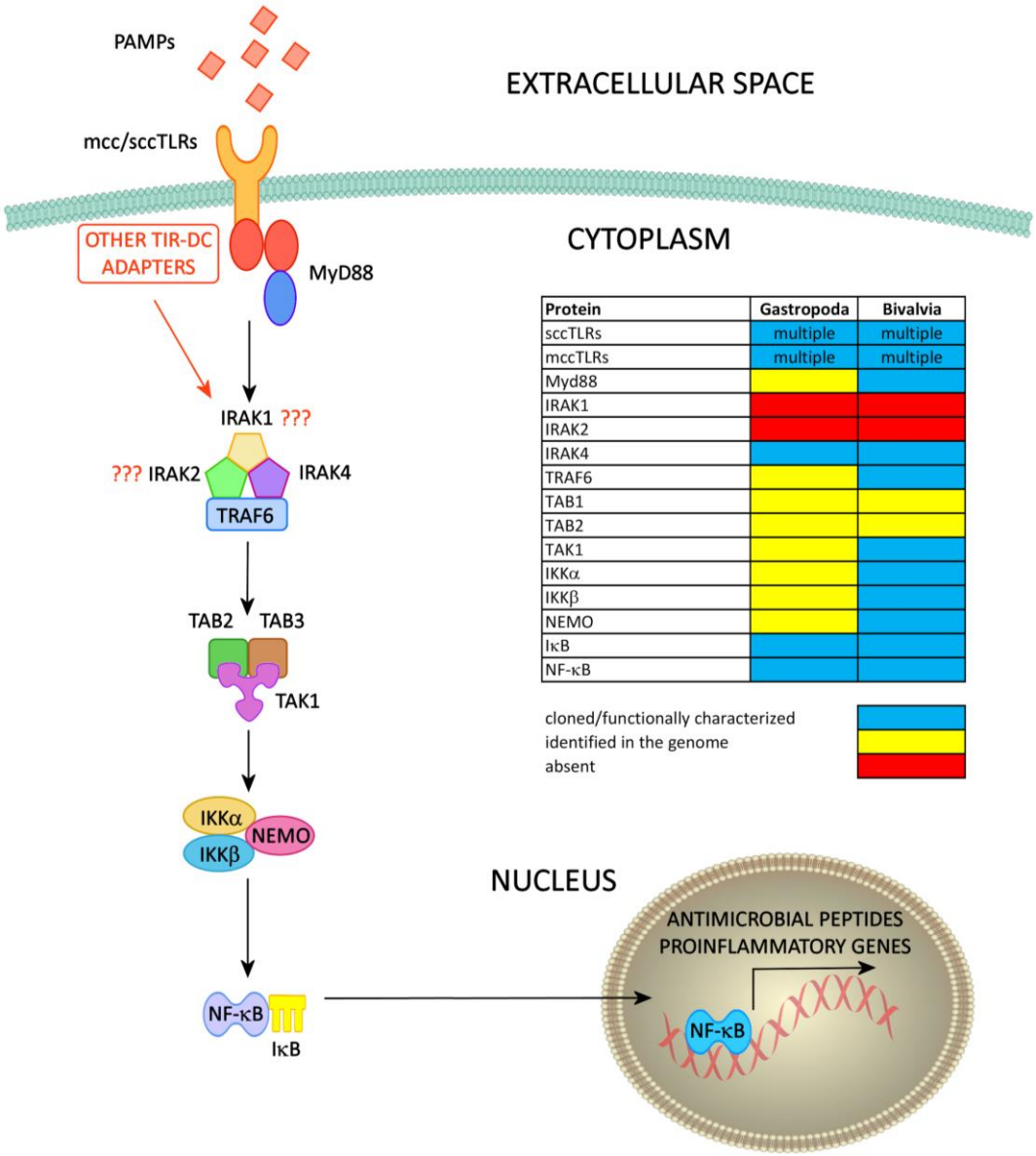
Overall, while most of the key components of the canonical intracellular signaling pathway activated downstream of TLRs seem to be well conserved between bivalve and gastropod molluscs, it remains to be investigated whether the high number and diversity of TLRs can trigger parallel intracellular signaling routes which are not shared with vertebrates. As briefly mentioned above, the remarkable sequence diversification of TLRs and the high number of intracellular TIR-DC proteins in these organisms could, at least in line of principle, allow a tailored immune response with the production of highly specific immune effectors.

As this response is likely to be driven by the interaction between TLRs and TIR-DC adaptors alternative to MyD88, the diversity of such molecules between bivalve and gastropod molluscs is certainly a topic worth of attention.

Antiviral response: STING as a major cytosolic viral sensor

While the molecular mechanisms adopted by molluscs and other invertebrates to manage infections by bacteria and eukaryotic parasites are starting to be unveiled, the genetic basis of their antiviral immunity remains nearly completely unknown. The reasons of this poor knowledge are multiple and mostly linked to the somewhat elusive nature of molluscan viral pathogens, their difficult isolation, identification and characterization. Only in the very recent years a series of technological improvements have finally permitted to gain an in depth view of mollusc-associated virome through the application of next generation sequencing technologies, but this field can still be considered to be at its very early stages. Taking into account the important economic losses inked to virus-induced mass mortalities in mollusc species of commercial interest (Barbosa Solomieu *et al.,* 2015), this field of study will most likely encounter a great expansion in the years to come.

Other authors have already provided an



Protein	Gastropoda	Bivalvia
sccTLRs	multiple	multiple
mccTLRs	multiple	multiple
Myd88		
IRAK1		
IRAK2		
IRAK4		
TRAF6		
TAB1		
TAB2		
TAK1		
IKKα		
IKKβ		
NEMO		
IκB		
NF-κB		

Fig. 3 Overview of the canonical intracellular immune signaling pathway activated downstream of TLRs upon PAMP binding. Only the main components are shown. Proteins whose existence has not been demonstrated yet in molluscs are marked by question marks.

excellent review of the complex molecular antiviral machinery present in oysters, whose components have been identified by homology with their vertebrate counterparts (Green *et al.,* 2015). This system comprises a broad range of membrane-bound and intracellular receptors and has a significant overlap with the intracellular signaling routes activated downstream of TLRs. The multifaceted antiviral response of molluscs possibly also involves autophagy, which could be mediated by the recognition of viral nucleic acids by TLRs located in endosomes, and the activity of the RNA-induced silencing (RISC) complex and RIG-like receptors (RLRs). While all these components have been only marginally studied in mollusks, they appear to be present, for the most part, in the genomes of both bivalves and gastropods.

Far for providing a complete account of the entire complement of gene-encoded molecules involved in antiviral response, this section of the review will mainly focus on one of the key components of the cytosolic system of viral sensing, the Stimulator of Interferon Genes (STING), which is missing in all the sequenced gastropod genomes.

STING is a key regulator of intracellular sensing of viral nucleic acids which is usually bound to the endoplasmic reticulum membrane through N-terminal transmembrane domains. Upon viral

sensing, STING dimerizes and migrates to the perinuclear region, where it interacts with TBK1 (a key molecule also in TLR signaling), thereby activating the IRF3 transcription factor and triggering the expression of interferon genes (Fig. 4) (Ishikawa *et al.,* 2009). STING can either bind directly single- or double-strand nucleic acids or collect signals derived from a multitude of intracellular receptors with somewhat redundant functions (Ma and Damania, 2016) and acting as a major hub coordinating immune responses to viral infection.

The different players of this cytosolic sensing pathway are sometimes difficult to be identified due to large sequence divergence between protostomes and vertebrates, and lineage-specific loss of the known vertebrate regulators upstream of STING (Gerdol and Venier, 2015). Also, while IRF-like sequences have been previously identified in mollusks (Wang *et al.,* 2013), no IRF-3 and interferon-like sequences have ever been described in invertebrate organism, suggesting that the terminal branch of this pathway might be largely divergent between vertebrates and invertebrates.

Despite these differences, STING homologs can be readily identified in many protostomes, including bivalve molluscs, although these proteins present remarkable structural differences compared to their vertebrate counterparts: (i) they lack N-terminal transmembrane domains for the anchoring to the ER membrane; (ii) they are associated to an N-terminal TIR-like domain, whose typical function is immune signal transduction (Fig. 4).

Also, the TIR/STING domain pair is repeated two times, suggesting that, unlike vertebrates, no dimerization is necessary for STING activation, as the homotypic interaction could possibly involve the two STING domains present within the same protein precursor. A large scale genomic comparative study has recently evidenced that similar TIR/STING proteins are also present in brachiopods and polychaetes, even though only a single TIR/STING domain pair is found in these organisms (Gerdol *et al.,* 2017). On the other hand, as mentioned above, no STING gene is present in the available genomes of gastropods and cephalopods. However, transcriptome data mining revealed that homologous sequences, with the brachiopod/polychaete domain configuration, are present in Caenogastropoda, implying that the loss of STING might have occurred only in Heterobranchia and Patellogastropoda (Fig. 4).

Another important difference between bivalves and gastropods within the STING pathway is linked to cGAS, an important DNA sensor containing a Mab-21 domain, which can catalyze the production of the second messenger cGAMP, activating STING (Ablasser *et al.,* 2013). Although no cGAS sequence has been ever formally described in mollusks, several Mab-21 domain containing proteins can be identified in their genomes. However, while only a single-copy cGAS-like gene is present in gastropods, the oyster genome encodes several dozen paralogous sequences, potentially offering a very broad array of STING activators with slightly different binding specificities.

Overall, this data seems to point towards a broad capability of recognition of foreign nucleic acids by bivalves through STING/cGAS, opposed to gastropods, where STING is absent (with the exception of Coenogastropoda) and its upstream cGAS-like partners display low sequence diversity.

Obviously, as no functional evidence has been provided so far about the involvement of molluscan STING and cGAS homologs in antiviral defense, the possible effects of the loss of this pathway in gastropods are a matter of speculation. By homology with vertebrates, where STING knockout determines an increased susceptibility to lethal viral infections (Ishikawa *et al.,* 2009), one might argue that gastropods are likely to be more susceptible to viral infections than bivalves, unless any compensatory, presently unknown strategy has been developed to cope with this loss.

Occurrence and diversity of antimicrobial peptides

One of the most striking differences between gastropods and bivalves is the scarce amount of reports in the molecular immunology literature about antimicrobial peptides (AMPs) in the former, opposed to the large number of publications produced over the past two decades for the latter (Li *et al.,* 2011). This disparity is so remarkable that one might wonder whether this is linked a more developed arsenal of microbe-killing peptides or, simply, to the greater efforts put so far in bivalve antimicrobial research in bivalves. As a matter of fact, the available genomic and transcriptomic data is in agreement with literature reports, pointing out that gastropod molluscs lack several AMP gene families which are present not only in bivalves, but often also in other protostomes (Table 1).

Mussels (*Mytilus* spp.) represent a remarkable example of the expansion and diversification of host defense peptides. Classical biochemical approaches carried out in the early ‘90s permitted to isolate several secreted cysteine-rich AMPs from circulating hemocytes, where these molecules are usually stored as inactive precursors (Charlet *et al.,* 1996; Mitta *et al.,* 1999). Thanks to the recent developments of NGS, we have learned that these peptides are produced by multi-genic families, named defensins, mytilins and myticins. Despite remarkable differences in amino acid composition and organization of the peptide precursor, these mussel AMPs pertain to the same cysteine-stabilized alpha-helix beta-sheet (CS-αβ) superfamily, which reunites diverse peptides characterized by a common structural motif, *i.e.,* the presence of an alpha helix, followed by two antiparallel beta-sheets, whose position in the three-dimensional space is stabilized by three (or four) disulfide bridges. While the role of these cysteine-rich AMPs is certainly linked to pathogen killing, with a preferential activity against Gram-positive bacteria, it has also been suggested that they may act as immune modulators, with a cytokine-like function (Balseiro *et al.,* 2011). Following the studies in mussels, defensin-like peptides, either characterized by the presence of six cysteine residues, like in arthropods or eight cysteine residues, were shown to be also present in

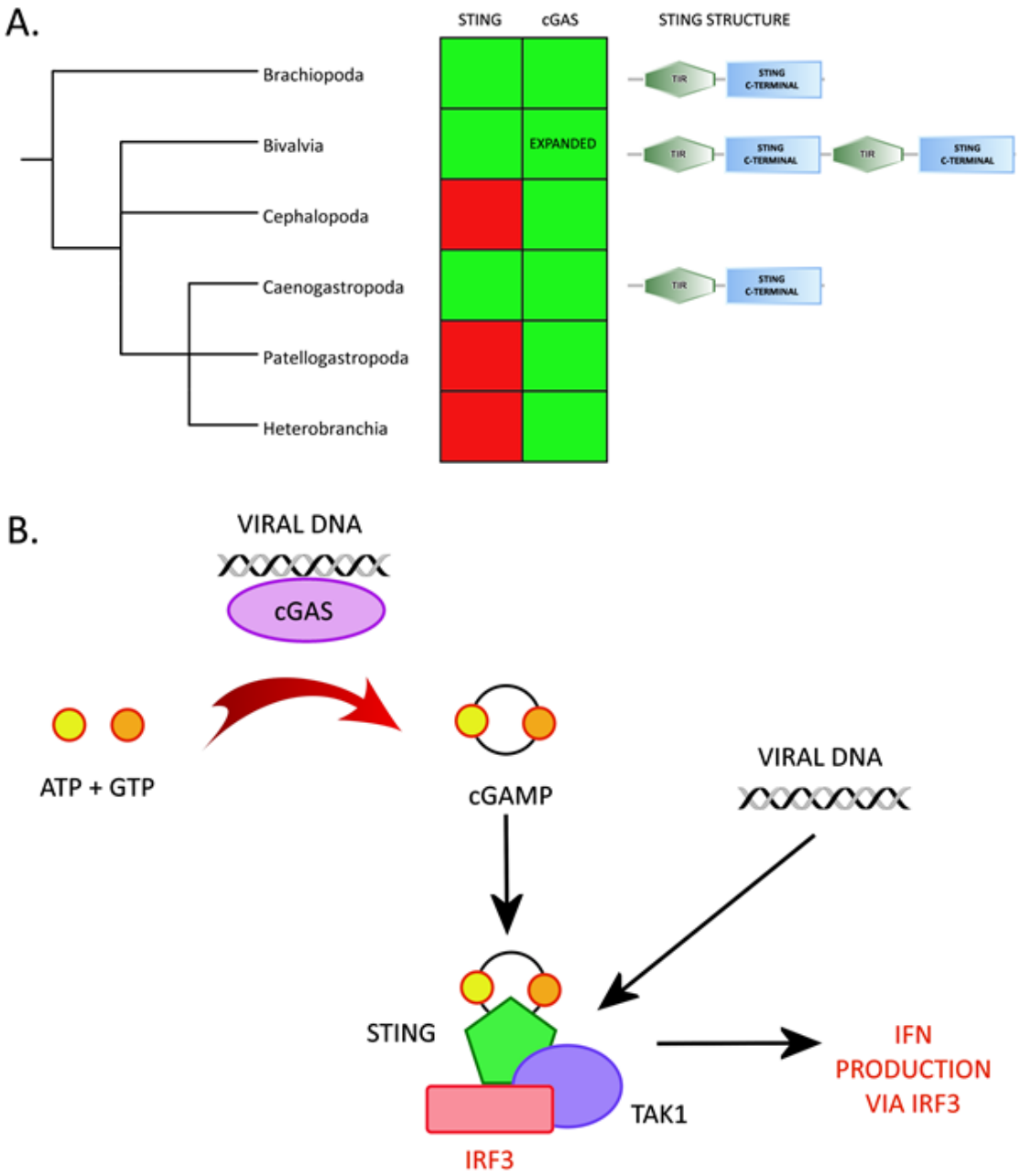


Fig. 4 Panel A: taxonomical distribution of TIR/STING and cGAS within Mollusca. Presence and absence are marked by green and red colors, respectively. Brachiopoda were included as an outgroup phyla for Lophotrochozoa. Whenever present, the domain organization of STING is shown. Panel B: schematic overview of viral sensing mediated by STING. STING can directly bind viral nucleic acids, forming a complex with TAK1 and IRF3, which is activated and translocated to the nucleus, where it triggers the expression of interferon genes. Alternatively, STING can be activated by the interaction with cGAMP molecules produced by cGAS upon the recognition of foreign DNA in the cytosol. Molecules whose homologs have not been reported yet in molluscs are marked in red.

oysters, clams and freshwater mussels, with either hemocyte or mantle tissue specificity (Gueguen *et al.,* 2006; Peng *et al.,* 2012; Wang *et al.,* 2015).

It is noteworthy that CS-αβ peptides similar to bivalve and arthropod defensins have been reported as missing in some large invertebrate taxa, which also include gastropods (Rodríguez de la Vega and Possani, 2005). This is confirmed by the absence of gene products sharing significant sequence similarity to any of the previously described invertebrate defensins in *L. gigantea*, *B. glabrata* and *A. californica*. However, this consideration cannot be extended to all gastropods, since a defensin peptide with six cysteines has been identified in the abalone *Haliotis discus*, pertaining to the ancestral gastropod clade Vetigastropoda (De

Zoysa *et al.,* 2010). This report adds further complexity to the evolutionary history of invertebrate defensins, whose common ancestry has been previously brought into question (Tarr, 2016).

Big defensins display an similar taxonomic distribution, characterized by an apparent absence in gastropods and multiple reports in diverse bivalve species (Zhao *et al.,* 2010; Rosa *et al.,* 2011). Despite their name, these AMPs are not related to the other invertebrate defensin-like peptides described so far, as they share the same fold of vertebrate β-defensins. Despite the lack of big defensins in the available gastropod genomes, a big defensin-coding sequence appears to be present in the transcriptome of the abalone *Haliotis tuberculata*, mirroring the presence of classical defensins in abalones and extending the known taxonomical distribution of these AMPs, which had so far been found only in bivalve mollusks, horseshoe crabs and amphioxus (Gerdol *et al.,* 2012).

As evidenced from sequence data mining, macins are the only class of CS-αβ peptides apparently present in all bivalves and gastropods species. This AMP family comprises multifunctional peptides which are thought to be involved both in bacterial killing and wound healing and which display some variations in their cysteine array, which may present 4, 5 or 6 disulfide bridges (Gerdol *et al.,* 2012). Consistently with reports in other protostomes (Tasiemski *et al.,* 2004), macins appear to be broadly expressed in various tissues and, in particular, they critically contribute to the antibacterial activity of the mucus of the giant snail *Achatina fucata* (Zhong *et al.,* 2013).

The repertoire of molluscan cysteine-rich peptides is growing at a fast rate as new AMP families are identified either by conventional or by -omic methods. Once again, mussels have been a major target for AMP discovery, as mytimycins (Sonthi *et al.,* 2011), myticusins (Liao *et al.,* 2013) and the enigmatic CRP-I family, whose function still remains to be fully elucidated (Gerdol *et al.,* 2015), have been described in the past few years.

Comparatively, only a little attention has been put in the discovery of linear AMPs devoid of cysteines, and this finds a possible explanation in the difficulty of the discovery of these fast-evolving gene products by sequence similarity-based searches. The most relevant case of linear AMPs described in molluscs so far are probably molluscidins, low-complexity and highly cationic short peptides composed by several dibasic repeats, found in both oyster and abalones (Seo *et al.,* 2013, 2016). Another small family of linear peptides, named CgPrp, rich in Pro and Arg residues, has been identified in oyster hemocytes. Although CgPrp peptides do not show any detectable antimicrobial activity, they are significantly and synergistically able to enhance the effectiveness of defensins (Gueguen *et al.,* 2009). As far as gastropods are concerned, potent proline-rich AMPs with no similarity to any other known peptide have been isolated from the hemolymph of the marine snail *Rapana venosa* (Dolashka *et al.,* 2011). While the research on linear cationic AMPs is still mostly based on classical biochemical isolation methods, the use of *in silico* approaches is currently proving to be a valid complementary tool in the discovery of novel AMPs in molluscs, as testified by the recent discovery of myticalins in *M. galloprovincialis*, a novel class of taxonomically restricted, hypervariable AMPs which display a broad spectrum of activity towards Gram+ and Gram- Bacteria (Gerdol *et al.,* 2016).

Conclusions

With over 100,000 extant species and their colonization of nearly all terrestrial, freshwater and marine environments, mollusks represent a unique case for the study of many aspects of evolutionary biology, including defense against potentially pathogenic microbes. Over 450 million years of independent evolution, gastropod and bivalves have clearly developed peculiar molecular mechanism to tackle these challenges and the nature of these unique adaptations are starting to emerge, also thanks to the contribution of the increasing number of fully sequenced genomes available.

We are quickly moving from the isolation and characterization of single molecules through classical biochemical methods to the possibility of mining entire genomes and transcriptomes, identifying dozens if not even hundreds of candidate immune receptors, signaling transducers, transcription factors and antimicrobial effectors. Obviously, these two approaches are complementary to each other, and the combination between data mining and functional approaches is quickly providing new hints about the placement of several missing pieces in the highly complex puzzle of the invertebrate immune system.

As far as molluscs are concerned, despite several recent discoveries of the utmost importance, such as the identification of a complete TLR signaling pathway and the discovery of molecular strategies providing immune memory, we are still missing large portions of the whole picture. Our knowledge of the functioning of viral sensing systems for example is still extremely limited and almost exclusively limited to the components which are homologous to those of vertebrates. At the same time, the nature of invertebrate cytokines is still elusive, despite the high conservation of the signaling routes leading to their production.

Most importantly, no global approach has yet been effectively implemented for defining how the different molecules involved in PAMP detection, in the activation of immune signaling and in the elimination of pathogens coordinate their activity. For example, while functional studies have permitted to elucidate the primary sequence and the binding specificity of several dozen molluscan lectins, it is presently unclear how such molecules transmit immune signals within the cell and, while some of these appear to work in a “seek and destroy” mode, thanks to the combination between PRR and pore-forming modules, others are likely to trigger a highly specific and finely regulated cellular response, which probably involves membrane receptors, intracellular partners and cytokine-like

Table 1 Summary of the main antimicrobial peptide families reported so far in bivalve and gastropod molluscs. A brief description of the taxonomic distribution, expression pattern and spectrum of activity is also reported, whenever available

AMP type/family	Bivalvia	Gastropoda
Defensins	Present in multiple species; 6/8 cysteine residues; hemocyte- or mantle-specific; activity against Gram+ bacteria	Absent in most species; present in abalones; 6 cysteines; expressed in whole body; unknown spectrum of activity
Mytilins	Taxonomically restricted to mussels, 8 cysteines; hemocyte-specific; broad spectrum of activity	Absent
Myticins	Taxonomically restricted to mussels, 8 cysteines; hemocyte-specific; activity against Gram+ bacteria; antiviral activity; might have immuno-modulating properties	Absent
Mytimycins	Taxonomically restricted to mussels; antifungal activity; hemocyte-specific; unknown spectrum of activity	Absent
Macins	Present in multiple species; 8/10/12 cysteine residues; expressed in whole body; unknown spectrum of activity	Present in multiple species; 8/10 cysteine residues; expressed in whole body; present in the mucus; broad spectrum of activity
Big defensins	Present in multiple species; 6 cysteines; expressed in whole body; broad spectrum of activity	Absent in most species; present in abalones; 6 cysteines; no expression data available
Other Cys-rich peptides	Myticusins, CRP-I; only reported in mussels so far; variable disulfide array; diverse pattern of expression and spectrum of activity	Not reported
Molluscidins	Only reported in oyster; linear peptide rich in dibasic repeats; gills-specific; broad spectrum of activity	Only reported in abalone; linear peptide rich in dibasic repeats; gills-specific; borad spectrum of activity
Pro-rich peptides	CgPrp, only reported in oyster; no antimicrobial activity but synergistically enhances the antimicrobial activity of defensins; hemocyte-specific. Myticalins, taxonomically restricted to mussels; gills-specific; broad spectrum of activity	Only reported in *R. venosa*; hemocyte-specific; unrelated to bivalve Pro-rich peptides; activity against Gram+ bacteria

molecules which still remain to be unveiled.

Overall, molluscs, with their divergent molecular strategies for PAMP sensing and pathogen killing, certainly represent fascinating models for the study of invertebrate molecular immunology in the years to come.

Acknowledgements

This work has been made possible through funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 678589, and from the University of Trieste FRA2015 program.

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