Big Defensins, a Diverse Family of Antimicrobial Peptides That Follows Different Patterns of Expression in Hemocytes of the Oyster Crassostrea gigas

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Abstract

**Background:** Big defensin is an antimicrobial peptide composed of a highly hydrophobic N-terminal region and a cationic C-terminal region containing six cysteine residues involved in three internal disulfide bridges. While big defensin sequences have been reported in various mollusk species, few studies have been devoted to their sequence diversity, gene organization and their expression in response to microbial infections.

**Findings:** Using the high-throughput Digital Gene Expression approach, we have identified in Crassostrea gigas oysters several sequences coding for big defensins induced in response to a *Vibrio* infection. We showed that the oyster big defensin family is composed of three members (named Cg-BigDef1, Cg-BigDef2 and Cg-BigDef3) that are encoded by distinct genomic sequences. All Cg-BigDefs contain a hydrophobic N-terminal domain and a cationic C-terminal domain that resembles vertebrate β-defensins. Both domains are encoded by separate exons. We found that big defensins form a group predominantly present in mollusks and closer to vertebrate defensins than to invertebrate and fungi CS-defensins. Moreover, we showed that Cg-BigDefs are expressed in oyster hemocytes only and follow different patterns of gene expression. While Cg-BigDef3 is non-regulated, both Cg-BigDef1 and Cg-BigDef2 transcripts are strongly induced in response to bacterial challenge. Induction was dependent on pathogen associated molecular patterns but not damage-dependent. The inducibility of Cg-BigDef1 was confirmed by HPLC and mass spectrometry, since ions with a molecular mass compatible with mature Cg-BigDef1 (10.7 kDa) were present in immune-challenged oysters only. From our biochemical data, native Cg-BigDef1 would result from the elimination of a prepropeptide sequence and the cyclization of the resulting N-terminal glutamine residue into a pyroglutamatic acid.

**Conclusions:** We provide here the first report showing that big defensins form a family of antimicrobial peptides diverse not only in terms of sequences but also in terms of genomic organization and regulation of gene expression.

Introduction

Big defensin is an antimicrobial peptide (AMP) initially characterized in a Chelicera, the horseshoe crab *Tachypleus tridentatus*, whose immune system has been extensively studied [1]. The big defensin purified from horseshoe crab hemocytes is a 79 amino acid peptide with antimicrobial activities against both Gram-positive and Gram-negative bacteria and fungi [2]. The polypeptide is composed of a highly hydrophobic N-terminal region and a cationic C-terminal region containing six cysteine residues involved in three internal disulfide bridges. Interestingly, after experimental trypsin digestion at Arg-37 residue, the two generated peptides were reported to display distinct activities: the N-terminal peptide is more active against Gram-positive bacteria whereas the C-terminal cationic peptide is more active against Gram-negative bacteria. Besides, the native full-length big defensin displays significant LPS-binding properties whereas the two separated regions do not [2].

The solution structure of horseshoe crab big defensin showed that the N-terminal hydrophobic sequence adopts a unique globular conformation consisting in a parallel β-sheet and two α-helices [3]. Interestingly, the C-terminal region of the horseshoe crab big defensin forms a β-sheet structure folded by three disulfide bounds, which is similar to the three-stranded antiparallel β-sheet structure of the human β-defensin HBD-2 and HBD-3 [4], antimicrobial peptides active against both Gram-positive and Gram-negative bacteria [5]. Such a structure is different from that of invertebrate defensins, which consists of an α-helix linked to an antiparallel two-stranded β-sheet by 3 to 4 disulfide bridges [6]. This last structure is common to all mollusk defensins and was solved in both mussel and oyster defensins [7,8].
Surviving infection with Vibrio: response to a Vibrio infection. Herein, we have improved our knowledge of AMPs from marine invertebrates have been the subject of intense diversifying selection [13,14]. It has been shown that the different members of the given AMP family can be expressed in various cells and tissues. For instance, members of the defensin family are continuously expressed both in oyster mantle (Cg-Defm) [8] and hemocytes (Cg-Defh1 and Cg-Defh2). [15]. Various proline-rich AMPs, named Cg-Prps, are also expressed in oyster hemocytes [16]. Besides, a high level of sequence diversity has been found both at the transcript and genomic levels. Both Cg-Defhs and Cg-Prps are multigenic families of AMPs [17]. Such diversity could greatly contribute to a broader antimicrobial response in oysters.

Interestingly, while big defensins have been largely studied in terms of primary structure and antimicrobial activities, all studies have been performed on one given sequence only. Surprisingly, little attention has been paid to their sequence diversity, phylogeny and gene organization and to their expression during the anti-infectious response. Herein, we have improved our knowledge of C. gigas antimicrobial response with the characterization of different members of the big defensin family.

Here, we have applied the high-throughput Digital Gene Expression (DGE) approach to identify genes potentially involved in oyster survival to Vibrio infections and have identified several EST sequences encoding big defensins named Cg-BigDef1, Cg-BigDef2 and Cg-BigDef3. We showed that they are encoded by different genomic sequences. Interestingly, whereas Cg-BigDef1 and Cg-BigDef2 were up-regulated in oyster hemocytes in response to a microbial challenge and to a pathogenic Vibrio infection, Cg-BigDef3 was constitutively expressed. We provide here the first evidence of big defensin diversity in terms of sequence, gene regulation and genomic organization. Finally, from a phylogenetic analysis of big defensin sequences identified here and found in the GenBank database, we show that big defensins form a group predominant in mollusks and distinct from the vertebrate and invertebrate defensin-related families.

Results

Identification of big defensin sequences in oyster response to a Vibrio infection

Our group has developed a high-throughput Digital Gene Expression (DGE) approach in Crassostrea gigas to identify hemocyte genes potentially associated to the oyster capacity to survive virulent Vibrio infections [18]. Thus, three hemocyte DGE libraries were generated from oysters Surviving infection with Virulent (SVir) and aVirulent (SaVir) Vibrio strains and from Non-infected (NInf) oysters. Library sequencing resulted in a total of 21,755 tag signatures differentially expressed (>2-fold change) which have been matched against the GigasDatabase [10]. From these analyses, a tag sequence (TAG1) mapped to two different EST sequences (GenBank: CU987401, AM83907) homologous to the horseshoe crab big defensin (Table 1). This tag displayed an occurrence of 111 in the NInf library and of 1866 in the SVir DGE library and 1058 in the SVir one and it was 24-fold more abundant in the infected oyster libraries than in the non-infected one. Another tag (TAG2) matched a third big defensin sequence (GenBank: AM863249) with an occurrence of 108 in the NInf library and only 8 and 19 in SVir and SaVir libraries, respectively (Table 1).

| Table 1. Assigned tag sequences specific to big defensins in oyster DGE libraries. |
|------------------------------------------|-----------------|----------------|----------------|----------------|----------------|----------------|
| DGE tag sequence | GenBank accession no. | GenBank description |
| TAG1 (CATGCGAGATTACTGC) | CU987401 | Big defensin (BDEF_TACTR: P80957) |
| TAG2 (CATGGATTAATCCCTC) | AM863249 | Big defensin (BDEF_TACTR: P80957) |

NInf: non-infected DGE library; SVir: surviving virulent Vibrio DGE library; SaVir: surviving avirulent Vibrio DGE library.

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highly hydrophobic N-terminal sequence and a cationic C-terminal sequence containing six cysteine residues at positions conserved in horseshoe crab big defensin and in vertebrate β-defensins (Figure 1a). Besides, putative mature big defensins are highly cationic with a calculated pI ranging from 8.6 to 9.2. Cg-BigDef1 shares 90% and 75% amino acid sequence identity with Cg-BigDef2 and Cg-BigDef3, respectively whereas Cg-BigDef2 and Cg-BigDef3 share 68% identity.

Big defensins form a diverse family of oyster defense peptides

In silico analysis of GigasDataBase led to the discovery of different isoforms for each oyster big defensin: seven were found for Cg-BigDef1 (GenBank: JF703137 to JF703143), three for Cg-BigDef2 (GenBank: JF703144 to JF703146) and eight for Cg-BigDef3 (GenBank: JF703147 to JF703154). Among the isoforms identified for Cg-BigDef1 and Cg-BigDef3, five and four of them differed at the amino acid level through six non-synonymous nucleotide substitutions, respectively. In contrast, the three Cg-BigDef2 isoforms only presented synonymous nucleotide substitutions. The 3' region, that codes the C-terminal domain of the mature peptide, is the most conserved region of the CDS with only few nucleotide substitutions. Besides, no indel events were observed in the isoforms of the different big defensins. Although many substitutions were found in the Cg-BigDef2, both signal peptide and propeptide cleavage sites remained conserved as well as the position of the six cysteine residues in the β-defensin-like domain.

Oyster big defensins are encoded by distinct genomic sequences

The genomic organization of the three Cg-BigDef forms was investigated by PCR-based cloning and sequencing of the encoding regions of oyster genomic DNA (GenBank: JF703155 to JF703160). Five oysters have been considered for this partial gene characterization which revealed that each of the three oyster big defensins is encoded by a distinct genomic sequence. Cg-BigDef1 and Cg-BigDef2 genomic organization was similar, revealing the presence of two exons interrupted by a single intron. For these forms, the first exon covers part of the 5'-UTR, the signal peptide, the propeptide sequence and the N-terminal domain of the mature peptide. The second exon covers the cysteine-rich C-terminal (β-defensin-like) region and part of the 3'-UTR sequence (Figure 1b). In contrast, in Cg-BigDef3, additional intron and exon were observed upstream the first exon common to the Cg-BigDef1 and Cg-BigDef2 genomic organization, revealing the presence of two exons interrupted by a single intron. For these forms, the first exon covers part of the 5'-UTR, the signal peptide, the propeptide sequence and the N-terminal domain of the mature peptide. The second exon covers the cysteine-rich C-terminal (β-defensin-like) region and part of the 3'-UTR sequence (Figure 1b).

The length and nucleotide composition of the exons differ among the three big defensin genomic coding sequences. Besides, for each Cg-BigDef, the exon length appears conserved among individuals whereas the intron lengths are variable (Figure 1b).
Big defensins cluster in a separated clade from other defensins families

The deduced amino acid sequences of Cg-BigDef1, -2 and -3 were compared with big defensin sequences from the horseshoe crab T. tridentatus (GenBank: PR0957), the amphioxus Branchiostoma belcheri tsingtaunense and B. floridae (GenBank: AAO18674, ADH03419), the scallop A. irradians (GenBank: ABC61319) and the clam Venusupes (Rudistes) philippinarum (GenBank: ADM25826).

Additionally, for this analysis, we identified several new translated EST sequences homologous to big defensins in different mollusk species in the GenBank database. These include sequences from the oyster C. virginica (BDEF_CRAVI: CV133136), the mussels *Mytilus galloprovincialis* (BDEF_MYTGA: FL490131), *M. californianus* (BDEF1_MYTCA: GE761911; BDEF2_MYTCA: GE759807) and *Hyriopsis cumingii* (BDEF_HYRUC: GW692819), the clam *V. philippinarum* (VpBD-EST: AM873974) and the abalone *Haliotis diversicolor* (BDEF_HALDS: GT870909).

Amino acid sequence identity of Cg-BigDef1, -2 and -3 with the big defensin from horseshoe crab *T. tridentatus* was in the range of 48 to 53%, and of 47 to 55% with the amphioxus species, *B. belcheri tsingtaunense* and *B. floridae*, as determined by ClustalW2 alignment (Figure 2a). Within mollusks, Cg-BigDef were 55 to 67% identical to mussel sequences (*M. galloprovincialis* and *M. californianus*), 57% identical to an oyster sequence (*C. virginica*), 55 to 59% identical to EST-derived sequences from clams (*V. philippinarum* and *Mercenaria mercenaria*), 39 to 45% identical to scallop sequences (*A. irradians* and *Mizuhaben jessoensis*), 41% identical to the deep sea hydrothermal vent mussel *Bathymodiolus azoricus*, and only 25 to 20% identical to the gastropod *H. diversicolor* sequence. However, less than 17% amino acid identity was observed between oyster big defensins and VpBD, recently reported as a big defensin in the clam *V. philippinarum* [12].

A phylogenetic tree was constructed with all big defensins mentioned above and defensin families from various species and phyla. In this tree, the defense peptides were split into five distinct groups: CSβ-containing defensins from fungi and invertebrates, big defensins from invertebrates, as well as α- and β-defensins, β-defensins and fish β-defensin-like peptides (Figure 2b). Oyster big defensins clustered together with other big defensin sequences, with exception of the clam VpBD sequence (GenBank: ADM25826) that showed no clear relationship with big defensins. Instead, this sequence clustered with β-defensin-like peptides from fish. Within the big defensin group, oyster big defensins appeared closely related to each other and with big defensins from *C. virginica*, *M. mercenaria*, mussels from the genus *Mytilus* and with a big defensin EST-derived sequence we identified in *V. philippinarum* (Figure 2b). Big defensins from horseshoe crabs, amphioxus and other mollusk species clustered in distinct groups. The phylogenetic tree also indicated that the big defensin cluster is closer to vertebrate α- and β-defensins than to invertebrate CSβ-containing defensins.

Cg-BigDefs are differentially modulated upon a bacterial challenge

The expression pattern of oyster big defensins was studied by quantitative PCR (qPCR) in response to a bacterial challenge. Oysters were injected with a mix of heat-killed Gram-positive and Gram-negative bacteria. A significant increase in Cg-BigDef1 (p < 0.025) and Cg-BigDef2 (p < 0.002) transcripts was observed in circulating hemocytes 12 h post-stimulation comparatively to a sterile sea water (SSW) control injection (Figure 3a). In contrast, no changes in Cg-BigDef3 transcript abundance were seen following injection of bacteria or SSW. However, for Cg-BigDef3, an unusual high variability in transcript abundance was observed between the oyster hemocyte pools in all experimental conditions, independently of the bacterial or SSW challenge (Figure 3a).

The expression of Cg-BigDef1 and Cg-BigDef2, which was modulated upon injection of heat-killed bacteria, was further analyzed in response to oyster experimental infections (intramuscular injection) with the virulent *Vibrio splendidus* LGP32 [21] and the avirulent *V. tasmanianus* LMG 20012T strains, belonging to the *V. splendidus* polyphyletic group [22]. In a control experiment, the injection of SSW (control) did not significantly modify the expression of Cg-BigDef1 and Cg-BigDef2 compared to unchallenged oysters. Conversely, the transcript abundance of Cg-BigDef1 and Cg-BigDef2 significantly increased after a *Vibrio* injection 8 h post-injection, reaching a peak at 24 h and slowly decreasing after 48, 72 and 120 h (Figure 3b). At the peak of expression (24 h), Cg-BigDef1 gene expression was 16-fold (p < 0.004) and 31-fold (p < 0.0001) higher for animals infected with the virulent and avirulent *Vibrio* strains, respectively, than in unchallenged oysters. Only at this time point-injection, a significant difference in gene expression (p < 0.011) was observed according to infection with the virulent or the avirulent *Vibrio* (Figure 3b). In the same conditions, Cg-BigDef2 gene expression was similar to that of Cg-BigDef1 (data not shown). This corroborated the DGE data (see above). A great variability in transcript abundance was observed for both Cg-BigDef1 and Cg-BigDef2, particularly upon *V. splendidus* LGP32 infection.

Cg-BigDef expression is restricted to circulating and tissue-infiltrating hemocytes

The localization of gene expression of the inducible Cg-BigDef1 and constitutive Cg-BigDef3 in unchallenged and *V. splendidus* LGP32-infected oysters was further studied by ISH using specific DIG-labeled riboprobes. In unchallenged oysters, the gene expression of Cg-BigDef1 and -3 forms was clearly restricted to hemocytes, both circulating, as seen in blood vessels and sinus, and infiltrating tissues. Cg-BigDef transcripts were detected in hemocytes infiltrating different oyster organs, such as the gills, the gonads and the digestive gland (Figures 4a and 4b). Consistent with our qPCR data, upon bacterial challenge of oysters, Cg-BigDef3 expression was not modulated whereas Cg-BigDef1 was strongly stimulated. Indeed, a striking increase in the number of Cg-BigDef1 positive cells was observed in hemolymph vessels and connective tissues (Figures 4c, 4d and 4e) of the oysters at 24 h after *V. splendidus* LGP32 injection compared to non-injected animals. No signals were observed on histological sections hybridized with sense riboprobes (Figure 4f).

Detection of Cg-BigDef1 in oysters subjected to bacterial challenge

The native Cg-BigDefs were finally investigated at the peptide level by RP-HPLC and mass spectrometry in oysters subjected to a bacterial challenge. Upon challenge, hemocytes expressing big defensins were seen highly infiltrating tissues including the gills (Figure 4e). We therefore compared the peptide profile of gill acid extracts at different times (12, 24 and 48 h) after an injection of heat-killed bacteria. Acid extracts were subjected to RP-HPLC, all RP-HPLC fractions being collected and subjected to MALDI-TOF MS.

Because the actual state of mature Cg-BigDefs was unknown, we performed a blind screen of all the RP-HPLC fractions for the presence of ions corresponding to putative maturation states of all the Cg-BigDef forms and isoforms characterized in this study. Potential maturationates included not only the oxidation of the
**Figure 2. Big defensins form a group predominantly present in mollusk species and closer to vertebrate defensins.** a: Multiple alignments of mature polypeptides of big defensins from bivalve mollusks, horseshoe crab and amphioxus. Identical amino acid residues are shaded with black backgrounds. b: Phylogenetic tree of different defensin groups, including big defensins, Cg-defs-containing defensins, δ-defensins, β-defensins and β-defensin-like peptides from fish. The tree was constructed using the Neighbour-Joining method in MEGA 4. Bootstrap sampling was reiterated 1,000 times. Sequences included in analyses of defensins were the following, where asterisks (*) indicate mollusk big defensin members newly identified in this study from GenBank: (i) big defensins: oysters Cossatostrea gigas (Cg-BigDef1*: AE922768, Cg-BigDef2*: AE922775, Cg-BigDef3*: AE922778) and C. virginita (BDEF_CRAVI*: CV733155), horseshoe crabs Tachypleus tridentatus (BDEF_TACTR: P80957) and Carcinus maenas (BREF_CAROA: CK086629), amphioxus Branchiostoma belcheri tsingtauense (BDEF_BRAFL: AA018674) and B. rerio (BREF_BRAFL: ADH03419), scallops Argopecten iradians (AIBD: ABG61319) and Mizuhopecten yessensis (MYBD: GH736001), clams Veneridae (Ruditapes philippinarum (WpBD: ADM25826, WpBD-EST*: AM873974) and Mercenaria mercenaria (BDEF_MERME: QG915266), mussels Mytilus galloprovincialis (BDEF_MYTA*: FL490131), *M. californianus* (BDEF_MYTGA*: FL490131), Norway rat *M. norvegicus* (DEF1A_CAVPO: P11478, MGD-1: P80571, MGD-2: AAD52656), abalones *Haliotis discus discus* (BDEF_HALDS: AC215982) and H. discus hannah (BDEF_HALHA: ABF699125), scorpion Androctonus australis (DEF4_ANDAU: P56668), tick Dermacentor variabilis (VSNA1: AAO24323), fruit fly Drosophila melanogaster (DEFL_DROME: P80957), mouse Mus musculus (CRYP-1: NP_034161), Norway rat Rattus norvegicus (DEFR2_RAT: Q62715), rabbit Oryctolagus cuniculus (DEF3_RABIT: P01376), domestic guinea pig Cavia porcellus (DEF1A_CAVPO: P11478), Homo sapiens (HNP-1: NP_004075, HNP-3: AAA35753, HNP-4: NP_001916); (ii) δ-defensins: spiny lobster Panulirus japonicus (PJD1: ACM62357, PJD2: ACM62358), chicken Gallus gallus (GAL4: NP_001001610), wild turkey Meleagris gallopavo (GPV-1: AAAG02913), R. norvegicus (RBD-4: NP_071989), water buffalo Bubalus bubalis (DEBF_BUBBU: AB36600), cattle Bos taurus (DEBF_BOSTA: CAC15400), goat Capra hircus (DEFB_CAPHI: ABF71365), H. sapiens (HBD-2: NP_004933, HBD-3: NP_061131); (iv) fish defensins: rainbow trout Oncorhynchus mykiss (DEF_ONCMY: ABR68250), zebrafish Danio rerio (DEF_DANRE: AM181358), fugu pufferfish Takifugu rubripes (DEF_TATRU: CAJ19280), spotted green pufferfish Tetraodon nigroviridis (DEF_TACRU: CAJS75564), Japanese flounder Paralichthys olivaceus (DEF_PAROL: ADA84138).

discilis bridges, but also the presence a pyrroglutamic acid at position 1 (instead of the glutamine) and the elimination of the C-terminal glycine leading to a C-terminal amidation. Only one fraction displayed ions with m/z values corresponding to double charged ([M+2H]2+, Cg-BigDefs) at all times after challenge. These ions were absent from the unchallenged oysters. Interestingly, this fraction, which eluted at 34% acetonitrile, corresponded to one peak that was almost absent from unchallenged oysters but significantly increased in absorbance after bacterial challenge (Figure 5). Ions of interest were detected at 12 h (m/z 5359), 24 h (m/z 5359) and 48 h (m/z 5358). Such masses correspond to the putative double charged ions of three isoforms of Cg-BigDef1, namely Cg-BigDef1-4 (GenBank: JF703141, [M+2H]2+ at calculated m/z 5359), Cg-BigDef1-5 and Cg-BigDef1-6 (GenBank: JF703142 and JF703143, [M+2H]2+ at calculated m/z 5350.5) assuming the following post-translation modifications: (i) elimination of the 13-residue propeptide region, (ii) cysteine bridge oxidation (loss of 6 Da), and (iii) cyclization of the N-terminal glutamine into a pyrroglutamic acid (loss of 18 Da).

**Discussion**

Here, we showed for the first time that oyster big defensins, Cg-BigDefs, form a family of antimicrobial peptides diverse in terms of sequences, genomic organization and gene expression regulation. By cDNA cloning we showed that Cg-BigDefs are composed of a 23-residue signal peptide followed by two cationic regions, namely a 13-residue propeptide sequence (calculated pI 8.6 to 9.7), and a C-terminal 87- to 94-residue sequence corresponding to the mature peptide (calculated pI 8.6 to 9.2). These two regions are separated by a propeptidase cleavage site Arg-X-(Arg/Lys)-Arg, a conserved motif for processing by furin, which in oyster hemocytes could be encoded by three EST sequences (GenBank: FP004575, CU989616, AM862886). The structure of oyster big defensin precursors differs from that of oyster defensins (Cg-Defs). Indeed, Cg-Defs derive from a precursor peptide composed of a signal peptide and a C-terminal cationic sequence, but lack the propeptide sequence. To date, nothing is known on the function of big defensin propeptide sequence. Because of its cationic charge,
the propeptide in big defensins is unlikely used for trans inactivation of the mature AMP, as described for human α-defensins [23]. One can speculate that, as shown for the pro-domain of insect attacin C [24], the oyster big defensin propeptide could rather generate a second AMP. Indeed, it is not only cationic but also predicted to fold into an α-helical conformation by PSIPRED v3.0 software (data not shown), two well-known properties for membrane disrupting AMPs [25].

We showed here that the oyster big defensin family is composed of three members, encoded by three distinct genomic sequences. Besides, additional sequence diversity was observed (isoforms) for each member. Our data suggest that the big defensin molecular diversity is generated by single point mutation or the presence of different polymorphic alleles within the population, rather than by alternative splicing, which is a common way to generate sequence diversity [26]. We showed here that the genomic sequences encoding the three Cg-BigDefs differ in terms of structure (Figure 1b). Therefore, the big defensin may be a multigenic family of diversified members, as known to occur for other defense peptides in animal species and in particular in oyster. Indeed, to date, our group has evidenced that oyster defensins were a multigenic family generating high molecular diversity of sequences expressed from hemocytes (Cg-Defhs) and from mantle (Cg-Defm) [13]. More than 30 different defensin isoforms were sequenced and about 13 copies of Cg-Defhs and Cg-Defm encoding genes were estimated in solely three animals analyzed. In the human genome, the β-defensin genes are organized in a cluster of a minimum of seven β-defensins and this cluster can be found from 2 to 7 or 12 copies according to individuals [27]. It is noteworthy that the polymorphism of immune genes including antimicrobials may result in variable expression level, potentially causative of variability in pathogen or disease susceptibility [28,29,30]. The unique diversity of oyster Cg-BigDefs has not been reported previously in other species. Indeed, to date, reports on big defensins have mainly focused on one sequence only [2,12].

Another major result from this study is that members of the Cg-BigDef family follow different gene regulation patterns in response to a bacterial challenge. The inducibility of Cg-BigDef1 and -2 was evidenced both by DGE approach and quantitative PCR on hemocytes from bacteria-challenged and Vibrio-infected oysters.

Figure 4. Localization of Cg-BigDef mRNA expression in unchallenged and Vibrio-infected oyster tissues by in situ hybridization (ISH). Histological sections of C. gigas were analyzed by ISH using Cg-BigDef-DIG-UTP antisense (a-e) and sense (f) riboprobes. In unchallenged oysters, Cg-BigDef1 (a) and Cg-BigDef3 (b) labeling appeared in hemocytes (arrows) located in blood vessels and infiltrating oyster tissues. In Vibrio-challenged oysters, strong hybridization signals were detected with Cg-BigDef1 antisense riboprobe in hemocytes located in blood vessel (c) and invading massively connective tissues of the oyster organs such as the digestive gland (d) and the gills (e). Control sections with the sense Cg-BigDef1 riboprobe were devoid of labeling (f). (Scale bars: 100 μm).

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Figure 5. Reversed-phase HPLC of gill acid extracts from unchallenged (black line) and immune-challenged oysters analyzed 12 h (green line), 24 h (blue line), and 48 h (red line) after an injection of heat-killed bacteria. Elution was performed with a linear gradient of 0-60% of acetonitrile in acidified water over 40 minutes at a flow rate of 0.6 ml/min (grey line). Absorbance was monitored at 214 nm. One fraction eluted with 34% acetonitrile (arrow) showed an increased absorbance over the time course. Ions compatible with the mass of a double-charged Cg-BigDef1 (asterisks) were identified by MALDI-TOF MS in this fraction only. They were found in challenged oysters at 12, 24 and 48 h (see m/z values) but not in unchallenged oysters.

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A. irridans was highly induced in circulating hemocytes after V. anguillarum challenge [12]. Cg-BigDef1 and -2 are the first AMPs found to be strongly induced in oyster hemocytes apart from the bactericidal/permeability-increasing protein, Cg-BPI [31]. Since no increase of Cg-BigDef transcript levels was evidenced following tissue injury (injection of sea water), the expression of the Cg-BigDef1 and -2 is likely induced by PAMPs (Pathogen-Associated Molecular Patterns). Interestingly, unlike Cg-BigDef1 and -2, Cg-BigDef3 gene expression seems to be constitutive and non-regulated in oyster hemocytes. This is similar to the regulation of oyster defensins (Cg-Defs) gene expression [15]. Future characterization of promoter regions of the three Cg-bigdef genomic sequences should help understanding their different expression patterns. In this study we also observed an unexplained individual variability of expression of Cg-BigDefs (mainly for Cg-BigDef3), which was not observed for Cg-Defs [15], and will require further attention.

Noteworthy, the differential regulation of Cg-BigDefs was observed in one same tissue, the hemocytes, as shown by in situ hybridization. Indeed, 24 h after Vibrio injection, only Cg-BigDef1 transcripts were seen to be induced in hemocytes as evidenced by the great increase in hemocyte labeling intensity and number of positive cells in tissues, compared to unchallenged oysters (Figure 4). No other tissues than hemocytes were found to express Cg-BigDefs. This contrasts with the Cg-BPI which is not only expressed in hemocytes but also in epithelia, where its expression is not regulated in response to a microbial challenge [31]. Interestingly, in the hard clam M. mercenaria, transcript levels of a big defensin homolog have been reported to increase in the mantle and gills after challenge with the protozoan parasite QPX (Quahog Parasite Unknown) [9], but probably following massive hemocyte infiltration of these tissues.

To our knowledge, this is the first report in a mollusk of an AMP family whose members are differentially regulated in hemocytes upon bacterial challenge. Like Cg-BigDefs, human β-defensins follow different regulation patterns in the intestinal epithelium. Indeed, while HBD-1 is constitutively expressed, HBD-2 is regulated upon bacterial infection [32]. Similar to the vertebrate intestinal tract, oyster tissues are densely populated by diverse microbial communities dominated by Vibrio species [33]. Thus, tight mechanisms of regulation must be established in the antimicrobial defenses for maintaining homeostasis and controlling infections [34]. Big defensins have a broad spectrum of antimicrobial activities that affects Vibrio species [2,12], which are common bacterial pathogens for oysters [21,35]. Therefore, we can assume that AMPs constitutively expressed as defensins and Cg-BigDef3 may represent a watchful waiting line of defense whereas AMPs transcriptionally regulated such as Cg-BigDef1 and -2 may intervene upon microbial invasion.

Our HPLC and MS data strongly suggested that the mature form of native Cg-BigDef1 results from cleavage of both the signal peptide and the propeptide sequence, as well as the cyclization of the resulting N-terminal glutamine residue into a pyroglutamic acid. This last modification, commonly reported in bioactive peptides/proteins, may contribute to the stabilization of the mature peptide and resistance to proteolysis [35]. That ions with a m/z value compatible with Cg-BigDef1 were only found in challenged oysters is consistent with the transcriptional regulation of Cg-BigDef1. In our acid extracts of oyster tissues, we could not observe any ion compatible with further cleavage of Cg-BigDef1 in two domains, namely the N-terminal hydrophobic domain and the C-terminal cationic domain. Altogether, this strongly suggests that mature Cg-BigDef1 is composed by both domains, a structure which was shown to provide wide antimicrobial activities and multifunctional properties to the horseshoe crab big defensin [2]. Interestingly, the C-terminal domain of big defensins present six cysteine residues whose arrangement is similar to those observed in vertebrate β-defensins [3], suggesting a phylogenetic relationship between β-defensin and big defensin peptides. Importantly, we show here for the first time that the hydrophobic N-terminal region and the C-terminal β-defensin-like domain are located in separated exons, a genomic architecture that has also been reported in multi-domain AMPs from crustaceans [37].

Finally, we showed that big defensins cluster in a separate clade distinct from other defensin families and closer to vertebrate defensins than to CSβ-containing defensins. This was concluded from the phylogenetic analysis of defensin sequences from a broad variety of species. All big defensins fell into one group. Only the clam big defensin VpBD [11], which does not display the conserved propeptide sequence observed in the big defensins, clustered with β-defensin-like peptides from fish (Figure 2b). However, in this clam species as well as in other bivalve mollusks including scallops and mussels we found several big defensins that properly clustered with oyster big defensins. Importantly, from our phylogenetic analysis, the big defensin family appears to be predominantly represented in mollusk species.

Conclusions
We show that big defensins represent in oyster a diverse multi-domain defense peptide family whose members are encoded by distinct genomic sequences and differentially regulated in hemocytes after bacterial challenge. These findings will help understanding the relationship and the respective implication of the constitutive and inducible antimicrobials in the oyster antimicrobial defense. In particular, attention must be paid on the diversity and variability of big defensin expression and their correlation with oyster survival capacities to pathogenic Vibrio infections.

Materials and Methods
Identification of big defensin homologues in oyster hemocytes by Digital Gene Expression (DGE) tag profiling
Crassostrea gigas DGE libraries were constructed with hemocyte mRNA precursors from non-infected and Vibrio-challenged oysters ([18], Rosa et al., in preparation). Briefly, a first DGE library (SVir DGE library) was constructed from pooled RNA samples of oysters Surviving infections with Virulent V. splendidus LGP32 [21] and V. aestuarianus LPf 02/41 [35], and a second library (SaVir DGE library) from individuals injected with the aVirulent V. splendidus-related strain V. tasmanianus LMG 20012T [22]. Hemocytes from Non-Infected animals were used for generating a control library (NInf DGE library). For each library, 7 μg of total RNA were used for sequence tag preparation using the Illumina’s Digital Gene Expression Tag Profiling Kit according to the manufacturer’s protocol (version 2.1B). Data from oyster DGE libraries were analyzed with BIOTAG software (Skuld-Tech, Montpellier, France) for tag detection, tag counting and for assessing DGE library quality, as described by Piquemal et al. [38]. About 14 millions of tags have been sequenced and 57,300 different tags have been identified, according to a level of sensibility established at 1 copy of mRNA per cell (1 copy for 300,000 molecules of mRNA). Obtained DGE tag signatures were matched against 29,745 unique transcribed sequences (7,940 contigs and 21,805 singletons) from different tissues of C. gigas (GigasDatabase; http://www.sigenae.org/aquafirst/). For tag to gene mapping, the virtual tags were extracted from all contigs and singletons. Only the full-length tags with 100% sequence identity
with oyster expressed sequence tags (EST) were assigned. EST-matched sequences from DGE libraries were analyzed for similarities using BLASTX at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov).

**Molecular cloning of oyster big defensins**

Three distinct EST sequences homologous to the big defensin antimicrobial peptide from *T. tridentatus* (GenBank: P80957) were identified in *C. gigas* cDNA libraries. Specific primers were then designed to confirm the nucleotide authenticity of each big defensin form identified in oysters (Table 2). Total RNA was extracted from oyster hemocytes using TRizol reagent (Invitrogen), treated with DNase 1 (Invitrogen) to eliminate contaminating genomic DNA and precipitated with 3 M sodium acetate. Following heat denaturation (70°C for 5 min), reverse transcription was performed using 1 μg of purified total RNA with 50 ng/μl oligo(dT)12–18 in a 20 μl reaction volume containing 1 mM dNTPs, 1 unit/μl of RNaseOUT Ribonuclease and 200 units/μl M-MLV reverse transcriptase in reverse transcriptase buffer according to the manufacturer’s instructions (Invitrogen). PCR reactions were carried out in a 25-μl reaction volume using 1 μl of synthesized complementary DNA (cDNA) as template. PCR conditions were as follows: 30 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min and a final elongation step of 72°C for 10 min. The amplification products were cloned and sequenced as described above.

**Table 2.** Nucleotide sequence of primers used in this study.

| Primer name | Forward primer (5'→3') | Reverse primer (5'→3') |
|-------------|------------------------|-----------------------|
| Primers for cDNA amplification | | |
| Cg-bigdef1 and -2 | TGTTAAGCTTATAGGACGTATC | GTAATCTCTGCTATACATAGT |
| Cg-bigdef3 | GGTAGTTGCCTGTTGCAAGAC | GGCTGATTAATCCATGCAAGC |
| Primers for genomic amplification | | |
| Cg-bigdef1g and -2g | AGCTTATAGGACGTATGAGG | GCCTGATTAATCCATGCAAGG |
| Cg-bigdef1g1 | GAGAAGACGTTATAGGCTGTCGTG | CCTGCATACAGTCATGAGG |
| Cg-bigdef1g2 | AGAAGAAGGTGAGACGACAG | TGCTGATTAATCCATGCAAG |
| Primers for quantitative real-time PCR | | |
| Cg-p40qt | AATCGTGCAGTTGCTGACAG | AATCAATCTCTGCTATGAG |
| Cg-bigdef1qt | TCTCCGTGCTTCTCTAATGG | GTCAATCTCTGCTATGAG |
| Cg-bigdef2qt | TCTCCGTGCTTCTCTAATGG | AATCAATCTCTGCTGACAG |
| Cg-bigdef3qt | AGAAGAAGGTGAGACGACAG | TGATCCGCACACACCAACC |
| Primers for in situ hybridization | | |
| Cg-bigdef1ish | TGTTAAGCTTATAGGACGTATC | GTAATCTCTGCTATACATAGT |
| Cg-bigdef2ish | AGAAGAAGGTGAGACGACAG | GACGTTGACGTATGACAG |

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**Partial gene characterization**

Genomic DNA (gDNA) was extracted from gills of five individual oysters using a standard phenol-chloroform extraction method followed by a treatment with RNase A (Invitrogen) and precipitation with 3 M sodium acetate. Genomic sequences for each oyster big defensin form were obtained by PCR amplification using gene-specific primers whose design was based on the cDNA sequences (Table 2). Amplifications were conducted in a final volume of 25 μl using 30 ng of gDNA under the following conditions: 10 min at 94°C, then 30 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 3 min and a final elongation step of 72°C for 10 min. PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gel and cloned and sequenced as described above.

**Oyster immune challenge and tissue collection**

Adult 2 year-old *C. gigas* oysters were purchased from a local oyster farm in Meze (Gulf of Lion, France) and kept in aquarium containing filtered sea water at 15°C at the Aquaculture Experimental Platform of IFREMER, Palavas-Les-Flots. The animals were fed twice with a mixture of live microalgae (*Nannochloropsis oculata*: 2.5×10^5 cells/ml; *Tetraselmis suecica*: 2.5×10^5 cells/ml) and bacterial mixture (*Microcosmus luteus*, *V. splendidus* and *V. anguillarum*) under 100 μl sterile sea water (SSW). Injection of SSW (100 μl) was used as control. Hemolymph was collected 12 h post-stimulation from the oyster posterior adductor muscle sinus using a 2 μl syringe equipped with a 23G-needle. Hemocytes were obtained by centrifugation (15 min, 1,000 xg, 4°C) of 3 hemolymph pools of 10 animals and directly processed for RNA extraction. Besides, gills from unchallenged and bacteria-challenged
oysters at 12, 24 and 48 h were harvested by dissection and immediately frozen and conserved in liquid nitrogen for biochemical detection of native Cg-BigDef. An experimental infection was further performed by injecting $5 \times 10^7$ CFU/animal of live oyster pathogen *V. splendidus* LMG 20012T. Unchallenged oysters (i.e. oyster at time 0 h) and oysters injected with 100 µl SSW were used as controls. Hemolymph was withdrawn 3, 8, 24, 48, 72 and 120 h post-injection and hemocytes were collected and pooled (3 pools of 10 individuals per conditions) for RNA extraction. Additionally, unchallenged animals and oysters at 24 h post-injection with *V. splendidus* LMG 20012 were sampled for in situ hybridization. All experimental infections were performed according to the IFREMER animal care guideline and policy.

Real-time quantitative PCR analysis

Total RNA extraction from oyster hemocytes and cDNA synthesis were performed as described above. Real-time quantitative PCR (qPCR) amplifications were performed in the LightCycler 480 (Roche) in a final volume of 6 µl containing 5 mM MgCl₂, 0.5 µM of each primer, 3 µl of reaction mix (LightCycler 480 SYBR Green I Master 2X) and 1 µl of each reverse transcribed RNA (diluted 1:19). Primer sequences are showed in Table 2. Each qPCR experiment was performed in triplicates and run under the following conditions: 95°C for 10 min; then 40 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 20 s and extension at 72°C for 25 s. Results are presented as changes in relative expression normalized with the *C. gigas* ribosomal protein L40 reference gene (*Cg-igpL40; GenBank: FP004478), using Pfaffl method [42]. Statistical significance was determined by Student’s t-test at $p<0.05$.

Analysis of *C. gigas* gill extracts by RP-HPLC and MALDI-TOF Mass Spectrometry

Native Cg-BigDef were searched in gills from unchallenged and stimulated oysters (10 animals per conditions). Frozen gills were ground to fine powder. Samples were then diluted in 10% acetic acid, homogenized, and left at 4°C for 20 min, before centrifugation at 8,000 x g (20 min at 4°C), and further purified with 0.05% trifluoroacetic acid (TFA). The RP-HPLC runs were carried out at 30°C on an Alliance system from Waters (Milford, MA, USA) coupled to a Photodiode array detector (Waters, Milford, USA). The separation was performed on an analytical C18 reversed-phase column (Vydac™, 218TP54, 4.6 x 250 mm, Protein – Peptide C18, 5 µm, Vydac Mojave, CA, USA). Extracts were purified using a linear gradient of 0–60% of acetonitrile in acidified water over 40 min at a flow rate of 0.6 ml/min. The column effluent was monitored for absorbance at 214 nm and the fractions were hand-collected following optical density, freeze-dried and conserved at $-20^\circ$C until use. Finally, all collected fractions were then analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) in a linear positive mode.

**In situ** hybridization

Tissues from 24 h *Vibrio*-challenged and unchallenged oysters were prepared for histology and **in situ** hybridization (ISH) analyses as described by Muñoz et al. [43]. Specific primers (Table 2) were used to prepare cDNA probes for the analysis of Cg-BigDef1 and Cg-BigDef3 mRNA in *in situ* expression. PCR products were cloned into a pCR®-Blunt II-TOPO® cloning vector that contains both Sp6 and T7 promoters. The recombinant plasmids containing Cg-BigDef1 or Cg-BigDef3 cDNA (GenBank: JF703143 and JF703149, respectively) were then used as templates for the preparation of the probes. Digoxigenin (DIG)-UTP-labelled antisense and sense riboprobes were generated from linearized cDNA plasmids by **in vitro** transcriptions using RNA labelling kits, T7 and SP6 RNA polymerases (Roche). DIG-labelled riboprobes were hybridized on oyster tissue sections as described previously [43]. Control consisted in replacing antisense riboprobes with sense riboprobes.

**Supporting Information**

**Figure S1** Nucleotide and deduced amino acid sequences (one letter code) of the three forms of big defensins from the oyster *Crassostrea gigas*: Cg-BigDef1 (a), Cg-BigDef2 (b) and Cg-BigDef3 (c). The predicted signal peptides are in bold and underlined. The putative propeptides are shadowed with grey background. Asterisks (*) mark the stop codon and the polyadenylation signals are double underlined. (TIF)

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**Author Contributions**

Conceived and designed the experiments: RDR EB. Performed the experiments: RDR AS JF PB DD-G EB. Analyzed the data: RDR PB DD-G EB. Contributed reagents/materials/analysis tools: PB EB. Wrote the paper: RDR PB DD-G EB.

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