
Molecular characterization of two isoforms of defensin from hemocytes of the oyster *Crassostrea gigas*

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Abstract:

Antimicrobial peptides (AMPs) are important components of the host innate immune response against microbial invasion. We previously characterized the first AMP from an oyster, a defensin, that was shown to be continuously expressed in the mantle of *Crassostrea gigas*. In this study, we report the cDNA cloning of two new isoforms of the defensin AMP family (Cg-defh1 and Cg-defh2) from the hemocytes of the oyster. The deduced amino acid sequences reveal two peptides of 73 amino acid residues with a mature portion consisting of 43 amino acid residues. Cg-Defh1 and Cg-Defh2 share 86% amino acid identity and belong to the "arthropod-molluscs defensin family". qRT-PCR analyses indicate that Cg-defh2 is continuously expressed in the hemocytes of *C. gigas*. In addition, after a bacterial challenge, the level of Cg-defh2 transcripts decreases dramatically in the circulating hemocyte population and this decrease can be correlated with an increase of Cg-defh2 transcripts in the gill and the mantle tissue, suggesting a possible migration of the hemocytes expressing Cg-defh2 towards the tissues implicated in the first defense barrier of the oyster. These results would suggest an important role of Cg-Defh2 in the oyster response to a microbial challenge.

Keywords: Mollusc bivalve; Marine invertebrate; Pacific oyster; Hemocytes; Antimicrobial peptide; Defensin

Introduction

It is widely known that antimicrobial peptides (AMPs) are major actors in innate immunity, conserved in evolution and present in all phyla of the living kingdom. These effectors present a great diversity in terms of structural features, biological properties and functions, and also in their tissue distribution and expression. [1, 2]. Defensins are compact cationic peptides, approximately 3-5 kDa in size, containing three or four disulphide bridges, and are active against a wide range of bacteria and fungi [3]. The vertebrate defensins can be grouped into three subfamilies, the α -defensins and β -defensins, which are distinguished on the basis of the connectivity of their six cysteine residues, and the cyclic θ -defensins [4]. In contrast with the classification of the vertebrate defensins based on their secondary structure, the grouping in clear distinct subfamilies of the invertebrate defensins is based on their biological properties, antibacterial *versus* antifungal [2]. Defensins are the most widespread family of invertebrate AMPs and more than 70 different defensins have been isolated in arthropods (insects, ticks, spiders, scorpions) and in molluscs [2]. In addition, the invertebrate defensins differ from the vertebrate defensins by their disulfide bridging [5].

To date, in molluscs, AMPs were only reported in the gastropod, *Dolabella auricularia* [6] and in the bivalves, the mussels *Mytilus edulis* [7] and *M. galloprovincialis* [8]. Based on a biochemical approach and molecular cloning, three groups of cationic cysteine-rich AMPs have been isolated from the hemocytes of unchallenged mussels, *M. galloprovincialis*: (i) defensin-like peptides, MGD-1 and MGD-2 [9]; (ii) myticins; and (iii) mytilins that have also eight cysteine residues but different specific cysteine arrays and amino acid sequences [10]. In the mussel, AMPs are only produced in hemocytes where they are stored and released following bacterial challenges [9]. Recently, two defensins from the oyster *Crassostrea virginica* [11] and *Crassostrea gigas* [12] were simultaneously discovered by two different groups. *C. virginica* defensin was purified from gill extract and contains 6 cysteines [11] whereas the defensin Cg-Def, from *C. gigas*, was isolated from mantle tissue using Expressed Sequence Tag approach and contains 8 cysteines [12]. These two defensins display sequence homology with members of the “arthropod-molluscs defensins family”. In our group, the *C. gigas* defensin from mantle has been fully characterized [12]. Cg-Def recombinant peptide was produced in *Escherichia coli* and displayed antibacterial activity. We observed that *Cg-def* gene is continuously expressed in the mantle. Additionally, the “CS $\alpha\beta$ type” 3D structure of Cg-Def was determined in aqueous solution by ^1H NMR spectroscopy and molecular modeling. In the present study, we report the cDNA cloning of two novel isoforms of defensin from *C. gigas* hemocytes and their gene expression analyses after bacterial challenge.

Materials and Methods

Animals, tissues collection and immune challenge

Adult oysters, *Crassostrea gigas* (3 years old; 12 cm), were purchased from a local oyster farm in Palavas (Gulf of Lion, France) and kept in sea water at 15°C. Oysters were stimulated by balneation with 5×10^8 bacteria/litre. The micro-organisms used for the challenge were the heat-killed bacterial strains *Micrococcus luteus*, *Vibrio splendidus* and *Vibrio anguillarum*. Hemolymph was collected at different times (24 and 48 h) from the pericardial cavity through the adductor muscle under an equal volume of anti-aggregant Modified Alsever Solution (27 mM Na-citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0) and immediately centrifuged at 700 g for 10 minutes (4°C) to separate the blood cells from plasma. After hemolymph collection, oyster tissues were harvested by dissection. Hemocytes and tissues (gills and mantle) were further treated according to different experimental procedures (see below). The experiments were done in triplicate and, to minimize individual variability, at least ten oysters were used in each experimental condition

RT-PCR and molecular cloning

Total RNA was extracted from the hemocytes of non stimulated *C. gigas* using Trizol reagent according to manufacturer's instructions (Invitrogen™) and treated with DNase Turbo (Ambion). Following heat denaturation (70°C for 5 min), reverse transcriptions was performed using 1 µg of total RNA prepared with 50 ng/µl oligo-(dT)₁₂₋₁₈ in a 50 µl reaction volume containing 1mM dNTPs, 1unit/µl of RnaseOUT (Invitrogen™) and 200 units/µl M-MLV reverse transcriptase in reverse transcriptase buffer. The cDNAs were amplified using primers Deffw 5' TTGCTGAGTAGAATGAAAGTATTCG 3' and Defrev, 5' CAGATTGCATAAAGATTTATTACACG 3' designed in the untranslated region of the cDNA sequence of *Cg-Def* (Genbank accession number CAJ19280) [12]. The amplification program consisted of 3 min at 95°C, followed by 34 cycles of 95°C for 30s, 55°C for 30s, 72°C for 30s min and a final elongation step of 72°C for 3 min. Amplified products were analyzed on 1,5 % agarose gels, cloned into pCR 2.1 TOPO TA cloning vector (Invitrogen™) and sequenced from both directions with T7 and T3 primers. The nucleotide sequences reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession numbers DQ400101 and DQ400102.

Sequence analysis

General homology searches were performed with the BLAST software on the NCBI home page (<http://www.ncbi.gov./Blast>). Deduced amino acid sequences were aligned by ClustalX (<http://www.ch.embnet.org/software/ClustalW.html>). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0. The tree was built by the Minimum Evolution method based on the alignment of the sequences using ClustalX (alignment was improved using the Seaview software). Bootstrap values (%) of 1000 replicates were calculated for each node of the consensus tree obtained.

Real Time PCR analyses

Real-time polymerase chain reaction (rtPCR) analysis was done to determine whether acute changes in *Cg-defh1* and *Cg-defh2* RNA abundance could be detected from hemocytes, gills and mantle tissues, sampled 24 and 48 hours post-stimulation. Pooled samples tissues total RNA (10 individuals per pool) were analysed from non stimulated and stimulated oysters at 24 and 48 hours post-challenge. For *Cg-defh1*, the forward and reverse primers were Defh1F (5'-AGTATTCGGACTTTTTACATTGGT-3') and Defh1R (5'-CCGCTCTACAACCGATGGATTG-3'). For *Cg-defh2*, the forward and reverse primers were Defh2F (5'-GTATTCGTA CTTCTTACATTAGC-3') and Defh2R (5'-GCTCTACAACCGATGGACCT-3'). The gene encoding the elongation factor (*e.f.*) was used as internal control. For *e.f.*, the forward and reverse primers were, EF (5'-ATGCACCAAGGCTGCACAGAAAG-3') and EFR (5'-TCCGACGTATTTCTTTGCGATGT-3'), respectively.

Total RNAs were treated with DNase (TURBO DNase, Ambion) to eliminate contaminating genomic DNA. The Dnase was removed by phenol chloroform extraction. First strand cDNA was synthesized using polydT primers (Oligo(dT)₁₂₋₁₈, Invitrogen). Reverse transcription was performed on 1 µg total RNA using the SUPERScript™ RNase H⁻ reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR amplifications were realized with the LightCycler™ (Roche) in the presence of SYBR-Green™ (Master SYBR Green™). Briefly, the following components were mixed to the indicated end-concentration: 5 mM MgCl₂, 0.5 µM of each primer, 1 µl of reaction mix (LC Fast Start Master SYBR Green I; Roche Diagnostics) in a final volume of 9.5 µl. Reverse transcribed RNA (0.5 µl) was added, as PCR template, to the LightCycler master mix and the following run protocol used: initial

denaturation at 95°C for 10 min; 95°C for 15 s; 62°C for 15 s; 72°C for 15 s with a single fluorescence measurement; melting curve program (73-95°C with a heating rate of 0.1°C per second and a continuous fluorescence measurement) and finally a cooling step to 30°C. For further expression level analysis, the crossing points (CP) were determined for each transcript using the LightCycler software. Specificity of RT-PCR product was analyzed on agarose gel and melting curve analysis. The copy ratio of each analyzed cDNA was determined as the mean of three replicates.

The relative expression ratio of and *Cg-defh1* was calculated based on the CP deviation of each RT-PCR product of RNA extracted from stimulated oyster *versus* the appropriate control sample (RT-PCR product of RNA from oyster non stimulated), and expressed in comparison to the reference gene (elongation factor, *e.f.*). The relative expression ratio of *Cg-def* was calculated based on the delta-delta method for comparing relative expression results defined as: $\text{ratio} = 2^{-[\Delta\text{CP}_{\text{sample}} - \Delta\text{CP}_{\text{control}}]} = 2^{-\Delta\Delta\text{CP}}$ [13].

In order to determine the expression pattern of *Cg-defh1* and *Cg-defh2* during bacterial challenge, two batches of oysters were prepared. In the first one, oysters were stimulated by bath with killed bacteria and in the second one, non-stimulated oysters (“naïve oyster”) were used as control. Then, qRT-PCR analyses were performed, with total RNA extract from hemocytes, mantle and gill, collected at two time points post-challenge (24 and 48 h).

Results and discussion

Characterization of two defensin cDNA from C. gigas hemocytes

Invertebrates rely solely on innate immune mechanisms that included both humoral and cellular response. Humoral immunity in marine invertebrates is characterized by antimicrobial agents present in the blood cells and plasma [14]. The involvement of AMPs in natural resistance to infection is sustained by their strategic location in phagocytes, in body fluids and at the epithelial level. We previously identified and characterized the first antimicrobial from the oyster *C. gigas*. *Cg-Def* defensin was isolated from *C. gigas* mantle tissue and was shown to be exclusively and continuously expressed in this tissue, suggesting a key role of this peptide in the antimicrobial defenses of the oyster by providing a first line of defense against colonization by pathogens [12]. In the present study, a PCR strategy was used to identify and clone two defensin isoforms from *C. gigas* hemocytes. PCR primers were designed from the 5' and 3'-untranslated regions of *Cg-def*. A single product of around 300bp was amplified by PCR. After cloning and sequencing, we obtained two different deduced amino acid sequences with 86% of identity and showing evident homology with the defensin AMP family. The full length nucleotide and amino-acid sequences of the two new isolated defensin isoforms from *C. gigas* hemocytes (*Cg-Defh1* and *Cg-Defh2*) are presented in Figure 1. The two cDNA sequences present a 195 bp region encoding a 65 amino acid peptide. The deduced amino acid sequence starts with a signal peptide and the cleavage site for signal peptidase is most likely located after the alanine residue preceding the glycine in position 23, as predicted by SignalP 3.0 software (data not shown).

The amino acid sequences of the mature peptides, *Cg-Defh1* and *Cg-Defh2*, were aligned with defensins from the “arthropod defensin family” available in GenBank, that contains defensins from arthropods and molluscs (Figure 2A). The position of the cysteines is well conserved, however, as observed with the defensins MGD-1 and MGD-2 from *M. galloprovincialis* hemocytes and *Cg-Def* from *C. gigas* mantle, *Cg-Defh1* and *Cg-Defh2* have four disulfide bonds. This additional bridge was proposed to render the peptide more stable in high osmolarity media such as in sea water [15]. In addition, it is noteworthy that, Seo et al., have recently isolated a defensin from the eastern oyster *C. virginica* and the corresponding 4265,0 Da peptide has 38 amino acids with only six cysteines [11]. *Cg-Defh1* and *Cg-Defh2* share 84 and 79% identity, respectively, with the mantle *Cg-Def* and both peptides show 51% identity with the *C. virginica* defensin. Less identity was observed with defensins from the

scorpion *Androctonus australis*, the dragonfly *Aeschna cyanea* and the tick *Dermacentor variabilis* (Figure 2A).

Phylogenetic tree was constructed with *Cg-Defh1* and *Cg-Defh2* amino acid sequences and the corresponding sequences from members of the invertebrate defensin family. The tree showed that the sequences were split into three clearly distinct groups (Figure 2B). The oyster defensins clustered together with mussel defensins and split up with the second group that comprises paleopteran insect (dragonfly) and arachnids (scorpion and tick) defensins. In addition, all defensins from neopteran insects clustered in the third group (Figure 2B) [16]. It is noteworthy that plectasin, the first fungal defensin described; shows a high degree of structural and sequence similarity to the defensins of invertebrates [17]. The similarity of plectasin to invertebrate defensins adds weight to a possible common genetic origin for the defensins of plants, fungi and protostome invertebrates. However, confirmation of this hypothesis will require the characterization of additional defensins from other fungi and/or invertebrates [17].

Real-Time PCR Analyses of Cg-def transcript levels after oyster bacterial challenges

qRT-PCR analyses were performed to determine the expression pattern of *Cg-defh1* and *Cg-defh2* in hemocytes, mantle and gill during bacterial challenge. qPCR analysis revealed that the amount of *Cg-defh1* transcript measured during the experiment was very low; then, it was difficult to measure acute expression profile pattern of *Cg-defh1* (data not shown). Concerning, *Cg-defh2*, qPCR analysis indicates that, in “naïve-oysters”, this defensin form is expressed continuously. However, significant decrease of *Cg-defh2* transcripts was evidenced in circulating hemocytes after a bacterial challenge. Indeed, very low level of *Cg-defh2* transcripts were detected 24 and 48 hours post bacterial challenge (Figure 3). Such results could explain why, in a previous EST project performed with RNA from hemocytes of oysters collected 12h post-bacterial challenge, we did not isolate any EST for defensins [18].

In addition, further investigation of the effects of the challenge on the tissue distribution of *Cg-defh2* revealed an increase of the level of *Cg-defh2* transcripts in the oyster mantle and gill, at 24 and 48 hours post-challenge (Figure 3). Because as for all experiments, the temperature melting curve and the size of the qPCR products were identical, we can assume that, we detected in the mantle and the gill tissues, transcripts of the same defensin expressed from hemocytes. These results would suggest that, after a challenge, the hemocyte population that expresses *Cg-defh2* could migrate towards the tissues implicated as a first defense barrier of the oyster. This pattern of expression is the same as the one observed for the defensin MGD-1 in the mussel *M. galloprovincialis* after a bacterial injection where hemocytes have been shown to concentrate at the site of injection [9]. Our data also corroborate the results obtained with the *C. virginica* defensin [11]. Indeed, this peptide was originally purified from the gills of the oyster but the authors proposed that they might originate from circulating hemocytes that are abundant in gill filaments [11]. Thus, it appears that in molluscs, defensin genes are not regulated as in insects, where the expression of the genes encoding antibacterial peptides is induced after bacterial challenge [19]. Consequently, the decrease of messenger concentration observed from the hemocytes could be due to an under-representative of the *Cg-defh2* producing cells in the circulation. Further studies investigating the ratio of the cells expressing *Cg-defh2*, after challenge, in the total population of circulating hemocytes are necessary to confirm this hypothesis.

Conclusion

The data in this report, which represent the first characterization of an antibacterial peptide from the hemocytes of an oyster, indicate a close relationship between the oyster defensins from hemocytes (*Cg-Defh1*, *Cg-Defh2*) and mantle (*Cg-Def*). Additional investigations of oyster immune peptides and their expression are underway to better understand

the role of these AMPs in the oyster immune response. However, preliminary results would suggest that the different isoforms of the defensins, *Cg-Defh1*, *Cg-Defh2* from hemocytes and *Cg-Def* from mantle could be detected in one individual. Further studies on the influence of microbial challenge on peptide production and localisation, together with a determination of their complete activity spectra will permit us to elucidate the reason for such diversity.

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Cg-Defh1

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ttgctgagtagaatgaaagtattcggactttttacattggtcggttctcctgatggtttct
      X X X X X L F T L V V L L M V S 16
gcagatatggcttttgcctggatttgggtgtccgctgaccagtataagtgtaacagtcac
  A D M A F A G F G C P R D Q Y K C N S H 36
tgccaatccatcgggttagagcgggctactgtgacgcagtcacactctggttaagatgt
  C Q S I G C R A G Y C D A V T L W L R C 56
acttgtaccgattgtaatggaaagaagtaagatctttcaagctgaataaaccagtcctga
  T C T D C N G K K * 65
atctgaccaagaactaaacgtgtaataaatctttatgcaatctg
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(A)

Cg-Defh2

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ttgctgagtagaatgaaagtattcgtacttcttacattagccggttcttctgatggtttct
      X X X X X L L T L A V L L M V S 16
gcagacatggctttcgcctggatttgggtgtccgggtgaccagtatgagtgtaaccgtcac
  A D M A F A G F G C P G D Q Y E C N R H 36
tgcaggccatcgggttagagcgggctactgtgacgcagtcacgctctggttaagatgt
  C R S I G C R A G Y C D A V T L W L R C 56
acttgtaccgggtgtagtggaaagaagtaagatcgttcaagctgaattgaccagtcctga
  T C T G C S G K K * 65
atctgaccaagaactaaacgtgtaataaatctttatgcaatctg
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(B)

Cg-Defh1

G	F	G	C	P	R	D	Q	Y	K	C	N	S	H	C	S	I	G	C	R	A	G	Y	C	D	A	V	T	L	W	L	R	C	T	C	T	D	C	N	G	K	K
G	F	G	C	P	G	D	Q	Y	E	C	N	R	H	C	S	I	G	C	R	A	G	Y	C	D	A	V	T	L	W	L	R	C	T	G	C	S	G	K	K		

Cg-Defh2

(C)

Fig. 1. Nucleotide and deduced amino acid sequences of Cg-Defh1 (A) and Cg-Defh2 (B) cDNAs and alignment of mature defensins (C). (A, B) The deduced amino acid sequences of the ORF are indicated above the nucleotide sequence. Asterisks indicate the stop codons. Amino acids are numbered on the right. The incomplete sequences of signal peptides are underlined and mature peptides are indicated in bold letters. The sequences of the primers used for Cg-defh1 and Cg-defh2 cDNA cloning are in italics. (C) Cysteines are in bold and identical residues are boxed.

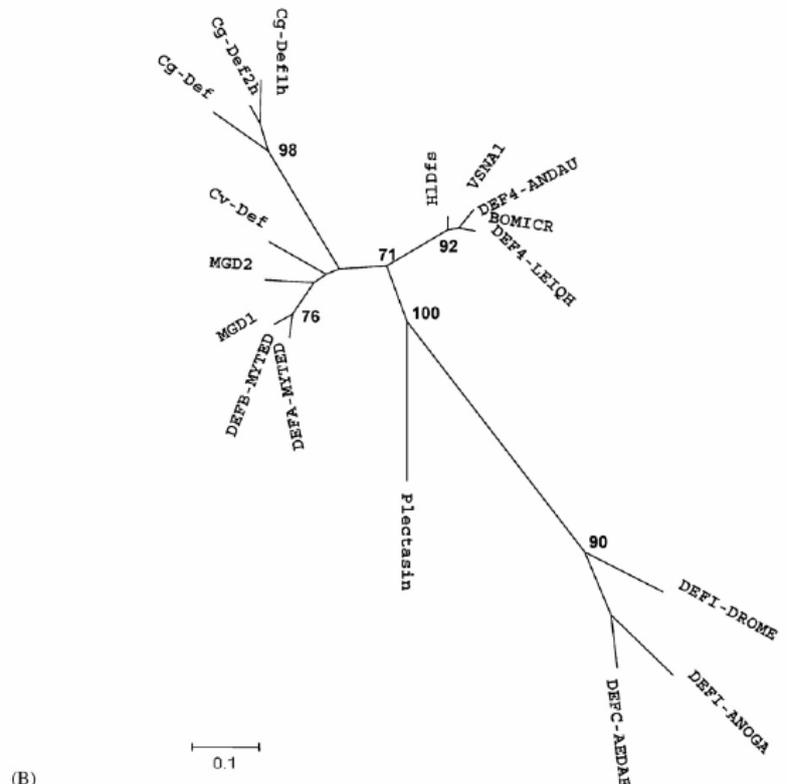
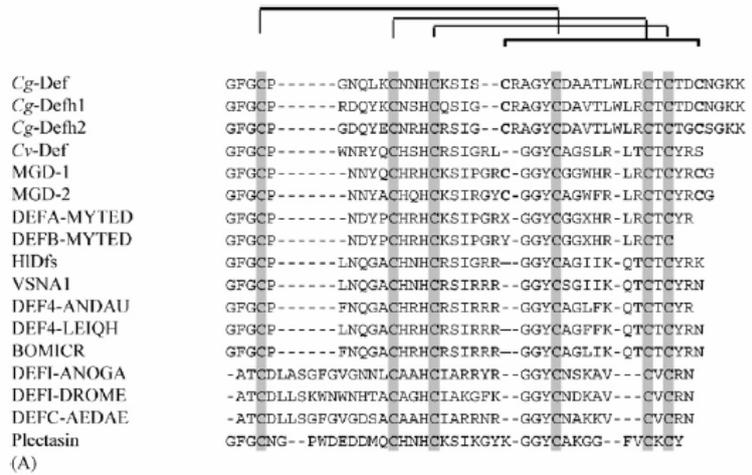


Figure 2. (A) Sequence alignment of defensins from arthropods and molluscs. Defensin obtained from hemocytes were alignment with others invertebrate defensins. Conserved cysteine residues are shaded. The two additional cysteine residues found in the *Cg-Def* mantle, *Cg-Def* A and B, MGD-1 and MGD-2 are in bold. The disulfide bonds are indicated. (B) Phylogenetic tree of invertebrate defensins. The tree was generated using an alignment made using CLUSTAL X. From this alignment a distance based phylogenetic tree was constructed using the Minimum Evolution method of the MEGA package. The bootstrap values of the branches are indicated. Oyster *Crassostrea gigas* (*Cg-Def* [AJ565499], *Cg-Defh1* [DQ400101] and *Cg-Defh2* [DQ400102]) and *Crassostrea virginica* (*Cv-Def*, [11]); mussels *Mytilus edulis* (*DEFA-MYTED*, [P81610], *DEFB-MYTED*, [P81611]) and *M. galloprovincialis* (*MGD-1*, [P80571]; *MGD-2*, [AAD52660]); tick *Dermacentor variabilis* (*varisin*, *VSNA1*, [AAO24323]), scorpion *Androctonus australis* (*DEF4-ANDAU*, [P56686]), the african malaria mosquito *Anopheles gambiae* (*DEFI-ANOGA*, [Q17027]), fruit fly *Drosophila melanogaster* (*DEFI-DROME*, [P36192]) and yellowfever mosquito *Aedes aegypti* (*DEFC-AEDAE*, [P81603]). *Haemaphysalis longicornis* (*HIDfs*, [BAD93183]), *Boophilus microplus* (*BOMICR*, [AAO48943]), *Leiurus quinquestriatus* (*DEF4-LEIQH*, [P41965]) and the saprophytic fungus *Pseudoplectanina nigrella* (*plectasin*, [CAI83768]). The accession numbers are in brackets.

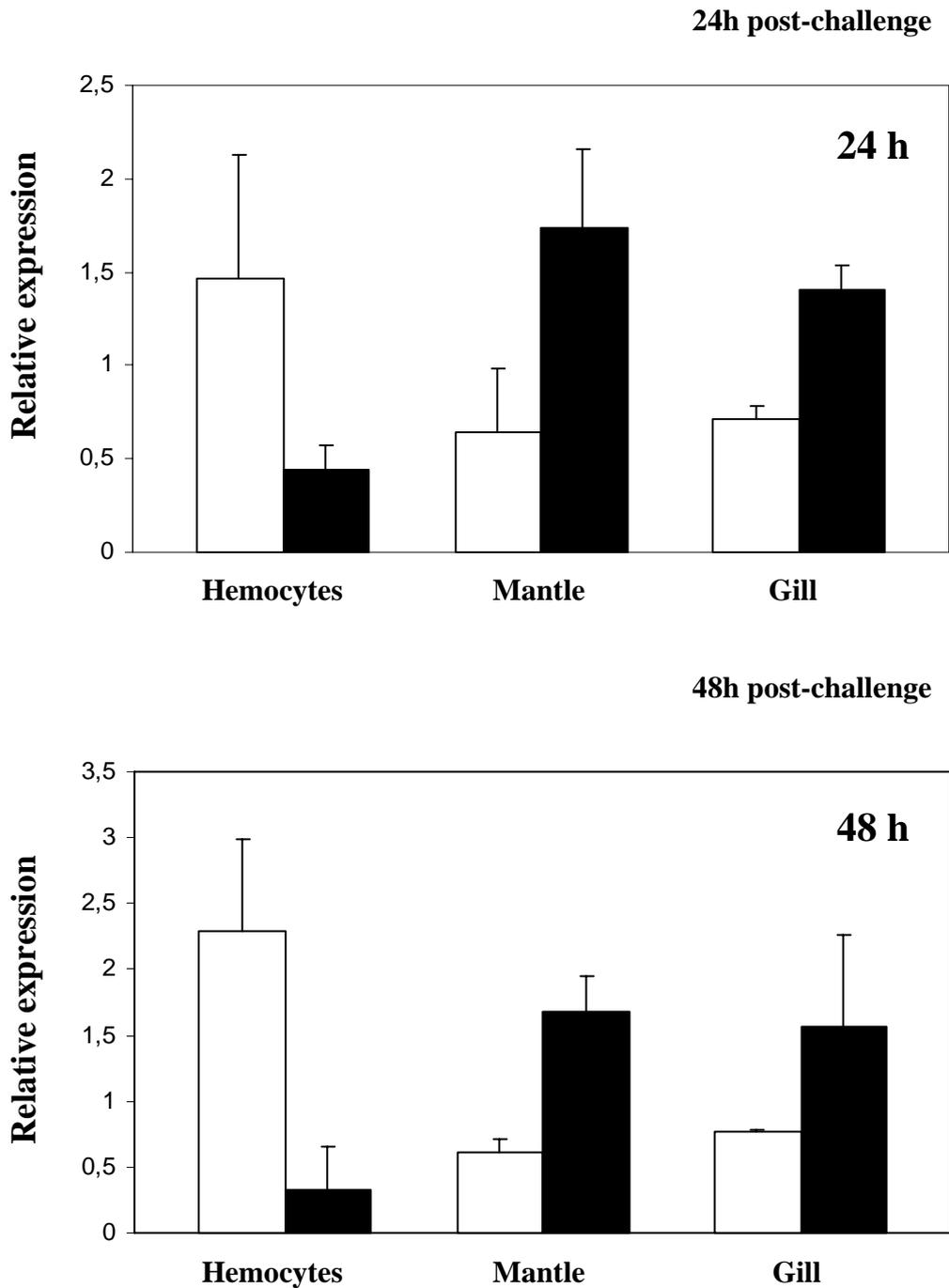


Figure 3. Expression of *Cg-defh2* mRNA analyzed by real time quantitative RT-PCR. *Cg-defh2* mRNA expression was measured in hemocytes, mantle and gills following bacterial challenge. Results are means \pm SE of three independent experiments realized on a pool of ten oysters from non-stimulated (white) and stimulated (black) oysters, at 24 and 48 h post-infection. Bars represent the relative *Cg-defh2* transcript levels normalized to elongation factor (*ef.*) transcript levels, as described in Materials and Methods. The $\Delta\Delta CP$ values for each experimental condition were obtained by subtraction of mean of all ΔCP s (relative expression of the mean = 1).