Chapter 6

Specificity of innate immunity in bivalves: a lesson from bacteria

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Keywords: innate immunity, bivalves, *Mytilus*, vibrios, hemocytes, serum, opsonins, mannose sensitive hemagglutinin

Running title: Specificity of bivalve immunity to bacteria

Abstract

Bivalves are a relevant ecological group, widespread in freshwater, estuarine and marine environments, with many edible species (such as oysters, mussels, clams). Like all other invertebrate groups, bivalves lack an adaptive immunity, but they are endowed with a potent and complex innate immune system (humoral and cellular defenses). Bivalve immunity displays a wide variety of sensitive receptors, selective effectors, and synergistic genetic regulatory networks that ~~afford~~ protection in a fluctuating environment.

As filter feeders, bivalves accumulate large numbers of microorganisms, mainly bacteria, that can either establish a commensal relationship with the host without causing diseases, or proliferate and invade soft tissues, resulting in high mortality. In this ~~light~~, understanding the relationship between bivalve immune system and bacteria has important implications not only for protection of economically important species, but also for human health ~~concern~~. Available data underlying the specificity of bivalve immune response to bacterial challenge will be summarized.

1. Innate immunity in bivalves: diversity and complexity

Bivalves (Mollusca, Lophotrochozoa) are a relevant ecological group, widespread in freshwater, estuarine and marine environments, with many edible species. Like all other invertebrate groups, bivalves lack an adaptive immunity: however, they are endowed with a potent and complex innate immune system (humoral and cellular defenses) similar to that of vertebrates [1]. The lack of acquired immunity and the capacity to form antibodies (specific response) does not mean lack of specificity: invertebrates have evolved genetic mechanisms capable of producing thousands of different proteins from a small number of genes; this diversity allows them to recognize and eliminate a wide range of different pathogens [2].

Bivalve hemocytes are responsible for cell-mediated immunity through the combined action of the phagocytic process with humoral defense factors such as agglutinins (e.g., lectins), lysosomal enzymes (e.g., acid phosphatase, lysozyme), toxic oxygen intermediates, and various antimicrobial peptides [3]. The morphology, ultrastructure and functions of bivalve haemocytes were reviewed by Hine [4]. Granular haemocytes (basophilic and acidophilic granulocytes) form a distinct group, whereas agranular haemocytes are heterogeneous in appearance and ultrastructure (blast-like cells, basophilic macrophage-like cells, hyalinocytes). Not all types occur in each bivalve species; scallops lack granulocytes, and the hyalinocyte is a poorly defined cell type in several groups; moreover, due to functional heterogeneity, the functions of each haemocyte type cannot be reliably extrapolated between species. In the last decade, the application of flow cytometry analysis and molecular characterization of different immune-related molecules has greatly improved our knowledge on functional characterization of hemocytes, underlying both common and distinct features of the immune system in different bivalve species [1,5-8].

In bivalves, innate immunity promotes generalized protection against not only pathogenic organisms (e.g. protozoa, bacteria, viruses), but also environmental stressors (e.g. presence of contaminants, algal toxins, air exposure, mechanical stress, high temperatures, changes in salinity) [9]. To afford protection in this fluctuating environment, bivalve immunity displays a wide variety

of sensitive receptors, selective effectors, and synergistic genetic regulatory networks. Moreover, as filter feeders, bivalves can accumulate large numbers of microorganisms from the aquatic environment; this may result in a concentration of potential pathogens, mainly bacteria, that can either establish a commensal relationship with the host without causing diseases, or proliferate and invade soft tissues, resulting in high mortality. For edible species (such as oysters, mussels, clams), understanding the relationship between the immune system and bacteria has two main implications: 1) to ensure a better protection in the intensive breeding of economically important species; 2) to control potential accumulation of human pathogens of concern for public health. In this chapter, available data underlying the specificity of bivalve immune response to bacterial challenge will be summarized.

2. Bivalves and marine bacteria

A rich and diverse microbiota is present in the aquatic environment, and bivalves can ingest many different kinds of bacteria, including some that can be pathogenic to the bivalve host [10]. Bacterial pathogens can affect larvae cultured in hatchery, and adults cultured in the natural environment; generally, they are most virulent ~~in~~ during larval stages. Larval pathogens include members of *Vibrio, Pseudomonas, Alteromonas, Moraxella,* and *Aeromonas* genera [11]. Vibrios are also involved in diseases of juvenile and adults bivalves together with, but to a lower extent, bacteria belonging to other genera [11-13].

Vibrios are Gram-negative marine bacteria widely distributed estuarine and coastal waters and sediments. The genus comprises human pathogens (*V. cholerae, V. vulnificus, V. parahaemolyticus*) and species pathogenic for aquatic animals[9] (*e.g., V. splendidus, V. aestuarianus*). Vibrios tend to be most common in warmer waters (above 17°C) and, depending on the species, they tolerate a range of salinities. A common trait is the presence of multiple lifestyles: a planktonic, free-swimming state and an adhering form on biotic and abiotic surfaces. Vibrios represent a high proportion of bacteria isolated from healthy and diseased bivalves, with 100-fold

higher concentration than in the surrounding water. For some of the bivalve-associated vibrios, the precise role as commensal, opportunistic or pathogenic organisms remains to be defined.

Vibrio species associated with bivalve larvae diseases include *V. anguillarum*, *V. alginolyticus*, *V. tubiashii*, *V. splendidus*, *V. pectenicida*, and *V. neptunius* [9-11]. Pathogen ~~entry~~ has been reported from brood stock, seawater and algal food [14]. Larval vibriosis is an aggressive and rapidly progressing infection that affects different species and best documented in oysters. Disease outbreaks are characterized by bacterial swarming around the velum, loss of larval motility, extensive soft tissue necrosis, and rapid mortality (up to 90% within 24 hours of initial exposure to the most pathogenic strains). Oyster larvae cannot repair damage to the mantle during the early stages of infection; this capacity increases with the increasing size of the juveniles [11]. The higher susceptibility of developing larvae to bacterial infection may be due to an immature immune system, and investment of energy in fast metabolic development for settlement. Metamorphosis was identified as a crucial stage when larvae increased the expression of immune-related genes and responded to environmental signals [15]; however, information on ontogeny of the immune response in different species is still scarce.

Vibriosis ha~~ve~~ been also described in juvenile and adult oysters, clams and mussels [11,12]. Vibrios are associated to the syndromes known as Brown Ring Disease (BRD) in adult clams [12,16] and Summer Mortality in juvenile oysters[13,17-19]. BRD affects both reared and wild clams (*Ruditapes philippinarum* and *R. decussatus*); it is caused by *V. tapetis*, that was shown to be capable to reproduce the disease in healthy animals due to production of secreted proteins [11,20]. An alteration of the calcification process of the inner surface of the valves and the presence of a brown deposit of conchiolin between the shell edge and the pallial line is typical of the disease [11]. The geographical origin of the clams and environmental factors (*i.e.* temperature and salinity) seem to play a role in sensitivity to infections [10,11]. Resistance to BRD in different clam species and stocks may be also related to the capacity of shell repair, as well as to the phagocytic activity of the hemocytes [21-22]. Information on virulence factors of *V. tapetis* is scanty; recently, several protein

fractions from extracellular products were shown to display biological activity towards clam hemocytes [20].

The physiological and/or genetic status of the oysters, and multiple stressors such as elevated temperature, low dissolved oxygen, and limited energy resources after spawning were associated with Summer Mortality events [13]. In addition, one or more infectious agents, such as herpes virus, OsHV1[19], and different *Vibrio* species (*e.g.*, *V. aestuarianus*, *V. splendidus* clade, *V. harveyi*) have been implicated as etiological agents [17,18]. High temperatures might be important stressors by affecting host physiology and susceptibility to infection, and supporting proliferation, spread and virulence of thermodependent *Vibrio* spp [23].

The so called *V. splendidus* clade constitutes a complex of phenotypically and genetically related species [24], with several members causing significant losses in the aquaculture industry worldwide. Studies with the *V. splendidus* LGP32 strain (recently assigned to the species *V. tasmaniensis*, belonging to the same clade [25]) indicated that its virulence is linked to the outer membrane protein ompU [26]. Other virulence factors are the metalloprotease Vsm [27], and an invasive serine protease Vsp specifically secreted through outer membrane vesicle production [28]. A recent 2-year sampling campaign in the northern Adriatic Sea (Italy) [29] showed that genes encoding OmpU protein and zinc metalloprotease are present in strains belonging to different species of the clade. Nasfi et al. [26] suggested that diverse clones of the *V. splendidus* clade can replace each other during different mortality outbreaks, probably favored by massive lateral transfer of virulence factors thus underlying the epidemiological risk of emergence of new virulent strains.

The species *V. aestuarianus* is ubiquitous in different geographic areas. It was associated with oyster Summer Mortalities and its pathogenic potential was shown in experimental oyster challenges [30,31]. Isolates show variable virulence likely linked to the varying toxicity of the bacterial extracellular products. Those produced by *V. aestuarianus* 01/032, a strain isolated during a mortality outbreak in an experimental hatchery, caused morphological changes and immunosuppression in *C. gigas* haemocytes *in vitro* [32]. These effects were ascribed to the capacity

of the strain to produce a Vam metalloprotease that affects hemocyte morphology and impairs phagocytic function. GbpA (N acetyl glucosamine binding protein) and/or MSHA (Mannose Sensitive Hemagglutinin) adhesins are present in a large proportion of *V. aestuarianus i*solates [29]; both ligands are involved in interactions with environmental surfaces (*e.g.*, chitin), which might contribute to its ~~the~~ persistence in the environment [9]. Moreover, ~~they~~ these adhesins may play a role in mediating surface interactions between bacteria and bivalve hemocytes, thus affecting the immune response (see below).

3. Immune recognition

Immune recognition is the first step of the immune response, allowing the discrimination of self-/not self-substances. Pattern Recognition Receptors (PRRs) on the hemocyte membrane and in hemolymph serum play a crucial role in activating the immune system to eliminate pathogens. PRRs selectively recognize a large family of conserved foreign molecules called Pathogen-Associated Molecular Patterns (PAMPs), such as lipopolysaccharides, lipoproteins, peptidoglycans, lipoteichoic acids, viral dsRNA, unmethylated bacterial DNA, zymosans and heat shock proteins. Several groups of distinct PRRs have been identified in bivalves, including lectins, peptidoglycan recognition proteins (PGRPs), Gram-negative binding proteins (GNBP), toll-like receptors (TLRs), scavenger receptors (SRs), rig-like receptors (RLRs) and NOD-like receptors (NLRs) [1,8,33]. The most studied PRRs show a high versatility and flexibility; however, some degree of specificity could be identified.

Calcium-dependent (C-type) lectins are a superfamily of proteins that can bind PAMPs through the recognition of carbohydrates, thus promoting their agglutination/immobilization and triggering successive immune functions, such as opsonization and phagocytosis. Multiple lectin-related transcripts have been identified in different bivalve species, that are up-regulated by immune challenge, showing a broad specificity towards microorganisms but a remarkable carbohydrate-binding specificity [34].

Toll-like receptors (TLRs) are involved in the molecular recognition of pathogens as well as in cell adhesion, signal transduction and cell growth [35]. The search for components of the Toll signaling pathway has recently lead to their discovery in many bivalve models; transcriptome analysis using Next Generation Sequencing (NGS) technologies lead to the identification of a vast repertoire of putative TLRs encoding sequences for *M. edulis* [33], *R. philippinarum* [36], and *C. virginica* [37]. TRLs and components of the Toll-activated pathways were up-regulated in the hemocytes of marine bivalves following single in vivo injection with different bacteria and PAMPs, with the majority of the challenges involving Vibrios as predominant marine bacteria [38].

The complement system pathway relies on several interacting proteins to recognize and then eliminate foreign microorganisms, with a pivotal role in the initiation of defence mechanisms, including agglutination, adhesion, opsonisation and cell lysis. Once activated, the complement system promotes target proteolytic reactions that operate following classical, lectin or alternative pathways. C1q, a subcomponent of the complement C1 complex, is considered to be a versatile PRP, binding directly to a broad range of PAMPs of bacteria, viruses, and parasites, as well as enhancing pathogen phagocytosis [39]. Some C1q proteins with specific ligand recognition properties have been described and characterized also in bivalves: similar expression changes were observed in the hemocytes challenged with both Gram+ and Gram− bacteria [40,41].

4. Immune signaling

Upon the successful recognition of foreign compounds, activated PRRs trigger different intracellular signaling pathways that are required for the immune response. This may lead to rapid activation of phagocytosis, ROS production, release of pre-existing enzymes of antimicrobial molecules, as well as to changes in transcription of immune or stress response genes at the nuclear level [42]. Among immune signaling pathways, the mitogen-activated protein kinase (MAPK), nuclear factor kB (NF-kB), the complement component and the Toll pathways have been investigated in bivalves. For a discussion of the NF-kB, complement and Toll pathways see refs. [1,8].

Studies on components of kinase-mediated transduction pathwaysfirst revealed specificities in the bivalve immune response to different bacteria. In *Mytilus galloprovincialis* hemocytes, exposure to different bacterial species and strains, heterologous cytokines and natural hormones, as well as organic environmental chemicals, underlined the role of conserved cytosolic kinases (such as MAPKs and protein kinase C-PKC) and kinase-activated transcription factors (such as Signal Transducers and Activators of Transcription-STATs, c-AMP Responsive Element- CREB), in the immune response [42]. In particular, *in vitro* studies showed rapid phosphorylation/ dephosphorylation of these signaling components in response to different stimuli, with specific time courses and resulting in activation of different functional immune parameters. Challenge with different bacteria (*E. coli* and *V. cholerae*) resulted in differential activation/inactivation of cytosolic kinases, as well as of the transcription factors STAT1 and CREB [42]. In particular, different strains of *E. coli* and *V. cholerae* (*E. coli* MG155, a wild-type strain carrying type 1 fimbriae, and its unfimbriated derivative, AAEC072 Δfim; *V. cholerae* O1 El Tor biotype strain N16961, carrying MSHA, and its ΔMSHA mutant) induced distinct patterns of phosphorylation of MAPKs, in particular of the stress-activated p38 and JNKs, as well of PKC isoforms, that were related to differences in bactericidal activity [43]. The lower anti-bacterial activity of hemocytes towards the mutant *E. coli* strain and wild-type *V. cholerae* compared with wild type *E. coli* was associated with a reduced capacity of activating MAPKs. Moreover, the ΔMSHA *V. cholerae* strain, that was the most resistant to the hemocyte bactericidal activity, induced downregulation of cell signaling, strong lysosomal damage and reduced hydrolytic enzyme release [44]. These results underlined how not only different bacteria, but also different bacterial strains can elicit specific responses in terms of activation of cytosolic components of kinase-mediated signaling: these effects were ascribed to specific surface interactions between hemocytes and bacteria. Interestingly, the differential effects on immune signaling and the resulting immune response observed *in vitro* well correlated with the capacity of mussels to clear different *E. coli* and *V. cholerae* strains from their hemolymph in vivo [45,46].

Differential responses were also observed in *Mytilus* hemocytes challenged with two different bivalve pathogens, *V. splendidus* LGP32 and *V. anguillarum* (ATCC 19264). Functional responses were first observed *in vivo*, after challenge with heat-killed bacteria [47]. The underlying mechanisms were investigated *in vitro*, with live bacteria, revealing differential activation of immune signaling by the two vibrio species [48]. *V. splendidus* LGP32 rapidly induced significant changes in hemocyte adhesion, lysosomal membrane stability, lysozyme release, extracellular ROS and NO production. These responses were associated with rapid and persistent activation of p38 MAPK and PKC isoforms. On the other hand, *V. anguillarum* showed a reduced capacity to stimulate functional immune responses, in line with reduced activation of p-38 MAPK and PKC with respect to *V. splendidus*.

Overall, these studies underlined the specificity of kinase-mediated signaling activated by bacterial challenge in mussel hemocytes: however, the identification of these signaling components, evaluation of protein expression and their activation state (phosphorylation), were based on the utilization of heterologous antibodies, and on the use of specific pharmacological inhibitors of their mammalian counterparts. Homologues to MAPK pathway constituents were first sequenced in oyster [49] and manila clam [50]. More recently, NGS analyses on *R. philippinarum* [51] and *C. virginica* [52] have helped to finally identify a functionally conservative set of regulated transcripts associated with MAPK pathways. Recent advancements in transcriptomics and data mining pipelines have also enabled the discovery of JAK/STAT homologues in *M. edulis* [33].

5. Immune Effectors

Defense responses involve phagocytosis of foreign materials, ROS production and release of hydrolytic enzymes, lectins and anti-microbial peptides by the hemocytes [3]. Bivalve hemolymph serum contains a wide range of different secreted components that participate in agglutination, opsonization, degradation, encapsulation of microorganisms, as well as in clotting and wound healing [3,9]. An overview of the most recent accomplishments in the fields of antimicrobial peptides, lysozymes, cytokines and acute phase processes that depend on perforins, immune cell activation

and antioxidant enzymes is given in Bassim et al.[8]. Additionally, proteins involved in metal homeostasis, such as ferritin and metallothionein, were identified in bivalves following exposure to pathogens or PAMPs and are thought to be part of the elicited anti-microbial processes [51].

Induction of many cellular and serum functional immune parameters by different stimuli, including *in vitro* and *in vivo* challenge with live and heat-killed bacteria and PAMPs, has been evaluated in a large number of studies on different bivalve species. Although quantitative differences could be observed in different experimental conditions, no clear evidence of specificity emerged from these studies, and therefore data are not reported this chapter. An exception to this is represented by antimicrobial peptides (AMPs), small cationic peptides with a remarkable structural diversity, engaged in the destruction of bacteria inside phagocytes, before being released into hemolymph to participate in systemic responses [1]. In *Mytilus*, different AMPs (including defensins, mytilins, myticins) share antibacterial and antifungal properties; on the other hand, Mytimycin (MytM), was identified as the first strictly antifungal protein from mollusks [52].

6. Specificity of the immune response to pathogenic vibrios : role of surface interactions and serum soluble components

The specificity of bivalve immune response has been investigated mainly in bivalve species susceptible to infection by certain *Vibrio* spp. and strains. However, a limited number of studies focused on the mechanisms underlying differential responses of bivalves to different pathogenic vibrios, in particular with those strains associated with oyster summer mortalities as described above. The first work describing specific mechanisms involved in the interactions between bivalve hemocytes and bacteria was that of Duperthuy et al. [26], with oysters and the oyster pathogen *V. splendidus* LGP32. In *C. gigas*, LGP32 uses the OmpU protein to attach and invade the hemocytes through Cg-EcSOD (extracellular superoxide dismutase), the major plasma protein, that acts as an opsonin mediating recognition and promoting phagocytosis. In this process, Cg-EcSOD is recognized through its RGD (Arg-Gly-Asp) sequence by hemocyte β-integrins, leading to

subversion of the cell actin cytoskeleton, inducing the expression of trafficking genes, and resulting in actin and clathrin polymerization. Capable of intracellular survival, LGP32 was shown to escape from host cellular defenses by avoiding acidic vacuole formation and by limiting ROS production.

A different situation was observed in *M. galloprovincialis*, that is resistant to LGP32 infection [53]. LGP32 was rapidly phagocytized by the hemocytes, where it induced lysosomal damage; when internalized, it remained viable and culturable within intracellular vacuoles apparently escaping lysosomal degradation through disregulation of phosphatidylinositol 3 Kinase (PI-3K) signaling, leading to impairment of the endo-lysosomal system. However, interactions with hemolymph soluble factors were not crucial in determining the effects of this strain on mussel hemocytes. Actually, in mussels, the major plasma protein is the 'extrapallial fluid major protein' (EP protein), that shares no sequence homology with CgEcSOD [54]. The effects of LGP32 in mussels were confirmed *in vivo*, following injection with bacteria, where no bactericidal activity towards *V.s.* was observed at different times post injection; this strain was actually able to grow within mussel hemolymph, leading to stressful conditions in the hemocytes. However, this effect was transient, and hemocytes showed the capacity to recover at longer times post-injection. Overall, these data indicated that the mechanisms involved in promoting LGP32 adhesion and invasion in oyster and mussel hemocytes may be profoundly different, resulting in different effects. Moreover, these data underlined the role of species-specific interactions of soluble hemolymph proteins with different vibrio strains

Previous studies showed that in mussels soluble hemolymph components can play a key role in mediating the interactions between bacteria and hemocytes. In *M. galloprovincialis*, serum soluble factors specifically bind mannose-sensitive bacterial ligands (i.e., type 1 fimbriae of *E. coli* and MSHA pilus of *V. cholerae*), thus promoting efficient adhesion to and killing by hemocytes[43-46]. Preliminary data indicated that a thermolabile protein fraction, with a MW>10kD may be involved in this process [55]. The possible interactions between mussel hemocytes, soluble opsonins and MSHA carrying bacteria are depicted in Fig. 1.

As mentioned above, MSHA adhesins are also present in a large proportion of environmental isolates of *V. aestuarianus* [29]. *V. aestuarianus* pathogenicity to *C. gigas* has been demonstrated by experimental challenge [32]; on the other hand, *V. aestuarianus* isolates were only moderately pathogenic to *M. galloprovincialis* [18,56]. The different sensitivity to infection of the two bivalve species may partly depend on their different capability to kill invading pathogens through the action of soluble hemolymph components [3,44].

The role of mannose-sensitive interactions in *V. aestuarianus* 01/032 sensitivity to killing by *M. galloprovincialis* and *C. gigas* hemolymph was recently investigated [57]. Although 01/032 bacteria adhered to hemocytes of both bivalves, they were sensitive to the bactericidal activity of whole hemolymph from mussel but not from oyster; in addition, adhesion to mussel (but not oyster) hemocytes was affected by D-mannose. The mussel hemolymph protein responsible for promoting mannose-sensitive interactions of *V. aestuarianus* 01/032 with the hemocytes, thus serving as an opsonin, was identified as the extrapallial protein precursor (EP) of *M. edulis* [57]. EP, the major plasma protein in *Mytilus,* is an acidic glycoprotein with a high histidine content that can bind Ca^{2+} and heavy metals [54]. Recently, by a MS-based approach, a complex and anomalous N-glycan structure was determined in *M. edulis* EP. Such unique structure and calcium and heavy metal binding properties suggest a possible role for this protein in multiple biological functions, including shell formation, metal ion transportation, and detoxification [58]. Interestingly, EP also shows a conserved domain homologous to MgC1q6, a complement component identified in *M. galloprovincialis* [41]. These data ascribe to mussel EP the additional role of mediating specific immune interactions against bacteria carrying D-mannose sensitive ligands.

7. Conclusions

Increasing knowledge on bivalve immunity is revealing a complex innate immune system able to recognize and eliminate a wide range of invading microorganisms in a fluctuating environment. Studies with different bacterial species and strains will help understanding the mechanisms

underlying the specificity of bivalve immune response, thus contributing to develop innovative solutions and tools for the prevention, control and mitigation of bivalve disease in farmed species.

8. References

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Legend to Figure 1 – Schematic representation of the interactions between *Mytilus* hemocytes, soluble hemolymph components and bacteria carrying the D-Mannose sensitive MSHA adhesin**.**