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## The new insights into the oyster antimicrobial defense: Cellular, molecular and genetic view



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### ABSTRACT

Oysters are sessile filter feeders that live in close association with abundant and diverse communities of microorganisms that form the oyster microbiota. In such an association, cellular and molecular mechanisms have evolved to maintain oyster homeostasis upon stressful conditions including infection and changing environments. We give here cellular and molecular insights into the *Crassostrea gigas* antimicrobial defense system with focus on antimicrobial peptides and proteins (AMPs). This review highlights the central role of the hemocytes in the modulation and control of oyster antimicrobial response. As vehicles for AMPs and other antimicrobial effectors, including reactive oxygen species (ROS), and together with epithelia, hemocytes provide the oyster with local defense reactions instead of systemic humoral ones. These reactions are largely based on phagocytosis but also, as recently described, on the extracellular release of antimicrobial histones (ETosis) which is triggered by ROS. Thus, ROS can signal danger and activate cellular responses in the oyster. From the current literature, AMP production/release could serve similar functions. We provide also new lights on the oyster genetic background that underlies a great diversity of AMP sequences but also an extraordinary individual polymorphism of AMP gene expression. We discuss here how this polymorphism could generate new immune functions, new pathogen resistances or support individual adaptation to environmental stresses.

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## 1. Introduction

Oysters are bivalve mollusks belonging to the Ostreidae family (Mollusca, Bivalvia, Lophotrochozoa). They are sessile filter-feeders living in shallow water from intertidal zones of bays, lagoons and estuaries. In these habitats, oysters are confronted and adapted to great changes in biotic and abiotic environmental conditions. Abiotic factors include temperatures and salinity fluctuations but also exposure to xenobiotics and water acidification due to human activities. Biotic factors include abundant and diverse populations of microbes. As filter feeders, oysters are in permanent contact and exchanges with microorganisms. Thus, they harbor on their

surfaces and inside their body cavities and hemolymph a dense microbiota which has been shown to be greatly dominated by *Vibrio* species [1,2]. Indeed, a large attention has been paid over the past years, to populations of vibrios as they are among the most abundant cultivable bacteria isolated from oyster tissues [3]. In healthy oyster, bacteria load, including vibrio, has been shown to vary over time according to individuals and to temperature, with hemolymph average concentrations of 5,7 colonies forming unit (CFU) per  $\mu\text{L}$  [2,4]. Hence, oyster must be seen as an organism associated to a microbiota (including mutualists, opportunists and pathogens), that has adapted effective cellular and molecular mechanisms for maintaining homeostasis and health status in stressful and changing environments. The multifactorial diseases affecting *Crassostrea gigas* oysters worldwide [5] are the outcome of an equilibrium collapse in the interplay between the biotic and abiotic environmental factors such as microorganisms and

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temperature [6], on the one hand, and the oyster physiology and immune responses on the other hand [7].

By focusing on *C. gigas*, we propose here to give cellular and molecular insights into the oyster antimicrobial defense system considering the genetic background of individuals.

## 2. Effectors of the antimicrobial defense

Oyster immunity involves not only hemolymph-mediated reactions, but also immune effectors produced by epithelial cells from various organs, including gills, mantle, digestive gland and intestine, which participate in the antimicrobial defense mechanisms.

### 2.1. Plasma proteins

As oysters have a semi-open circulatory system, hemolymph is typically an important interface between the immune system and the microorganisms that enter the oyster body. The oyster hemolymph is devoid of clotting reaction by means of plasma gelation but the hemocytes display remarkable spontaneous reaction of aggregation resulting in cellular clot [8]. Aggregation is reversible, the hemocytes can further disperse and re-enter the circulating system. *In vitro* hemocyte aggregation has been shown to be inhibited by recombinant tetraspanin [9] which participates to cell-adhesion molecular complexes of mammalian white cells. To date, no respiratory protein has been characterized in Ostreoidae bivalves but strikingly, the oyster plasma is characterized by the over representation of proteins that present homologies with extracellular metalloenzyme Superoxide Dismutases (EcSODs). Named dominin [10], cavortin [11] or EcSOD [12,13], these proteins could belong to a complex family of multifunctional molecules [5]. For instance, one member of this family, Cg-EcSOD, has LPS-binding properties and acts as an opsonin for pathogenic vibrios (see below) [13,14]. To date, there is no compelling evidence that all SOD-related proteins retained SOD activities [10,11]. Thus, these circulating proteins may play major roles, even now underexplored, in the interplay with the oyster hemolymph microbiota but also in the antimicrobial defense reactions, by mediating microbe recognition and promoting phagocytosis.

### 2.2. Oyster hemocytes

The hemocytes (blood cells) are immunocompetent cells but they are also involved in many physiological processes such as wound and shell repair, nutrient transport and digestion, gonad resorption. As the oyster circulatory system is semi-open, hemocytes are not confined to the vessels and they invade or reside in many other tissues [15]. Thus, infiltrating hemocytes are present in all cavities, tissues and epithelia of oyster body where they can also fulfil defense functions. The term hemocytes refers to a diversity of circulating cells that is best highlighted by the lack of unified classification, although they have been extensively studied since early 1970's. Indeed, the lack of molecular and functional genetic tools has precluded any in-depth characterization of cell lineage ontogeny and discrimination of functionally distinct cell types.

#### 2.2.1. Hemocyte lineage

Although a clear definition of distinct cell types is still missing, a consensus about three main cell populations, i.e. blast-like cells, hyalinocytes and granulocytes, appears in the literature as they can be distinguished either by microscopy or flow cytometry analyses [15,16]. Among hyalinocytes, also named agranular cells, a subset of professional phagocytes, which are sometimes described as macrophage-like cells, harbor a potent phagocytosis activity; they account for 30–40% of the total populations of hemocytes [14].

Attempts to define cell lineages and functional subsets have been reported using classical May-Grünwald Giemsa (MGG) staining, peroxidase or phosphatase staining, electron microscopy, or flow cytometry analyses [16–19]. From works in the different oyster species, some authors reported basophile and eosinophile granulocytes similar to their mammalian counterparts [16]. Other authors have drawn hypothesis of a cell maturation process in one single lineage from blast-like cells, hyalinocytes to granulocytes [20]. Hence the different hypotheses range from one unique lineage to numerous functionally distinct cell types from different lineages [16,20]. One caveat for most of these different reports is that most of the tools that have been used so far for hemocyte characterization were developed to analyze mammals blood cells (like the MGG staining for example) and thus have to be interpreted with caution in other species. Another caveat is that depending on the maturation stage or the functional activities of one cell type, the cell morphologies and physico-chemical characteristics may change, as for example the internal complexity of professional phagocytes that increases upon phagocytosis when analyzed by flow cytometry. Altogether, the actual number of cell lineages and functionally specialized subsets of hemocytes remain to be carefully examined and accurately determined.





#### 2.2.2. Hematopoiesis

As for the distinction of the different cell types, the ontogeny of oyster hemocytes remains to be fully characterized. Different hypotheses about their hematopoietic origin have been elaborated over the past decades. Cheng (1981) proposed that hemocytes could originate from the differentiation of connective tissue cells [17]. Tirapé and colleagues (2007) described that the expression of *Cg-tal* (Tal1/SCL) [21], a family of transcription factors involved during embryonic hematopoiesis in vertebrates [22] was only detected in cells emerging from blood vessel endothelium, which is reminiscent of the hematopoietic cell emergence from the hemogenic endothelium in vertebrate embryos [23,24]. More recently, a study from Jemaà and colleagues (2014) using BrdU to localize mitotic cells within the oyster tissues suggested that some hemocyte progenitors emerge from particular structures at the basement of the gill epithelium [25], which is reminiscent of assumptions made earlier by Cuénot in 1891 [26]. Altogether, this sum of potentially contradictory pieces of work highlights the lack of knowledge about hematopoiesis in oysters and more largely in bivalves. Although the recent progress in molecular biology has dramatically advanced our knowledge on the immune-function of hemocytes, little is known about oyster hemocyte life cycle and cell lineage origin. This discrepancy is probably due to the lack of dedicated molecular tools for cell lineage analysis and/or the lack of long-term cell culture systems for studying cell differentiation and maturation. Such tools gave access to a comprehensive knowledge of hematopoiesis in other animals from drosophila to human. The recent release of the full *C. gigas* genome should help to develop the required tools.

### 2.3. Antimicrobial peptides/proteins (AMPs)

Several gene-encoded antimicrobial peptides and proteins (AMPs) sharing common molecular features with AMP families described in other kingdoms of life have been characterized in oysters (Table 1). These host defense effectors are usually small cationic (less than 10 kDa), amphipathic peptides showing a broad diversity of amino acid sequence and structural conformations [27,28]. However, some large cationic proteins with antimicrobial properties have also been described (Table 1). Based on common structural features or conserved sequence motifs characteristics for AMP families, oyster antimicrobials have been classified as

**Table 1**  
Antimicrobial peptides and proteins from the oysters *C. gigas* (Cg) and *C. virginica* (Cv).

AMP family	Name	Localization	Expression (microbial challenge)	3D structure	Antimicrobial activity	References
<b>Cationic antimicrobial peptides</b>						
Defensins	Cg-Defm	Mantle	Constitutive		Gram (+)/nM range (MIC)	Gueguen et al., 2006 Gonzalez et al., 2007 Duperthuy et al., 2010
	Cg-Defh1/-2	Hemocytes	Constitutive		Gram (-)/μM range (MIC)	
	AOD (Cv)	Gills	ND	ND	Gram (+)/μM range (MEC) Gram (-)/μM range (MEC)	Seo et al., 2005
Proline-rich peptides	Cg-IgPrp Cg-stPrp	Hemocytes	Repressed	ND	Synergistic activities on Gram (+) and Gram (-) <sup>b</sup>	Schmitt et al., 2012
Big defensins	Cg-BigDef1,	Hemocytes	Induced		ND	Rosa et al., 2012
	-2 Cg-BigDef3	Hemocytes	Constitutive		ND	
Molluscidin	Cg-Molluscidin	Mantle Gills	Constitutive	ND	Gram (+)/μM range (MEC) Gram (-)/μM range (MEC)	Seo et al., 2013
<b>Cationic antimicrobial proteins</b>						
Bactericidal/permeability increasing proteins	Cg-BPI/-2	Hemocytes Epithelia	Induced Constitutive		Gram (-)/μM range (MIC)	Gonzalez et al., 2007
Macrophage expressed gene 1 like protein	Cg-Mpeg1	All tissues	Constitutive	ND	Gram (+)/μM range <sup>e</sup> Gram (-)/μM range <sup>e</sup>	He et al., 2011
<b>Multifunctional proteins displaying antimicrobial activities</b>						
Ubiquitin	Cg-Ubiquitin	Gills (infiltrating hemocytes?)	Induced?		Gram (+)/μM range (MEC) Gram (-)/μM range (MEC)	Seo et al., 2013
Histones	Cv-H2B	Gills	ND	ND	Gram (+)/μM range (MEC) Gram (-)/μM range (MEC)	Seo et al., 2011
	Cg-H1/H5	Gills (infiltrating hemocytes)	ND	ND	Gram (+)/μM range (MIC) Gram (-)/μM range (MIC)	Poirier et al., 2014

Antimicrobial activities are expressed as Minimal Inhibitory Concentrations (MIC) or Minimal Effective Concentrations (MEC).

ND = not determined.

<sup>a</sup> Three-dimensional structure of Cg-Defm [PDB: 2B68].

<sup>b</sup> Synergistic activity with Cg-Defm and Cg-BPI.

<sup>c</sup> Three-dimensional structure of horseshoe crab (*Tachypleus tridentatus*) big defensin [PDB: 2RNG] (Kouno et al., 2008).

<sup>d</sup> Predicted structure of bactericidal/permeability-increasing protein (BPI) [PDB: 1BPI] (Beamer et al., 1998).

<sup>e</sup> Partial growth inhibition at ~1 μM.

<sup>f</sup> Predicted structure of Cg-Ubiquitin according to mouse Tab2-Nzf [PDB ID: 3A9] (Vijay-Kumar et al., 1985).

defensins, big defensins, proline-rich AMPs and bactericidal/permeability-increasing (BPI) proteins. More recently, two molecules with antimicrobial activities, the Macrophage expressed gene1-like protein and the Molluscidin peptide have also been characterized in oysters. An overview of the characteristics of the main oyster antimicrobials is proposed in Table 1 and their amino acid sequences are presented in Table 2.

### 2.3.1. Defensins (Cg-Defm)

Defensins are by far the best described AMPs from oysters. Since their first isolation from mussels [29,30], defensins have been identified in diverse species of mollusks. The first defensin characterized in oysters has been purified from acidified gill extracts of the American oyster *Crassostrea virginica* [31]. Then, in *C. gigas*, three representative members of defensins have been identified by genomic approach as expressed in the mantle epithelia, Cg-Defm [32], and in the hemocytes, namely Cg-Defh1 and Cg-Defh2 [33]. A broad diversity of defensin sequences was found in *C. gigas* (see “AMP genomic diversity and evolution” section below).

- **Gene organization.** Each Cg-Def is encoded by a separate gene with different genomic organization. The mantle defensin genes (Cg-defm) display two structures, (i) two exons separated by a unique intron [32], a genomic organization similar to that of the mussel and scorpion defensin genes [34], and (ii) three exons separated by two introns. Hemocyte defensin genes (Cg-defhs) only display the latter structure, in which the second intron

separates the two last residues of the mature peptide apart from the rest of the sequence [35].

- **Precursors.** All oyster defensins are expressed as precursors consisting in a hydrophobic signal peptide (prepeptide) immediately followed by the 4.6–4.7 kDa cationic mature peptide (pI 8.5–8.7). The absence of specific sorting/retention signal suggests defensins are secreted outside the cells.
- **Structure.** Mollusk defensins are small (4–5 kDa) cationic molecules containing six to eight cysteine residues engaged in three to four intramolecular disulfide bridges. They are all predicted to contain an  $\alpha$ -helix linked to an antiparallel two-stranded  $\beta$ -sheet by disulfide bridges, making the so-called cysteine-stabilized  $\alpha$ -helix/ $\beta$ -sheet motif (CS $\alpha\beta$ ) [36]. The American oyster defensin (AEO) isolated from *C. virginica* [31] contains 6 cysteines whereas in *C. gigas*, only defensins with eight cysteines were found [32,33,35]. The tridimensional structure of a mantle defensin from *C. gigas* (Cg-Defm) was solved, showing that the CS $\alpha\beta$  motif is indeed stabilized by four disulfide bridges (cysteine pattern: C1–5C2–6C3–7C4–8) [32]. The fourth disulfide bridge is a unique feature from mollusk defensins. It has been proposed to be implicated in the stabilization of the mature peptide to the high osmolarity environment found in the sea water [37].
- **Antimicrobial activities and mechanism of action.** The *C. virginica* defensin was shown to be active against both Gram-positive and Gram-negative bacteria. Thus, in a so-called ultra-sensitive radial diffusion assay (URDA), the Minimal Effective Concentration (MECs) of AEO were below 3.5 μM [31]. Until

**Table 2**  
Amino acid sequences of most representative variants of *C. gigas* antimicrobial peptides and proteins. Sequences correspond to the mature peptides. The number of amino acids (aa), molecular weight (kDa) and estimated isoelectric point (pI) of the molecules are indicated on the right of each sequence.

AMPs	Amino acid sequences	No aa	Mass (kDa)	pI
<b>Defensins</b>				
Cg-Defh1	FGFCPRDQYKCNHSCQSGCRAGYCDAAVTLWLRCTCTDCNGKK	43	4.76	8.50
Cg-Defh2	FGFCPRDQYKCNHSCQSGCRAGYCDAAVTLWLRCTCTDCNGKK	43	4.67	8.51
Cg-Defm	FGFCPRDQYKCNHSCQSGCRAGYCDAAVTLWLRCTCTDCNGKK	43	4.64	8.73
<b>Big defensins</b>				
Cg-BigDef1	QAQALLPIASVAGLTVSAPVFAALVTVYGAYALRYNRRRENSYQRIRSDHSHSCANNRGRWCRPTCFSEHYTDWFNNDVCGSYRCCRPGRSG	94	10.70	9.06
Cg-BigDef2	QAQALLPIASVAGLTVSAPVFAALVTVYGAYALRYNRRRENSYQRIRSDHSHSCANNRGRWCRPTCFSEHYTDWFNNDVCGSYRCCRPGRSG	87	9.85	8.61
Cg-BigDef3	QAQALLPIASVAGLTVSAPVFAALVTVYGAYALRYNRRRENSYQRIRSDHSHSCANNRGRWCRPTCFSEHYTDWFNNDVCGSYRCCRPGRSG	87	9.77	8.84
<b>Proline-rich peptides</b>				
Cg-IgPp	GPIRRPKPRPRPE	15	1.80	12.18
Cg-sIPp	GPIRRPKPRPRPE	13	1.55	12.00
<b>Bactericidal/permeability increasing proteins</b>				
Cg-BPI	KTPGLQTRITDRALAYEVALDLSLQVTKQQIPDQHQSGDVKFDITGMNVKQFTKPSRSVLIQNVGLSWSTSGTGLAIHGDFKYYKRYK GIKISDHGFDLKAINGINFOJIKIEIGMDGTGRPTMKAVGCSNVSADIKFHGGAWVYNLFSGQLENKIKDMVGGGGLLCKQLNLTLD VNGMKSLOKLPVTVQAKRFELLYRFLSKPFSQTKFMETVYHKGVEVYVNAVVDAPFAAPPLIKSSDTSRMIMYIWLSDYVVTMSYNALK YNQLQYVNTKDLPSGVLNITTCPSQTCIGIKIIGTKFPNTVMILYMKSTSPNMTAQNSTVNVASGDIVFAAQPGGKYVFLTL SATMSTTISLMIENEKVFVKVLPISVTVKDSKIPVSPPEGLNFVKGIVSVPEPKLNELGAAGFPPLVNSVHFTNTQLTVAKDTLLIATDLKYSG KNPGJKRSITSNGLNVAVALDLSLQVTKQQIPDQHQSGDVKFDITGMNVKQFTKPSRSVLIQNVGLSWSTSGTGLAIHGDFKYYKRYK KKGIRSDHGRFDLTCGGTITAINLIGMDSMGRPTISSTSCSVRSTGIKHFHGGRAWYLNPRGKVAKKLSIEGEGLLC KQLTKLVNVDGARAKLAQLPVSKVFAKRFELLYRFLSKPFSQTKFMETVYHKGVEVYVNAVVDAPFAAPPLIKSSDTSRMIMYIWLSDYVVTMS YTFASHGFLTYVNTDKNPGGILNITCKNMGCKLJKIKGQMFNPSQVMLNRSSTMPNMTAFNATTKVDADGEIKFYATKGGKGVDFYL TLSANMSTTISITVKNKVFARVVKLPINVHVKDSKIGHLNDFMLNFKVKKIITTFVPEPKLNELGCGNGRPLPIGSGVHFANTELLIHQNS LLIATDVKYLQ	458	50.14	9.37
Cg-BPI2	KTPGLQTRITDRALAYEVALDLSLQVTKQQIPDQHQSGDVKFDITGMNVKQFTKPSRSVLIQNVGLSWSTSGTGLAIHGDFKYYKRYK GIKISDHGFDLKAINGINFOJIKIEIGMDGTGRPTMKAVGCSNVSADIKFHGGAWVYNLFSGQLENKIKDMVGGGGLLCKQLNLTLD VNGMKSLOKLPVTVQAKRFELLYRFLSKPFSQTKFMETVYHKGVEVYVNAVVDAPFAAPPLIKSSDTSRMIMYIWLSDYVVTMSYNALK YNQLQYVNTKDLPSGVLNITTCPSQTCIGIKIIGTKFPNTVMILYMKSTSPNMTAQNSTVNVASGDIVFAAQPGGKYVFLTL SATMSTTISLMIENEKVFVKVLPISVTVKDSKIPVSPPEGLNFVKGIVSVPEPKLNELGAAGFPPLVNSVHFTNTQLTVAKDTLLIATDLKYSG KNPGJKRSITSNGLNVAVALDLSLQVTKQQIPDQHQSGDVKFDITGMNVKQFTKPSRSVLIQNVGLSWSTSGTGLAIHGDFKYYKRYK KKGIRSDHGRFDLTCGGTITAINLIGMDSMGRPTISSTSCSVRSTGIKHFHGGRAWYLNPRGKVAKKLSIEGEGLLC KQLTKLVNVDGARAKLAQLPVSKVFAKRFELLYRFLSKPFSQTKFMETVYHKGVEVYVNAVVDAPFAAPPLIKSSDTSRMIMYIWLSDYVVTMS YTFASHGFLTYVNTDKNPGGILNITCKNMGCKLJKIKGQMFNPSQVMLNRSSTMPNMTAFNATTKVDADGEIKFYATKGGKGVDFYL TLSANMSTTISITVKNKVFARVVKLPINVHVKDSKIGHLNDFMLNFKVKKIITTFVPEPKLNELGCGNGRPLPIGSGVHFANTELLIHQNS LLIATDVKYLQ	457	50.3	9.74

now, native defensins could not be purified from *C. gigas* tissues in sufficient amounts to enable complete biochemical characterization and determine their spectrum of activity. Antibacterial and antifungal activities of Cg-Defensins were studied with recombinant peptides [32,38]. All oyster defensins were shown to be mainly active against Gram-positive bacteria against which they displayed low to very low minimal inhibitory concentrations (MICs) in the range of 0.01–6  $\mu$ M. Conversely, they did not display significant antimicrobial activity against Gram-negative bacteria including oyster pathogens (MICs  $\geq 10 \mu$ M). This is likely due to their capacity to strongly inhibit peptidoglycan biosynthesis [38], which is readily accessible at the cell wall of Gram-positive bacteria and hidden by the outer membrane of Gram-negative bacteria. Studies on *Staphylococcus aureus* showed that antibacterial activity is observed in absence of membrane disruption and results from the high affinity binding of Cg-Defensins to the cell wall precursor lipid II [38]. Interestingly, at high concentrations (10  $\mu$ M and above), Cg-Defensins can also present membrane-disruptive properties as observed against the Gram-negative *Vibrio tasmaniensis* LGP32 [39].

### 2.3.2. Big defensins (Cg-BigDefensins)

Big defensins (BigDefensins) are antimicrobial polypeptides (8–11 kDa) only found in marine invertebrates (Arthropoda, Mollusca and Cephalochordata) [40]. They are cationic molecules with pI ranging from 8.6 to 9.2. The first BigDef was purified from the hemolymph cells (amebocytes) of the horseshoe crab *Tachyporus tridentatus* (Chelicerata) [41]. In *C. gigas* oysters, BigDef homologues were identified more recently through a genome-wide transcriptomic study [40]. Oyster big defensins (Cg-BigDefensins) form a diverse family of AMPs composed of three representative members, namely Cg-BigDef1, Cg-BigDef2 and Cg-BigDef3. In *C. gigas*, Cg-BigDef expression is restricted to hemocytes, both circulating and infiltrating oyster tissues.

- **Gene organization.** Each of the three Cg-BigDefensins is encoded by a separate gene. The genomic organization of *Cg-bigdef1* and *Cg-bigdef2* genes is similar, with two exons interrupted by a single intron. In contrast, in *Cg-bigdef3*, additional intron and exon are observed upstream the first exon common to the other *Cg-bigdef* genes. In all *Cg-bigdef* genes, the  $\beta$ -defensin domain is exclusively encoded by the last exon [40].
- **Precursors.** Oyster big defensins are expressed as prepropeptides, which start with a predicted 23-residue signal peptide (prepeptide), followed by a propeptide region of 13 residues and a cationic 94- or 87-residue mature polypeptide of 10.7 kDa (Cg-BigDef1), 9.8 kDa (Cg-BigDef2) or 9.7 kDa (Cg-BigDef3). Similarly, in the horseshoe crab, BigDef is produced as a precursor molecule that is further processed into a 8.6 kDa mature polypeptide [41].
- **Structure.** The solution structure of the horseshoe crab BigDef is the only one available to date. It showed that big defensins are composed of an N-terminal globular and hydrophobic domain connected to a C-terminal domain containing  $\beta$ -sheet structures and folded by three disulfide bridges. Interestingly, the arrangement of the disulfide bridges is identical to that of vertebrate  $\beta$ -defensins ( $\beta$ -defensin domain: C<sub>1-5</sub>C<sub>2-4</sub>C<sub>3-6</sub>) [42]. Based on mass spectrometry data, it has been proposed that oyster big defensins undergo post-translational modifications like the conversion of the N-terminal glutamine residue of the native Cg-BigDef1 into a pyroglutamic acid [40].
- **Antimicrobial activities.** The antimicrobial activities of oyster BigDefensins have not been characterized to date. However, in



another bivalve mollusk, *Argopecten irradians*, BigDefs were reported to be active against both Gram-positive and Gram-negative bacteria and fungi [43] as well as in the horseshoe crab *T. tridentatus*, where, the native BigDef was also shown to display a significant LPS-binding activity [41].

### 2.3.3. Proline-rich peptides (Cg-Prps)

Proline-rich AMPs (PrAMPs) have been identified in vertebrates including mammals and amphibians as well as invertebrates such as insects and crustaceans. They form a group of diverse peptides that display a high content in proline and arginine residues (typically from 25 to 50%). They display short Pro–Arg motifs, which have been proposed to be implicated in their antimicrobial activity. PrAMPs are also characterized by their mode of action, which usually does not involve the lysis of bacterial membranes but rather the penetration into Gram-negative bacteria by translocating into the cytoplasm via a permease/transporter-mediated uptake. Thereby, many PrAMPs act by interfering with essential cellular functions intracellularly [44]. In *C. gigas* oysters, a cDNA sequence showing homologies to PrAMPs was identified as expressed in hemocytes [45]. A high number of Cg-Prp (*C. gigas* proline-rich peptide) forms were then identified as expressed by oyster hemocytes. They display two lengths (16 or 18 amino acids), with shorter peptides differing from the original Cg-Prp by the deletion of a conserved Pro–Arg motif [35]. Consequently, the original form was renamed as long Cg-Prp (Cg-IgPrp; 1.8 kDa) and the new form as short Cg-Prp (Cg-stPrp; 1.5 kDa) [46]. Cg-Prps are highly cationic peptides with a theoretical *pI* of 12–12.1.

- **Gene organization.** Cg-Prps are a multigenic family with genes containing or not an intron. The presence/absence of an intron is observed in the genes encoding both the long and short peptide forms.
- **Precursors.** PrAMPs from *C. gigas* (Cg-Prps) derive from 59 to 62 amino acid precursors composed of an N-terminal signal peptide followed by an acidic region (putative propeptide) and a C-terminal cationic proline-rich region, containing two repetitions of a Pro–Arg motif [45]. This organization is conserved in all precursors of Cg-Prps [35].
- **Antimicrobial activities.** Native Cg-Prps could not be purified from oyster hemocyte extracts until now. Therefore, synthetic Cg-Prps were used to explore their spectrum of antimicrobial activity. Both long and short Cg-Prps were poorly active against Gram-positive bacteria and were not active against Gram-negative bacteria. However, Cg-IgPrp displayed strong synergy with Cg-Defs and Cg-BPI against both Gram-positive and Gram-negative bacteria [45,47]. The underlying mechanisms remain unexplored.

### 2.3.4. Cg-Molluscidin

Besides the well-known AMP families mentioned above, a novel antimicrobial peptide has been purified for the first time from the gills of *C. gigas*. It does not show sequence homology with already known AMPs. This 5.5 kDa peptide, which shows a calculated *pI* of 11.28, was named Cg-Molluscidin [48]. Cg-Molluscidin transcripts were found predominant in oyster mantle tissue, then in gills and other organs, but it is likely that Molluscidin could be expressed by infiltrating hemocytes. No data are yet available on gene organization of this new AMP.

- **Precursor.** Native Cg-Molluscidin (55 amino acids) was shown to derive from a precursor molecule by the only elimination of its N-terminal methionine. This precursor is devoid of signal

peptide or anionic proregion. From the mass spectrometry data, the native Cg-Molluscidin would not undergo any further posttranslational modification [48].

- **Structure.** The primary structure of Cg-Molluscidin is characterized by a high percentage of two residues, Ala (15) and Lys (23). The sequence is organized in 10 dibasic residue repeats including Lysine–Lysine or Lysine–Arginine and separated by hydrophobic amino acids, Ala (15 amino acids), Val or Gly [48].
- **Antimicrobial activities.** Native Cg-Molluscidin was shown to be active against both Gram-positive bacteria in a so-called ultrasensitive radial diffusion assay (URDA). Under these conditions, the Minimal Effective Concentration (MECs) of Cg-Molluscidin was in the range of 1.3–31.3 µg/mL against Gram-positive bacteria and 0.4–2.3 µg/mL against Gram-negative bacteria including *Vibrio parahaemolyticus*. No antifungal or hemolytic activities were recorded for Cg-Molluscidin [48].

### 2.3.5. Bactericidal/permeability increasing protein (Cg-BPI)

Bactericidal/permeability-increasing proteins (BPIs) and lipopolysaccharide-binding proteins (LBPs) are components of the immune system that have been mainly characterized in mammals. Although highly similar (45% sequence identity), only BPIs display antimicrobial activity. By genomic approaches, LBP/BPI-related genes have been found in a number of non-mammalian vertebrates, invertebrates like nematodes and mollusks, and protists [49]. A homologue of the human BPI (hBPI) protein with a calculated molecular mass of 50.1 kDa, was identified in *C. gigas* oysters by a screening of a hemocyte EST library [50]. Cg-BPI expression is constitutive in oyster epithelia and induced by microbial challenge in oyster hemocytes [50]. More recently, a homolog of Cg-BPI named Cg-BPI2 was also identified in a *C. gigas* hemocyte EST library [51]. It shares 56.4% amino acid identity with Cg-BPI. It was proposed to originate from Cg-BPI by gene duplication but it would follow a distinct expression pattern [51]. To date, Cg-BPI gene organization has not yet been determined.

- **Precursors.** Cg-BPI is expressed as a precursor protein composed a 19-residue signal peptide followed by the mature protein [50]. Cg-BPI2 displays the same organization of Cg-BPI precursor, with a 22-residue signal peptide, followed by the mature protein [51].
- **Structure.** As deduced by molecular modeling, Cg-BPIs display the typical structural features of hBPI with an N- and C-terminal β-barrel type domains connected by a proline-rich central domain [50,51]. The N-terminal domain of Cg-BPI contains the LPS-binding regions characterized in hBPI and the Lys and Arg residues required for LPS-binding. This domain also contains the cysteine bridge of hBPI at conserved position, three extra cysteine residues being identified (both in the N- and C-terminal domains) whose folding remains unknown [50].
- **Antimicrobial activities and mechanism of action.** The full-length recombinant Cg-BPI was shown to be a monomeric protein (50.1 kDa) highly active against the short-chain LPS *Escherichia coli* strain SBS363, against which it displayed bactericidal activity. It was 30 times less active against the long-chain LPS *E. coli* ML35. Consistent with its spectrum of activity directed against Gram-negative bacteria, recombinant Cg-BPI displayed both LPS- and Lipid A-binding activities. Like hBPI, it permeabilized the cytoplasmic membrane of *E. coli* ML35 [50]. The activity of the recombinant Cg-BPI2 N-terminal β-barrel domain was also shown to be exclusively active against Gram-negative bacteria [51]. Thus, like in human BPI, the N-terminal domain of *C. gigas* BPIs is sufficient for its antibacterial activity.

### 2.3.6. Macrophage expressed gene 1-like (*Cg-Mpeg1*)

Lastly, a macrophage expressed gene 1-like protein has been identified in *C. gigas* by a genomic approach (GenBank: EF627979; HQ228218) and proposed to have antimicrobial properties [52]. The *Cg-Mpeg1* (81.8 kDa) is a member of the membrane attack complex and perforin (MACPF) protein superfamily, a large group of pore-forming proteins widely conserved in the animal kingdom from mammals to low vertebrates. *Cg-Mpeg1* shows 47–48% identity with three abalone (gastropod) *Mpeg1* sequences [53] and, 39% and 35% identity with sequences from mammals and sponges, respectively [52]. *Cg-Mpeg1* would be constitutively expressed in various tissues of healthy oysters and *Cg-Mpeg1* transcripts were predominantly detected in gonads and gills [52]. Gene organization remains to be determined.

- **Precursor.** *Cg-Mpeg1* is encoded as a putative 752 amino acid precursor carrying an N-terminal signal peptide of 19 amino acids.
- **Structure.** The *Cg-Mpeg1* mature protein is predicted to contain a membrane-attack complex/perforin (MAPCPF) domain and a C-terminal transmembrane segment [52].
- **Antimicrobial activities.** A sequence of *Cg-Mpeg1* including the MACPF domain has been expressed in *E. coli* system. The recombinant MACPF domain could inhibit the growth of both Gram-positive and Gram-negative bacteria including *Vibrio alginolyticus* [52]. It is unknown whether *Cg-Mpeg1* creates membrane damages in bacteria.

## 2.4. Other antimicrobial molecules

### 2.4.1. Lysozymes

Lysozymes are cationic hydrolytic enzymes widely distributed in the animal kingdom and found in a variety of cells, tissues and secretions from bacteria to humans. After being considered for a long time as hydrolytic enzymes involved in the degradation of bacteria cell wall, lysozymes are now considered as antimicrobial proteins. Bivalve lysozymes are members of a large family of proteins, referred to as invertebrate-type lysozymes [54]. These enzymes are believed to be involved in host defense due to their lytic properties on the peptidoglycan, the major component of the bacterial cell wall. However, this property could also be used by some lysozymes for digestive functions in oysters [55,56]. In *C. gigas*, lysozyme activities were reported in gills, mantle and digestive diverticula [57], then different lysozymes sequences have been identified in *C. gigas* by genomic approaches. Three lysozyme cDNAs have been characterized in *C. gigas*. CGL-1, -2 and -3 are encoded by 530–536-bp cDNA sequences with an open reading frame of 429-bp deducing 142 amino acid residues [58,59]. The precursors carry an N-terminal signal peptide of 20 amino acids. Comparatively in *C. virginica*, cv-lysozyme 3 cDNA was identified as 663-bp sequence with a 564 bp open reading frame encoding 187 amino acids [56]. A predicted signal peptide was 18 amino acid residues.

Three different genomic sequences coding for lysozymes can be found in *C. gigas* genome scaffolds (GenBank: JH816436, JH816734, JH819154), confirming that lysozymes comprise a multigenic family. Lysozyme expression has been detected and localized in digestive cells of digestive tubules, gills and mantle [56,58,60,61]. However, CGL-1 was shown to be differentially expressed in hemocytes following *C. gigas* infection with the pathogenic *V. tasmaniensis* LGP32 compared to a non-virulent one [62]. Besides, lysozyme activity has been shown to increase in hemolymph following a co-stimulation of LPS and an oyster recombinant tumor necrosis factor, rCg-TNF1 [63]. Altogether, these results support a role of lysozyme in the oyster antimicrobial defenses, which

requires to be further investigated. Indeed, based on *in vitro* activity of recombinant CGL-1 and -3, lysozymes seems to display a weak activity (10–40 µg/mL) specifically against Gram-positive bacteria.

### 2.4.2. *Cg-Ubiquitin*

Ubiquitin is a structurally conserved polypeptide found in almost all tissues of eukaryotic organisms that regulates several processes, the most widely recognized being protein degradation [64]. Thus, in an essential function, ubiquitin molecules are covalently attached to target proteins for their subsequent degradation via the proteasome. Several additional functions of vertebrate ubiquitins have been described, such as alteration of cellular location, stability or activity of the target proteins, and lately, antibacterial and antifungal activities [65,66].

In oysters, the antimicrobial activity of ubiquitin has been recently reported [67]. *Cg-Ubiquitin* was purified from acidified gill extracts of *C. gigas*. The polypeptide had a molecular weight of 8.4 kDa and the N-terminal region is identical to ubiquitin sequences reported from other species. *Cg-Ubiquitin* transcripts were found to be expressed at high level in the mantle tissue, and to a lesser extent in gills, digestive gland, adductor muscle and labial palps [67]. *Cg-Ubiquitin* is encoded as a 76 amino acid precursor fused to the ribosomal protein S27. The precursor included a six amino acid motif and three conserved lysine at the functional sites, which might participate in the formation of the ubiquitin–protease complex. *Cg-Ubiquitin* also displays an 80-amino acid carboxyl ribosomal protein S27 extension with a classical zinc finger motif [67]. By homology modeling, *Cg-Ubiquitin* was deduced to display three secondary structural motifs, including three  $\alpha$ -helices and four  $\beta$ -strands separated by 7 loop regions. The C-terminal region of the *Cg-Ubiquitin* mature polypeptide terminated with an Arg residue instead of a Gly–Gly doublet, characteristic of ubiquitin molecules in other species [67].

The purified *Cg-Ubiquitin* was shown to be active against Gram-positive (MECs, 3.4, 7.8 and 40 µg/mL) and Gram-negative bacteria (MECs, 1.9–12.0 µg/mL) using the ultrasensitive radial diffusion assay (URDA) developed by the authors. *Cg-Ubiquitin* was bacteriostatic and did not permeabilize the bacterial membranes. No antifungal or hemolytic activities were observed for this polypeptide [67].

### 2.4.3. Histones

Histones play an essential architectural role in chromatin formation and their post-translational modifications play a key role in gene regulation. Since 1942 [68], histones have also been shown to carry antimicrobial activities against a wide range of pathogens including Gram-negative and Gram-positive bacteria, fungi, virus and protozoa [69]. These proteins and their derivative peptides have shown a wide range of actions in antibacterial processes, including permeabilization of bacterial cell membrane, binding to bacterial DNA and/or RNA [69], and neutralization of the LPS toxicity [70]. Recently, H2B histones active against Gram-positive and Gram-negative bacteria strains have been isolated from the American oyster *C. virginica* [71]. Similarly, antimicrobial H1-delta and H5 histones have been purified from gills of *C. gigas* oysters injured or infected with the oyster pathogen *V. tasmaniensis* LGP32 [72]. They showed potent antimicrobial activities against several strains of Gram-positive and Gram-negative bacteria with MICs below 0.7 µM. Moreover, in response to infection by the protozoan parasite *Perkinsus marinus*, the abundance of H4, H3.3 and H2B histone transcripts increased in total extract of *C. virginica* gill tissue [73]. Similarly, histone H4 protein levels of *C. virginica* appeared to increase in hemocyte lysates and extracellular hemolymph of oysters infected by *P. marinus* [74]. Finally, antimicrobial histones of *C. gigas* accumulated in gills after injury and infection while they

were absent from gills of unchallenged oysters [72]. This phenomenon correlated with a massive infiltration of hemocytes in the gills of infected oysters. Altogether, these studies support a role of histones in the antimicrobial defense of oysters. In agreement, release of extracellular histones was recently associated to a novel defense reaction in oyster referred to as ETosis [72] (see hemocyte reactions below).

### 3. The antimicrobial response to infection

The oyster antimicrobial response to infection needs to be reconsidered with the recent evidence that these bivalves naturally host diverse and abundant microbial communities [1] that may contribute to homeostasis, host protection and fitness in rapidly changing environments [75]. In a view similar to that of the interaction between the mammalian gut immune system and the abundant and diverse intestinal microbiota, the existence in oysters of an abundant microbiota associated to its epithelial surfaces and hemolymph questions about the modulation of the immune response. How is the equilibrium between bacteria and healthy oysters maintained? How are the oyster antimicrobial responses to opportunistic pathogens triggered and regulated? This leads to interesting tracks of reflection on the meaning of the “self versus non-self” theory in immunity [76] as well as on the fine-tuning of the interplay between the immune system and microbial organisms [77].

With the development of genomic studies, significant breakthroughs have been achieved in the identification of immune-related genes involved in defense mechanisms including recognition, cell signaling and cell communication that are known to activate or trigger cell-mediated reactions such as phagocytosis and production of antimicrobial molecules [5,62,78]. In particular, various elements of the Rel/NF- $\kappa$ B pathway have been described in oyster to be involved in immune response [79–82] (see also this issue Yu), but still, we have no evidence that this pathway controls the regulation of oyster AMP expression. Nonetheless, differences have to be expected with the best described invertebrate, *Drosophila*, in terms of AMP regulation. Indeed, most of the knowledge on the signaling pathways controlling AMP expression has been acquired in *Drosophila*, which has an almost sterile hemolymph. Unlike in *Drosophila*, oyster AMPs appear to be predominantly constitutively expressed by hemocytes and epithelia. Moreover, the release of peptidoglycan and other microbe associated molecular patterns (MAMPs) by its abundant microbiota does not trigger an overwhelming of the immune system. To date, it is unknown whether those AMPs whose expression is induced by infection are induced by specific signals released by pathogens or by an overload of MAMPs in the oyster blood stream and tissues.

#### 3.1. Involvement of AMPs in the response to infection

Whereas genomic approaches developed from various oyster tissues have greatly contributed to progress in the characterization of AMPs in oysters, it is noteworthy that these studies do not provide clear understanding on the regulation of expression and function of these immune effectors. As in oysters, most of the known AMP families are expressed by hemocytes that migrate to infection sites and infiltrate tissues [47], monitoring AMP expression in tissues, *i.e.* differentiating AMP transcription/translation in tissues from infiltration of tissues by AMP-expressing hemocytes, is a particularly challenging task that requires the use of immunohistochemistry and/or *in situ* hybridization. Currently, such data are available for defensins, big defensins, Prps and BPI, while, to date, Mpeg-1 and Molluscidin expression has only been monitored in tissue extracts (usually by quantitative PCR).

#### 3.1.1. AMPs in healthy oysters

In oysters, antimicrobial proteins and peptides are mainly constitutively expressed (Table 1). By using *in situ* hybridization *Cg-defhs*, *Cg-prps* and *Cg-bigdef3* were shown to be expressed in hemocytes where, comparatively, transcripts of *Cg-BPI* and *Cg-Bigdef1* and -2 were barely detected. *Cg-defm* was expressed in mantle only while *Cg-bpi* was expressed in epithelia of a broad series of organs [47]. Besides, *Cg-molluscidin* transcripts have been detected by PCR in mantle, gills and labial palps and at lower levels in digestive glands and adductor muscles of healthy oysters, but no data is available on hemocytes [48]. Finally, *Cg-mpeg1* would be constitutively expressed in the same tissues, the highest level of transcripts being detected in gills and gonads, then in digestive glands followed by the mantle, heart, adductor muscle and hemocytes [52].

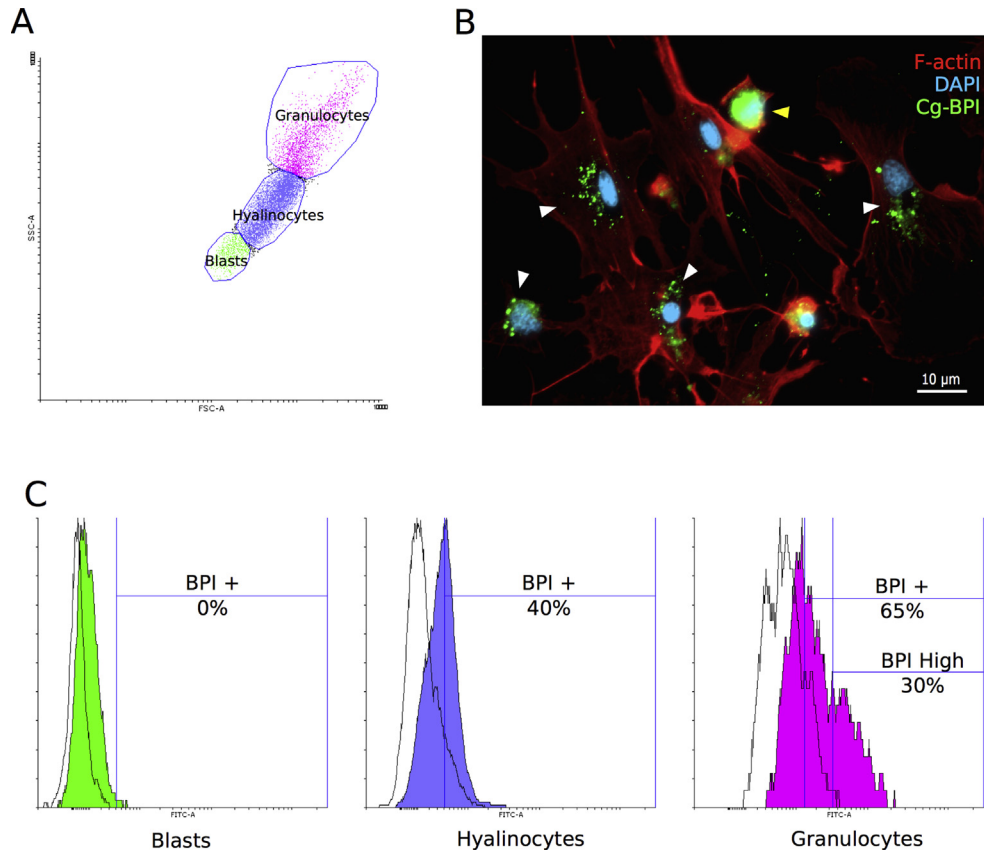
To date, only few data are available on the subcellular localization of AMPs in hemocytes and tissues. *Cg-BPI* and *Cg-Prps* appear to be stored in cytoplasmic granules in same hemocytes where *Cg-Defhs* are also immunocolocalized but uniformly distributed throughout the cytoplasm [32,47]. We recently showed that 40% of total hemocytes express *Cg-BPI*, although different subsets of hemocytes express it at different levels (Fig. 1). A subset corresponding to 40% of the hyalinocytes express *Cg-BPI* at a low level and the protein is stored in particular vesicles localized in the Golgi apparatus region. In granulocytes, two different subsets can be identified based on *Cg-BPI* expression. Most granulocytes express *Cg-BPI* (65%) with 30% of them expressing it at a high level, and the protein is stored in large cytoplasmic granules in those cells (see Fig. 1 and [47]).

#### 3.1.2. AMPs in diseased and injured oysters

Various infectious and non-infectious challenges have been used to study AMP expression in oysters. Still, most often, injection of bacteria and injury has been used, respectively. By using *in situ* hybridization and qPCR, *Cg-bpi* and *Cg-bigdef1* and -2 were shown to be strongly induced by bacterial challenge in hemocytes [40,50]. Apart from them, *Cg-Mpeg1* was reported to be induced in this tissue, but weakly and only 6 h after challenge [52]. On the contrary, as described above, expression of *Cg-defhs* and *Cg-bigdef3* is not regulated in response to microbial challenge. Injured or infected tissues often show an increase in AMP transcript abundance consistent with their immune function. This increase can be due to the accumulation of AMP-expressing hemocytes. This has been particularly well illustrated for the *Cg-Defh*-expressing hemocytes following an injection of sea water or bacteria into the oyster adductor muscle. The decrease in *Cg-Defh* transcript abundance in circulating hemocytes was seen to be concomitant with an increase in their abundance at the site of injury, and to a lesser extent in surrounding tissues of the mantle and gills [47,50]. Interestingly, neither *Cg-BPI* nor *Cg-Prp*-expressing hemocytes would migrate towards the injection site. Indeed, *Cg-Prp* expression seems to decrease in both, circulating and infiltrating hemocytes after a vibrio challenge [47]. *Cg-Molluscidin* transcript abundance have been reported to increase significantly in gills after microbial challenge with vibrios [48]. However, it is still unknown whether this is due to hemocyte infiltration or tissue expression. Discrepancy in AMP expression and localization following challenge can be observed in literature, mainly due to the omission of the migratory behavior of hemocytes and their abilities to infiltrate oyster tissues where they may aggregate.

Studies on AMP family expression have revealed the existence of different chemotactic behavior among hemocyte populations or between hemocyte developmental stages. Most of all, they highlight (i) the major role of the hemocyte populations in the AMP-mediated defense reactions and (ii) the complexity of this tissue





**Fig. 1. Bactericidal/permeability increasing protein is expressed at different level by different hemocyte subsets.** A. Cytometry analysis of SSC and FSC of hemolymph whole cells reveals the three main cell populations: blasts, hyalinocytes, and granulocytes. B. Immunofluorescent labelling of Cg-BPI protein in hemocytes reveals subset of hyalinocytes and granulocytes with low Cg-BPI content (white arrowhead) and high Cg-BPI content (yellow arrowhead). C. Cytometry analysis of Cg-BPI labelling of each hemocyte populations show that about 40% of all hemocytes express the BPI that correspond to 40% of the hyalinocytes and 65% of the granulocytes. Interestingly among the granulocytes, 30% of them express Cg-BPI at a higher level (BPI-High) than the other granulocytes thus defining two granulocyte distinct subsets for the level of Cg-BPI expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in terms of functions and mechanisms of regulation of immune-related gene expression, which could be related to existence of various cell lineages. As an example, while, upon infection, constitutively expressed AMPs like Cg-Defhs and Cg-Bigdef3 are transported through the migratory behavior of hemocytes, Cg-BPI and Cg-Bigdef1 and -2 are strongly transcribed in hemocytes infiltrating tissues [40,50].

Therefore, hemocytes must be seen as vehicles that drive given AMPs toward damaged or infected organs where other AMPs like Cg-Defm and Cg-BPI are constitutively expressed. Thus, AMP colocalization may occur in epithelia of various organs contributing to synergism and to local active antimicrobial reaction. One can assume that synergism takes place also during phagocytosis for AMPs colocalized in some hemocyte populations as reported for Cg-Defhs and Cg-Prps, and for Cg-Defhs and Cg-BPI [45,47]. Indeed, strong synergism against the oyster pathogen *V. tasmaniensis* LGP32 was reported between hemocyte defensins (Cg-Defh1 and -2) and mantle defensin Cg-Defm [47].

One striking feature of oyster antimicrobial defense to infection or injury is the absence of systemic humoral reaction characterized by the massive release or secretion of AMPs into the blood stream. In contrast, great amounts of AMPs are released following microbial infection in many invertebrates. In insects, microorganisms induce the expression of AMPs in epithelial cells and in the fat body, which are immediately and massively secreted in the blood stream [83]. In shrimp, the constitutively expressed penaeidins, which are stored in cytoplasmic granules, are released through an original

phenomenon of intracellular degranulation followed by the lysis of the hemocytes [84]. In mussels, AMPs are released by active degranulation process [85].

The lack of evidence of AMP release in oysters strongly contrasts with their detection in cells and tissues. Such low concentrations of extracellular AMPs support the hypothesis of a controlled antimicrobial defense that enables oysters to host and interact with a beneficial microbiota in its hemolymph and body. It also suggests that AMPs function primarily in hemocytes or epithelial cells. From our current knowledge, the AMP-mediated control of pathogens in oysters is likely based on phagocytosis as described for human neutrophil peptides [86] or mussel mytilins [87] in a process that benefits from the hemocyte migratory behavior. Besides, the constitutive expression of AMPs at surface epithelia may contribute to control the commensal microbiota and confer protection against potential pathogens [88].

### 3.2. Hemocyte reactions

Hemocytes play a central role in the oyster defense, they are able to respond to pathogen invasion or tissue damages through chemotaxis and phagocytosis [15,17]. Their microbicidal activities are based not only on the production of antimicrobial peptides/proteins as discussed above [32,40,50] but also on hydrolytic enzymes [89] and Reactive Oxygen Species (ROS) [90,91]. As described above, the very active recruitment of hemocytes is best highlighted by their massive infiltration in injured and/or infected tissues

[15,72,92]. Hemocyte migration leads to a local concentration of immune effectors believed to prevent host tissue invasion [47].

### 3.2.1. Phagocytosis

One of the best described immune function of hemocytes is phagocytosis of microorganisms (Fig. 2). The central role of phagocytosis in the immune response has been evidenced by (i) the overrepresentation of transcripts of phagocytosis-related genes in hemocytes of oysters surviving infections by virulent vibrio strains as compared to oysters receiving the same doses of a non-virulent strain of vibrio [62], and (ii) by the identification of pathogenic vibrios that either avoid phagocytosis like *Vibrio aestuarianus* O1/32 [93] or resist intracellular killing like *V. tasmaniensis* LGP32 [14]. The phagocytosis avidity of hemocytes is enhanced by opsonisation of the microorganisms by plasma proteins like Cg-EcSOD, which promotes  $\beta$ -integrin-mediated phagocytosis [14]. Concomitant to phagocytosis, hemocytes generate a significant respiratory burst resulting in the production of a variety of intermediate ROS [72,90,91], in a major microbicidal reaction in oysters. The production of ROS relies on NADPH oxidases as well as a transmembrane enzyme termed DUOX for (Dual Oxidase) that were evidenced in the *C. gigas* genomic resource database [94]. The expression of a DUOX-like gene in hemocytes was associated to a successful response of oysters against virulent vibrios [62].

After engulfment of microorganisms, the phagosome undergoes maturation with acidification and sequential fusion with endosomal and lysosomal compartments including granules, which contain diverse families of antimicrobial peptides/proteins [45,47]. The release into the phagosome of microbicidal compounds leads to the rapid neutralization/degradation of the engulfed microorganisms. Among the hydrolytic enzymes that are released into the maturing phagosome, lysozymes are known play an important role in microbial destruction due to their lytic properties on the peptidoglycan of

the bacteria cell wall [95]. Although unknown to date, it is likely that AMPs and lysozymes stored in hemocyte cytoplasmic granules are delivered to the phagosome to kill phagocytosed bacteria.

### 3.2.2. Extracellular trapping through ETosis

Recent studies have shown that phagocytes from metazoans can control microbes through the release of DNA extracellular traps (ETs) carrying antimicrobial peptides and histones (Fig. 2). Antimicrobial activities of histones have been described since 1942, but the mechanisms facilitating histone release has long remained unidentified. In 2004 Brinkmann et al. described a new antimicrobial mechanism relying on the release by mammalian neutrophils of extracellular DNA networks carrying histones and granular antimicrobial proteins including AMPs and hydrolases [96,97]. Those ETs are able to entrap and eventually kill bacteria, fungi, parasites and viruses [96,98,99]. ET formation depends on ROS production [100], and can be induced by different immunological stimuli including microbe-associated molecular patterns or host inflammatory mediators associated with tissue damage [96,100]. This phenomenon of cell death responding to infection or damage was referred to as ETosis.

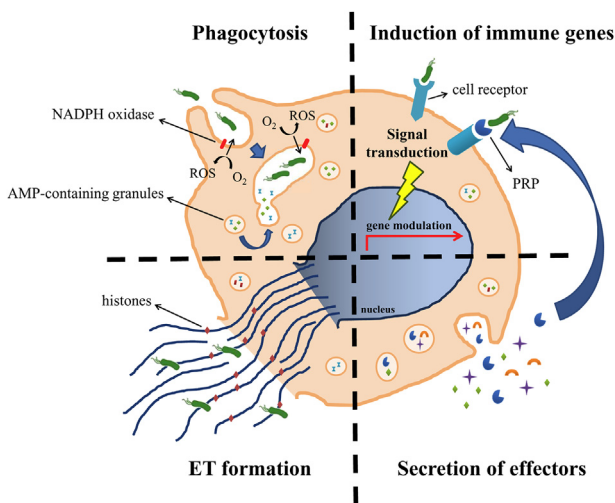
Evidence of ETs in the defense of invertebrates including *C. gigas* oysters is only recent [72]. In 2008, a first report on the insect *Galleria mellonella* suggested a role of extracellular nucleic acids in the immune response of invertebrates [101]. Three recent studies on mollusks, crustaceans and cnidarians demonstrated the actual role of ETs in the immune response of marine invertebrates and identified ETosis as a very ancient and evolutionary conserved host defense reaction. In oysters, hemocytes were shown to form ETs associated with antimicrobial histones, rapidly after an exposition to diverse microbial agents or tissue damage both *in vitro* and *in vivo* [72]. Similar to vertebrate neutrophils, the formation of ETs by the oyster hemocytes was dependent on the production of ROS [102]. However, unlike in other species, PMA failed to trigger the oxidative burst and the formation of ETs by oyster hemocytes. While in vertebrates ETs are formed mostly by neutrophils, the population of oyster hemocytes able to form ETs in oysters remains to be identified.

## 4. Unexplored functions of oyster AMPs

Until now, the main host defense function recognized for AMPs has been the direct killing of microorganisms. However, over the past years, an abundant literature has shown that AMPs from vertebrates support a broad series of immune functions. Indeed, there is new evidence that they also display immunomodulatory functions involving chemotactic activities, induction and/or inhibition of cytokine production, wound healing and modulation of immune cells responses (reviewed in Ref. [103]). Still, little attention has been paid to the multiple functions of invertebrate antimicrobial peptides. There are already some evidences that mollusk AMPs can serve immune functions other than antibacterial/antifungal, like myticin C from *Mytilus galloprovincialis*, which has antiviral and chemotactic activities [104] in addition to the known antibacterial properties of myticins [105]. However, these functions have been explored in heterologous systems and their role in mollusk defense has not been investigated to date. We review here a series of functions that deserve to be explored to better characterize the immune functions of oyster AMPs.

### 4.1. Antiviral properties

Antiviral activities of invertebrate AMPs have been poorly explored in homologous systems mainly due to the lack cell lineages facilitating the design of antiviral assays. At this stage it is still



**Fig. 2. Schematic representation of functions of oyster hemocytes. Phagocytosis:** After recognition, foreign microorganisms are phagocytosed and can be further destroyed by (i) the production of reactive species of oxygen (ROS) through the NADPH oxidase complex and/or (ii) defense molecules (antimicrobials, hydrolytic enzymes and other effectors) stored in granules that fuse with phagosome; **Extracellular trapping:** After an exposition to microbial agents or tissue damage, hemocytes release extracellular DNA traps (ETs) associated to histones and granular antimicrobial peptides/proteins, which can entrap microorganisms. **Induction of immune genes:** Hemocytes can be activated by various mechanisms including tissue damage, stress and microbial recognition by both soluble and cellular pattern recognition proteins/receptors (PRPs) leading to the induction of expression of some immune genes; **Secretion of effectors.** Immune effectors can be stored in hemocyte cytoplasmic granules or vesicles and released into the plasma, the extracellular spaces or oyster body cavities either continuously or following a microbial challenge or tissue damage.

unknown whether oyster AMPs can be antiviral. Interestingly, plectasin from the fungus *Pseudoplectania nigrella*, which shares a similar structure and mechanism of antibacterial action with Cg-Defs [38,106] was recently found to have antiviral activity. Studies on the antiviral properties of oyster defensins should be highly motivated by the severe viral diseases affecting oysters [107].

#### 4.2. Antifungal properties

Many C $\alpha$  $\beta$  defensins from invertebrates and plants have been characterized for their antifungal properties [108]. Some are strictly antifungal, while others like Cg-Defs seem to be potent antibacterials [47] with antifungal properties [32]. Antifungal properties have also been evidenced for other families of antimicrobials. For instance, the BPI antimicrobial protein from the gastropod mollusk *Biomphalaria glabrata* was recently proposed to protect the snail eggs from fungal infections [109]. The antifungal properties of Cg-BPI remain to be studied.

#### 4.3. Chemokine activities

Until the recent study on myticin C [104], little attention had been paid to the chemotactic properties of mollusk AMPs. However, diverse families of cysteine-rich AMPs can act as chemokines [110]. It is therefore tempting to speculate on the potential chemotactic activity of oyster defensins. Such an activity should also be investigated for Cg-BPI. Indeed, it has been shown that cellular responses are induced by human LBP, which is highly similar to BPI in terms of structure and LPS-binding properties. In particular, it participates in the acute mobilization of circulating neutrophils to sites of tissue injury [111]. Such an activity could also contribute to the massive migration of hemocytes to sites of infection/injury in oyster.

#### 4.4. Opsonization

Finally, an important function to be tested for mollusk AMPs is their capacity to promote phagocytosis by oyster hemocytes. Indeed, AMPs have the capacity to bind to the surface of bacteria and this process could enhance hemocyte phagocytosis. This has been shown for hBPI, which opsonizes bacteria, enhancing neutrophil phagocytosis [112]. As already shown for the extracellular superoxide dismutase Cg-EcSOD [14], such an opsonin activity deserves further investigation among oyster AMPs.

### 5. Genetic view of oyster antimicrobial defense

#### 5.1. AMP sequence diversity and evolution

##### 5.1.1. Genomic structural organization

Whereas *C. gigas* genome has been recently sequenced [78], our knowledge about oyster AMP gene organization in oyster genome remains quite limited. Numerous DNA and RNA sequences have been characterized among the different AMP families (Table 3), revealing a great intra- and inter-individual diversity. Cg-Def family appears to be the most diversified with 89 different mRNA sequences and 25 gDNA ones, available on GenBank (Table 3). However, it is noteworthy that we failed to evidence in the published database, OysterBase (<http://www.oysterdb.com/>), any full sequences of AMP encoding genes, but only mRNA sequences were found. Because whole *C. gigas* genome has been sequenced from one individual oyster [78], it is likely that the high level of diversity in terms of sequences and genetic structure variation (see below) contribute to difficulties for AMP sequence annotation. To date, it has been shown that Cg-Defs, Cg-Prps and Cg-BigDefs are the products of multigenic families displaying a variety of gene

**Table 3**

Number of DNA and RNA sequences characterized in *Crassostrea gigas* among families of antimicrobial peptides and proteins. The accession numbers available in GenBank are indicated.

	No nucleotide sequences	GenBank accession No
<b>Cg-Defs</b>		
mRNA	89	FJ669323 to FJ669352; JF766718 to JF766742; JF766743 to JF766768, DQ400101, DQ400102, AJ565499, AJ582628, AJ582629
gDNA	25	FJ669403-FJ669423; AJ582630, AM050547
<b>Cg-BigDefs</b>		
mRNA	23	JF703137 to JF703143, JF703144 to JF703146; JF703147 to JF703153, JN251121, JN251123, JN251124, JN251127, JN251129, JN251130
gDNA	14	JF703154 to JF703160; JN251122, JN251125, JN251126, JN251131, JN251132, JF703157, JF703158
<b>Cg-Prps</b>		
mRNA	80	FJ669353 to FJ669402; JF766783 to JF766798; JF766775 to JF766782; JF766769 to JF766773
gDNA	35	FJ669252 to FJ669286
<b>Cg-BPIs</b>		
mRNA	29	FJ669296 to FJ669322; AY165040, HM992925
gDNA	nd	
<b>Cg-Lysozymes</b>		
mRNA	4	AB179775, AB288344, AB288345, AB307634
gDNA	3	JH816436, JH816734, JH819154

nd: non determined.

structures and gene copy number variations. The number of Cg-def gene copies was shown to be highly variable (14–53 copies) among individual oysters as well as for Cg-prp which displays from 4 to 18 gene copy numbers according to individuals [35,113]. The genomic structural organization of oyster AMP families remains a vast domain to be investigated.

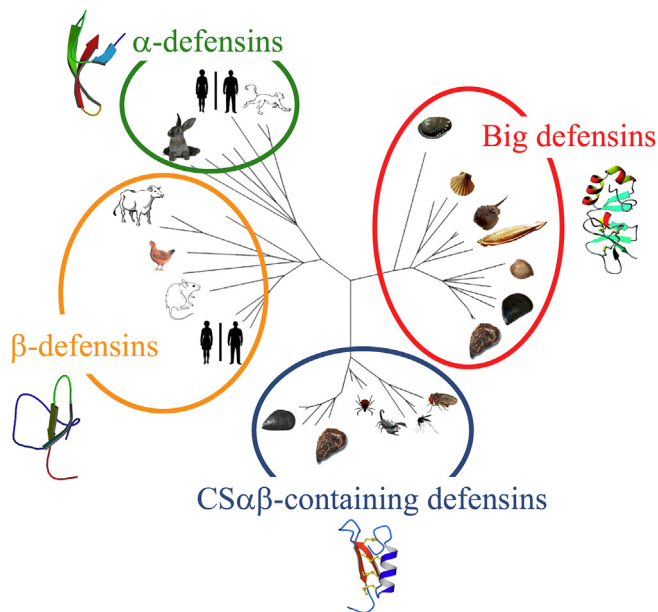
##### 5.1.2. Phylogenetics

Although sharing similar nomenclature, oyster defensins (Cg-Defs) and big defensins (Cg-BigDefs) have no clear phylogenetic relationships to each other (Fig. 3). Indeed, Cg-Defs are cysteine-rich peptides containing a C $\alpha$  $\beta$  (cysteine-stabilized  $\alpha\beta$ ) motif widespread in invertebrate defensins, like in arthropods and mollusks, but also in fungi and plants, and, remarkably, in toxins from scorpions [27,114]. However, the presence of eight cysteine residues was only reported in bivalve mollusk species [30,32,33]. This common structural fold strongly suggests there is a common ancestor to those different molecules, which has further evolved towards different functions, from toxins to antibacterials (fungi and mollusk defensins), antifungals (plant defensins), and as more recently shown antivirals (fungi defensins) [115]. On the other hand, invertebrate big defensins, including Cg-BigDefs, are more related to the  $\beta$ -defensins from vertebrates [40]. Indeed, in vertebrates, defensins adopt a three-stranded antiparallel  $\beta$ -sheet structure which is connected by a pairing of 6 cysteine residues. The cysteine pairing of invertebrate big defensins is similar to that of vertebrate  $\beta$ -defensins but totally differs from that found in the C $\alpha$  $\beta$  motif. Importantly, this cysteine pairing is similar in invertebrate big defensins and vertebrate  $\beta$ -defensins. It was recently proposed that vertebrate  $\beta$ -defensins originate from invertebrate big defensins [116].

##### 5.1.3. Sequence diversity

In the last years, high levels of sequence polymorphism were reported to be characteristic of several immune genes from invertebrates [117]. A number of studies into the origin and evolution of innate immune molecules evidenced numerous mechanisms of diversification and directional selection pressures over these genes,





**Fig. 3. Phylogenetic tree of animal defensins.** Human  $\alpha$ -defensin 1 (DEFA1) [PDB: 3LO4], chicken  $\beta$ -defensin (AvB2) [PDB: 2LG5], oyster mantle defensin (Cg-Defm) [PDB: 2B58] and horseshoe crab big defensin [PDB: 2RNG].

related to adaptive molecular evolution [118]. In *C. gigas*, high levels of sequence diversity have been detected for some oyster AMP families, notably Cg-Defm and Cg-Prps [35]. Phylogenetic analyses showed that Cg-Defm and Cg-Prps sequences were clustered into distinct groups forming clearly structured phylogenies. For Cg-Defm, three separate but constraint groups in which the three original forms where the most representatives are observed, while Cg-Prp phylogeny showed more diverse groups suggesting an ongoing or recent process of neo- or sub-functionalization. From the same study, the diversity of Cg-BPI sequences was shown to be less diverse when compared to the peptides, but the finding of a new variant of Cg-BPI suggests the existence of a greater diversity than previously thought [51]. Furthermore, Cg-BigDefm have been shown to display a diversity of sequences for the three of their members, presenting several isoforms of Cg-BigDef-1, -2 and -3 variants [40].

The variability on the sequences of *C. gigas* AMPs appears to be generated by a combination of different genetic mechanisms previously described in the diversification of several antimicrobial molecules in *Drosophila* [119]. The rapid evolution of AMPs is well documented [120–122] and suggests that hosts exposed to diverse pathogens may evolve a broader repertoire of antimicrobials that enhance their defensive potential [123]. The rapid evolution of AMPs has been related with the general hypothesis of co-evolution or “arms race”. In this theory, pathogens evolve continuously to escape from the immune response of hosts and, consequently, the immune system of hosts evolves to improve new barriers against pathogens [124]. Thus, it appears that oyster AMPs could be under strong selective forces shaping their sequence variations. This is supported by evolutionary analyses which revealed several negatively and positively selected sites for Cg-Defm and Cg-Prp [35]. Evidences of diversification in oyster AMPs also motivate the search for novel immune functions (neo-functionalization). It is indeed reasonable to speculate that such small and stable molecules, capable to resist to proteolysis in body fluids and tissues, are ideal candidates to mediate immune functions and are therefore the subject of intense selective pressures in the plastic association between the oyster and its microbiota.

## 5.2. Individual polymorphism of gene expression and genetic structural variation

The recent progress in genomics has opened new perspectives for exploring the oyster immune system. On the one hand, the first sequencing of *C. gigas* genome [78] and various transcriptomic studies allowed to identify a vast range of potential effectors and key actors of oyster immunity [62,94,125–127]. On the other hand, the development of genomic technologies such as high throughput RT-qPCR analyses allowed to explore the expression of numerous immune-related genes at the individual levels revealing an extraordinary polymorphism in basal expression of some of them in this species [128]. Indubitably, analyses performed at individual level may give new light on the genetic bases of oyster immune response and reveal its diversity and complexity. From these studies it is also clear that AMP-encoding genes show a great inter-individual variability of expression, as shown for *Cg-defm*, *Cg-prps*, *Cg-bpi* and *Cg-bigdefm* [40,113,129].

It is known that gene expression polymorphism may result from epigenetic modifications or genetic structural variation such as gene **Copy Number Variation** (CNV). CNV is a type of polymorphism characterized by differences in the number of copies of a particular gene in the genotype of an individual. CNV can be associated to variable phenotypes, including susceptibility to diseases [130]. In oysters, CNV has been evidenced for the AMP-encoding genes *Cg-defm* and *Cg-prps* with mean values of 18 and 48 gene copies respectively, whereas *Cg-bpi* has been found encoded by a single gene copy in all the individuals analyzed [35]. However, until now, the number of gene copies of *Cg-bpi2* and other oyster antimicrobials (*Cg-BigDefm*, *Cg-Molluscidin* and *Cg-Mpeg1*) in individual oysters remains unknown. In a recent work, CNV has been correlated to the variability of AMP gene expression in oysters. For *Cg-defm*, a positive correlation was found with variation of 14–48 gene copies among 14 individuals analyzed, whereas for *Cg-prps*, the variation ranged from zero to 18 copy numbers [113].

Noteworthy, inter-individual variability has been observed in the basal expression of *Cg-bigdefm* using high throughput RT-qPCR analyses. Among 163 individual oysters, variability in basal gene expression reached up to 30, 27 and 7-fold for *Cg-BigDef1*, *Cg-BigDef2* and *Cg-BigDef3*, respectively. In addition, *Cg-BigDef* expression could not be detected in 39 individuals. Further analyses at genome level revealed that this lack of expression was likely associated to the absence of encoding *Cg-bigdef* sequences [129] as previously observed for *Cg-prps* [113]. In such an extreme case of CNV, known as Presence–Absence Variations (PAV), large DNA segment present in some individuals can be entirely missing in others [131]. PAV is known in humans but also in plants for which the genetic diversity has been exploited for domestication. In humans, PAV has been described for the *DEFA3* gene that encodes the human neutrophil peptide-3 (HNP-3) [132]. The presence of the encoding gene is variable depending on the human geographical populations and this variability has been suspected to be implicated with differences of susceptibility to infectious diseases [133]. In a larger extent, CNV has been associated to disease susceptibility particularly in the case of human defensins [134,135].

To date, there are no evidences about a possible impact of such structural genome variations on the oyster susceptibility to infectious diseases. No relationship was observed between the Presence–Absence of *Cg-bigdefm* and the capacity of the oysters to survive experimental *Vibrio* infections [129]. In plants, it is assumed that PAVs may generate new pathogen resistances but most of all that it could contribute to individual adaptation to environmental stresses [136].



## 6. Conclusions

From the recent results and knowledge we reviewed here, the role of AMPs in the oyster immune system must be reconsidered taking into account that oysters must be seen as an organism associated to a microbiota. In such a context where the oyster can host an abundant and beneficial microbiota, AMPs cannot be considered only as repressive weapons. It is likely that in oyster, where AMPs are present at low concentrations [46], they are primarily involved in unexplored immune functions.

We have now evidences that the oyster antimicrobial defense is characterized by local responses ensured by hemocytes instead of systemic or humoral response as shown in other invertebrates. With their great capacity to move and infiltrate all oyster tissues and organs, the hemocytes, which carry AMPs and other immune effectors, may provide monitoring and immediate response to damages or injuries (Fig. 2). The mechanisms that underlie hemocyte communication, recruitment and their activation remain largely unknown and the potential role of AMPs in such immune modulatory functions requires a particular attention.

One major advance over the past years is the evidence in *C. gigas* of a great AMP sequence diversity and extraordinary polymorphism of basal AMP gene expression among individuals. The relationship between the AMP gene expression level and genome structural variations such as gene copy number or Presence-Absence Variation has been highlighted for the first time in an invertebrate. These results open indubitably new perspectives for investigating the oyster immune system and in particular the role that antimicrobials may play in the interaction of the immune system with the oyster commensal microbiota (from mutualists to pathogens) and the capacity of the oyster metaorganism (the oyster and its associated microbiota) to survive infections or diseases.

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