First evidence of the activation of Cg-timp, an immune response component of pacific oysters, through a damage-associated molecular pattern pathway

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Abstract:

In a previous work, we characterized a Crassostrea gigas cDNA (Cg-timp) encoding a protein which presents all the features of vertebrate tissue inhibitor of metalloproteinase (TIMP). The expression pattern of this gene led us to propose that Cg-timp is an important factor in oyster wound healing and defense mechanisms. Here we describe the analysis of Cg-timp expression in oysters challenged by live or dead bacteria as well as by bacterial secretory/excretory products and metalloproteinase. Surprisingly, bacterial secretory/excretory products activate Cg-timp gene expression whereas heat-inactivated ones do not. To address the question of the signal transduction pathway involved in Cg-timp gene activation, we isolated and sequenced Cg-timp promoter and upstream region. A 1-kb genomic DNA fragment flanking the 5′-end of the gene contains several regulatory elements and notably three NF-κB binding sites. The potential involvement of these motifs in Cg-timp gene regulation is discussed.

Keywords: Oyster; Crassostrea gigas; Mollusk immunity; Metalloproteinase; Tissue inhibitor of metalloproteinase; Damage-associated molecular pattern

Abbreviations: AP-1, activator protein 1; DAMP, damage-associated molecular pattern; LPS, lipopolysaccharide; TIMP, tissue inhibitor of metalloproteinase
1. Introduction

The vast majority of the cells that constitute a tissue or organ are in constant contact with each other and/or with the extracellular matrix (ECM). The ECM is a complex structure composed of many macromolecules and serves as a structural support media for the cells it surrounds. The interaction between the cells and the ECM, which is dynamic and multi-facet, thus influences cell shape, behavior, and ultimately the cell fate [1,2]. Timely degradation of the ECM is therefore crucial for controlling cellular differentiation, migration or growth that are required during diverse tissue remodeling events of embryonic and postnatal development, and in adult remodeling processes such as wound repair and angiogenesis [3]. The major enzymes that are involved in these processes are members of a family of zinc-dependant endopeptidases that catalyze the turnover of ECM components, the matrix metalloproteinase (MMP) family [4]. The activities of MMPs are tightly controlled by endogenous inhibitors called TIMPs (Tissue Inhibitors of Metalloproteinase) [5]. The local balance between MMPs and TIMPs is believed to play a major role in extracellular matrix remodeling, thus in a wide range of physiological processes.

The ECM components also serve as docking sites for microbial pathogen invasion [6]. Indeed, many molecules produced by microorganisms have been identified for their involvement in ECM component modifications. It is thought that these modifications are the essential initial events in the pathogenesis of many infections. Bacterial proteases, for instance, may directly activate latent MMP or inactivate protease inhibitors, and thus contribute to tissue damage and bacterial spread across tissue barriers [7,8].

Oyster populations are subjected to numerous infectious diseases caused by viruses [9], bacteria [10-12] or protozoan parasites [13,14]. Investigations realized on the pathogenic mechanisms of these infections led researchers to point out the role played by proteases. For instance, studies of the eastern oyster pathogen Perkinsus marinus showed that this protozoan produces extracellular proteases (ECP) which compromise oyster immune response [15] and enhance the protozoan’s ability to propagate within its host [16,17].

Several years ago, while searching for genes involved in oyster immunity, we isolated a cDNA encoding a protein which presents all the features of vertebrate TIMPs. We demonstrated that the gene encoding the oyster TIMP, Cg-timp, is only expressed in hemocytes [18]. Mollusk hemocytes are phagocytic cells involved in many functions including wound healing, shell repair, as well as internal defenses [19]. We also observed that Cg-timp mRNAs were accumulated in hemocytes of oysters subjected to shell damage or bacterial challenge, strongly suggesting the involvement of Cg-timp in wound healing as well as in defense mechanisms [18].

To further progress in the understanding of mechanisms regulating Cg-timp gene expression, we undertook the characterization of (i) the bacterial compounds involved in these processes and (ii) the promoter upstream region of the Cg-timp gene. We observed that Cg-timp mRNA accumulation in oyster hemocytes was indiscriminately induced by proteases produced by Gram-negative bacteria or extracted from a Gram-positive bacterium. These results strongly suggest that Cg-timp activation, in response to infection, was (i) independent of a non-self recognition step of bacterial compounds and (ii) relied on host-tissue damage occurring during a pathogen attack. To address the question of the signal transduction pathway involved in Cg-timp gene activation, we sequenced its promoter and upstream region. We identified two groups of DNA binding motifs which, in other organisms, play a role in conditional expression (wound healing for AP-1 or immune response for κB motif). The implication of these DNA binding motifs in Cg-timp gene expression is discussed.
2. Materials and methods

2.1 Animals, bacterial growth and immune challenge

Three- to four-year old oysters (Crassostrea gigas Thunberg) were collected from a commercial farm (Palavas, Gulf of Lion, France) and kept in sea water at 15°C. To minimize individual variability, at least 10 oysters were used in each experimental condition. Oysters were challenged by filing the shell and injecting into the adductor muscle either 100 µl of bacteria culture medium (saline peptone water, SPW) or 100 µl of a mixture of four pathogenic Vibrio strains (V. anguillarum, V. metshnikovii, V. tubiashii and V. S322 grown separately overnight at 24°C in SPW) or 100 µl of Vibrio culture supernatant filtered through 0.2 µm filters. Concentration of bacterial cultures was calculated from the optical density at 550 nm (1 unit OD550 corresponds to 5 x 10^8 bacteria/ml). Bacteria were collected by centrifugation (10000 g, 5 min), washed twice and resuspended in fresh SPW. Oysters were also challenged with Bacillus polymixa metalloproteinase (Sigma P6141) dissolved in TNC (50 mM Tris HCl pH 7.5, NaCl 200mM, CaCl2 5 mM). After injection, oysters were placed back to sea water tanks for 12 hours. Duration of immune challenge was chosen according to previous results [18].

2.2 RNA isolation and analysis

Hemolymph from challenged and unchallenged oysters was collected with sterile syringes from the pericardial cavity through the adductor muscle and immediately centrifuged at 1000 g for 10 minutes at 4°C. Hemocyte pellets were resuspended in Trizol™ reagent (1 ml of reagent per 10^7 cells) and total RNAs were extracted following manufacturer’s instructions. Total RNAs (10 µg per lane) were separated by denaturing 1.2 % agarose/formaldehyde gel electrophoresis, and blotted onto Hybond-N membranes (Amersham Pharmacia Biotech). RNAs were visualized by staining the membranes with methylene blue. Membranes were prehybridized for two hours at 65°C in 50% formamide, 5 X SSC (750 mM NaCl, 75 mM Na3 citrate 2H2O), 8 X Denhardt's (2% BSA, 2% Ficol, 2% PVP in 3x SSPE), 50 mM NaH2PO4 pH 6.5, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridizations were carried out overnight at 42°C in the same buffer containing the radiolabeled probe. To produce the full length Cg-timp probe, we used the 1301 bp cDNA fragment coming from the hydrolysis of Cg-timp phagemid by restriction enzymes EcoRI and XhoI. Ribosomal RNA probe was prepared by PCR as previously described [20]. The cDNA and PCR products were purified and radio-labeled ([α-32P] dCTP) using the random priming kit from Invitrogen™. After hybridization, membranes were washed twice for 15 min at room temperature in 2 X SSC containing 0.1% SDS and twice for 20 min at 60°C in 1 X SSC containing 0.1% SDS. Finally membranes were exposed to auto radiographic films (KODAK X-OMAT). Signal intensities were quantified with the Storm system technology from Molecular Dynamics.

2.3 Zymography detection of proteolytic activity of Vibrio culture supernatants.

Culture supernatants of the four Vibrio strains were mixed, filtered through 0.2 µm filters, and inactivated at 100°C for different periods of time (as indicated on Fig. 2A). The samples were analyzed (16 µl/lane) by SDS-PAGE (12%) containing 0.1% type A gelatin (Sigma G2500). After electrophoresis, gels were washed for 2 hours at room temperature in 2.5% triton X-100, to allow protein refolding (SDS removal), and incubated overnight at 37°C in 50 mM Tris HCl, pH 7.5, 200 mM NaCl, and 5mM CaCl2. Finally, gels were stained with Coomassie brilliant blue and destained with methanol/ acetic acid.
2.4 Real Time PCR Assay

To compare Cg-timp gene expression between challenged and unchallenged oysters, the relative abundance of Cg-timp mRNA was normalized to the amount of an endogenous reference, the gene encoding elongation factor 1 (e.f) [21].

Primer design and Reverse Transcription: For Cg-timp the forward and reverse primers were TimpF (5’-TGAGGCGATACACTTCTCTTATT-3’) and TimpR (5’-ACCCCTTGAATATGTCTCTTTCTT-3’) respectively. For e.f. the forward and reverse primers were EFF (5’-AGTCACCAAGGCTGCACAGAAG-3’) and EFR (5’-TCCGACGTTTCTTGTGCGATGT-3’) respectively.

LightCycler real-time PCR: First strand cDNA was synthesized with polydT primers (Oligo(dT)12-18, Invitrogen). Reverse transcription was performed on 1 µg total RNA using 200 units of SUPERScript™ RNase H’ reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. cDNAs were diluted 2.5 times in H2O and kept at -20°C until use. PCR amplifications were realized with a LightCycler™ (Roche) in the presence of SYBR-Green™ (Master SYBR Green™, Roche). 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) and 0.5 µl diluted reverse transcribed RNA, in a final volume of 10 µl. PCR conditions were as follow: initial denaturation at 95°C for 8 min; 40 cycles of denaturation at 94°C for 15 s, annealing at 62°C for 5 s and elongation 72°C for 10 s with a single fluorescence measurement; melting curve analysis (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 expression level analysis, the crossing points (CP) were determined for each transcript using the LightCycler software. Specificity of RT-PCR products were analyzed by agarose gel electrophoresis and melting curve analysis. The copy ratio of each analyzed cDNA was calculated as the mean of three replicates.

Relative quantification: The relative expression ratio of Cg-timp was calculated based on the CP deviation of each RT-PCR product of RNA extracted from stimulated oyster versus the appropriate control sample, and expressed in comparison to the reference gene e.f. The relative expression ratio of Cg-timp was calculated based on the delta-delta method for comparing relative expression results [22].

2.5 Screening of an oyster genomic library.

A Crassostrea gigas genomic DNA library, constructed in the λ Dash II vector (Stratagene®) and kindly provided by Pr. P. Favrel (University of Caen, France) was screened with radiolabeled Cg-timp cDNA. Hybridizations of nylon filters (Amersham Hybond-N) were performed overnight at 65°C in 5X Denhardt’s, 0.1% SDS, 5 X SSPE (3 M NaCl, 0.2 M NaH2PO4, 20 mM EDTA, pH 7.0), and 100 µg/ml denatured salmon sperm DNA. Filters were first rinsed in 2 X SSC, 0.1% SDS at room temperature, then washed twice at 65°C for 15 min in each of the following solutions: 1 X SSC, 0.1% SDS; 0.2 X SSC, 0.1% SDS, and 0.1 X SSC, 0.1% SDS. Over the 200.000 plaque-forming units that were screened, 6 hybridized with the Cg-timp probe. They were fully isolated after three successive rounds of screening. After Southern blot analysis of the six phages, genomic fragments containing Cg-timp were cloned into plasmidic vectors and sequences determined on both strands.
3. Results

3.1 Cg-timp expression was mainly induced by secretory/excretory compounds produced by bacteria.

In a previous work, we showed that Cg-timp expression was induced by bacterial challenge, suggesting its involvement in defense mechanisms [18]. To further characterize the compounds responsible for this gene induction, Northern blot analyses were carried out using total RNAs extracted from hemocytes of oysters subjected to different challenges. When oyster shells were filed or when oysters were injected with SPW, we observed a faint accumulation of Cg-timp RNAs in comparison with untreated oysters (Fig. 1, lanes C, F and SPW). This Cg-timp RNA accumulation was probably due to damages inflicted to the shell during the filing and to the adductor muscle during injection. In oysters challenged with Vibrio culture supernatants or live Vibrio, Cg-timp RNAs were accumulated by a factor of 2.7 and 3.3 respectively, in comparison to oysters challenged with culture medium (Fig. 1, lanes Sup and L over lane SPW). In contrast, oysters challenged with heat killed bacteria (15 min at 100°C), demonstrated a weaker Cg-timp RNAs accumulation (1.6 fold) (Fig. 1, lane HK over lane SPW). These results suggest that Cg-timp RNA accumulations are not triggered by bacterial cell wall compounds (still present in heat-killed bacteria), but instead by secretory/excretory substances produced by live bacteria and released in the culture medium.

3.2 Proteases secreted by Gram negative bacteria were able to trigger Cg-timp mRNA accumulation.

Proteases produced by microorganisms have been largely described as part of the toxic factors used by pathogens to invade the host [8]. To investigate the production of proteases by the four Vibrio strains used in this study, culture supernatants were analyzed by zymography on SDS-PAGE. We observed that the four supernatants contained protease activities that could be inactivated by chelating agents like EDTA, suggesting that these proteases belong to the metalloproteinase family (data not shown). To investigate if these proteases may be involved in Cg-timp gene activation, the mixture of the four bacterial culture supernatants was filtered to eliminate all bacteria, and heat inactivated. Effectiveness of proteases heat inactivation was tested by zymography analyses (Fig. 2A). Surprisingly, proteases produced by the Vibrio strains were very stable since a residual protease activity was still detected in the supernatant after a two-hour treatment at 100°C.

Then, culture medium, untreated and heat-inactivated supernatants were used to challenge oysters. Total RNAs were extracted from hemocytes and Cg-timp RNA accumulation was measured by real-time PCR. In oysters challenged by untreated supernatants, we observed a 3.6-fold increase in Cg-timp mRNAs compared to oysters challenged by culture medium. Conversely, in oysters challenged by heat inactivated supernatants, Cg-timp transcripts increased only by a factor of 1.6 (Fig. 2B). These results strongly suggest that proteases produced by bacteria and secreted into the culture medium are involved in Cg-timp gene induction.

3.3 A metalloproteinase extracted from a Gram-positive bacterium was able to induce Cg-timp mRNA accumulation.

In order to confirm the role of proteases in Cg-timp gene induction, a challenge experiment was conducted using a commercial metalloproteinase purified from a Gram-positive bacterium (Bacillus polymixa) to avoid the risk of cross-contamination from LPS. Northern blot analyses were carried out on total RNAs extracted from challenged and unchallenged oysters. Seven batches of ten oysters were used: three of them as controls and the others were subjected to injection of increasing amounts of metalloproteinase. Once again,
when oyster shells were filed or when oysters were injected with the buffer used to dissolve the metalloproteinase (TCN buffer), we observed a faint accumulation of Cg-timp RNAs compared to untreated oysters (Fig. 3, lanes C, F and TCN). These accumulations were of the same order of magnitude than in the experiment previously described (Fig. 1) and were also probably due to shell damage and adductor muscle lesions.

When oysters were challenged with metalloproteinase, we observed a dose-dependant accumulation of Cg-timp transcripts (Fig.3). Compared to oysters challenged by TCN, Cg-timp accumulation ranged from 2.2 to 8.6 in oysters challenged by 10 ng to 10 µg of metalloproteinase, respectively. The induction efficiency of this metalloproteinase purified from Gram-positive bacteria demonstrates the implication of proteases in the induction of Cg-timp mRNA accumulation.

3.4 Structure of Cg-timp gene and promoter analysis.

Variation of mRNA abundance can originate from modifications of either mRNA stability or gene transcription rate. Analysis of 5’ and 3’ UTRs of Cg-timp mRNA, using the UTRResource web site (http://bighost.area.ba.cnr.it/BIG/UTRHome/), failed to reveal any known regulatory elements, suggesting that Cg-timp expression was regulated at the transcriptional level as vertebrate timp genes. To further progress in the understanding of regulation of Cg-timp expression, we undertook the characterization of the Cg-timp gene and its upstream region.

To isolate Cg-timp gene, we screened a C. gigas genomic library using the Cg-timp cDNA as a probe (as described in materials and methods). We isolated and sequenced a 4.5-kb genomic DNA fragment containing the Cg-timp gene and its 5’ upstream region (GenBank accession number AY780357). The gene consists of five exons and four introns spanning 3.5 kb (Fig. 4A). This genomic organization seems to be a common feature to timp genes since it is conserved in vertebrates and invertebrates [23,24].

The analysis of the 5’ upstream region of Cg-timp gene using the Eukaryotic Promoter Database (EPD; http://www.epd.isb-sib.ch) enabled us to localize putative TATA-box and GC-box (Fig. 4B). The position of the transcription start site (TSS, 26 nts downstream the TATA-box) was deduced from Cg-timp cDNA sequences obtained during an EST program [21]. Transcription factor consensus binding sites were identified within Cg-timp promoter using TSEARCH software (http://molsun1.cbrc.aist.go.jp/research/db/TSEARCH.html). Numerous families of DNA binding motifs were detected and we focused on two of them due to their implication in the regulation of mammalian timp gene (AP-1) or immune genes (κB-motifs) (Fig. 4B).

Two AP-1 (Activator Protein 1) binding sites were identified in the upstream region of the Cg-timp gene (positions: -1085 to -1079 and -530 to -524). The two binding sites are different, the first one (-1085 to -1079) is the consensus sequence (5’-TGACTAA-3’) whereas the second one (-530 to -524) is noncanonical (5’-TGAGTAA-3’). We performed the same analysis on the 5’ upstream region of the Drosophila Timp gene (BACR28B01) and identified two AP-1 binding sites which are also different. The first one (-1340 to -1334) corresponds to the consensus sequence (5’-TGACTAA-3’) whereas the second one (-972 to -966) is noncanonical (5’-TGACTAT-3’). Interestingly, AP-1 sites present in the proximal promoter region of mammalian Timp-1 genes are identical to the noncanonical Cg-timp AP1 binding site (5’-TGAGTAA-3’) and nevertheless critical for basal and inducible transcription [25].

The second family of DNA binding sites, κB-motifs, detected in Cg-timp upstream region corresponds to binding sites for Rel/NF-κB transcription factors known to be involved in immune response both in invertebrates and mammals [26]. Nucleotide sequences and positions of κB-motifs found in Cg-timp upstream region as well as their comparison with
consensus κB-motifs from insects and mammals are presented in table I. Interestingly, the κB-motif at position -679 to -670 strongly matched with insect and mammal consensus motifs. Sequence analysis of the upstream region of the Drosophila Timp gene (BACR2801) revealed the presence of three κB-motifs matching the insect consensus sequence. In vertebrates, the presence of κB binding sites in the promoter of Timp genes has only been reported once in the mouse timp-3 gene [27].

4. Discussion

In this study, we investigated the mechanisms involved in the activation of Cg-timp expression during a bacterial infection of the Pacific oyster Crassostrea gigas. We observed that the injection of Vibrio culture supernatants triggered Cg-timp mRNA accumulation as efficiently as live Vibrio did, whereas heat-killed bacteria were twice less efficient. These observations suggest that Cg-timp mRNA accumulation was induced by secretory/excretory molecules produced by Vibrio and probably not by bacterial cell wall compounds such as LPS (still present in heat-killed bacteria). Zymography analyses of Vibrio culture supernatants revealed the presence of proteases highly resistant to heat inactivation (protease activity still detected in supernatants after 2 hours at 100°C). Although a residual protease activity was still present in these supernatants, their ability to induce Cg-timp mRNA accumulation was reduced in comparison to non-inactivated supernatants. These observations could explain Cg-timp mRNA accumulation in oysters injected with heat-killed bacteria. In the latter experiment, bacteria were killed by 15 min incubation at 100°C, a treatment that does not fully inactivate bacterial proteases (Fig. 1 and 2A). These results seem to indicate that protease activity is involved in Cg-timp activation.

Proteases, and more precisely metalloproteinases, have been described as key components of bacterial infectivity [8]. They play a role in host tissue degradation to facilitate pathogen dissemination, but also in host immune effector inactivation (e.g., antimicrobial peptide degradation [28,29]). To further assess the involvement of metalloproteinases in Cg-timp mRNA accumulation, oysters were injected with a commercial metalloproteinase purified from Bacillus polymixa. We observed a dose-dependent increase in Cg-timp mRNA accumulation. As the metalloproteinase used in these experiments was purified from a Gram-positive bacterium, Cg-timp mRNA accumulation could not be attributed to contaminating LPS. In addition, injection of LPS (up to 1 µg per oyster) did not entail any Cg-timp transcripts accumulation (data not shown). Altogether, these results demonstrate the implication of proteases in the induction of Cg-timp mRNA accumulation.

Host defense mechanisms can be divided in three essential steps: detection of infecting microorganisms, activation of intracellular signaling pathways, and triggering of effector mechanisms. It is currently admitted that the first step relies on non-self recognition through a set of pattern-recognition receptors that bind conserved bacterial pathogen-associated molecular patterns (PAMPs) such as LPS, mannans or glycans [30]. In the oyster, we observed that Cg-timp mRNA accumulation in hemocytes was indiscriminately induced following the injection of proteases produced by Gram-negative bacteria or a metalloproteinase extracted from a Gram-positive bacterium. These results strongly suggested that Cg-timp activation, in response to infection, was independent of a non-self recognition step of bacterial compounds. This hypothesis was further sustained by the observation that shell damages or lesions of the adductor muscle during injection induced, to a lesser extent, Cg-timp mRNA accumulation. Moreover, oysters which are filter-feeding organisms with an open circulatory system, contain bacteria in their body fluids and soft tissues. Contrary to vertebrates or other invertebrates like arthropods or nematodes, the presence of bacteria in the oyster hemolymph seems to be a normal feature [31]. In this context, the activation of oyster
defense mechanisms would not be triggered by PAMPs, or at least not only by PAMPs; otherwise their defense mechanisms would be constitutively activated. Altogether, these results strongly suggest that Cg-timp gene expression might be triggered by damage-associated molecular patterns (DAMPs) as defined in the Matzinger damage model [32] [33], rather than by PAMPs.

In order to get first insights into the possible transcription factors involved in Cg-timp gene activation in response to DAMPs, we sequenced Cg-timp promoter and upstream region to identify the presence of transcription factor binding sites. We focused on two groups of DNA binding motifs which, in other organisms, play a role in conditional expression as wound healing, cell differentiation and proliferation (AP-1), or in immune response (κB-motif). The promoter-proximal region of mammalian Timp genes contains several AP-1 binding sites critical for basal and inducible transcription in these organisms [25,34,35]. Recent data emphasize the importance of Fos and Jun as AP-1 factors that induce Timp-1 expression through a TGF-β pathway [36]. In mammals, Jun is a major component of the transcription factor complex AP-1 which regulates the expression of multiple genes essential for cell proliferation, differentiation and apoptosis. TGF-β has been shown to have profound effects on extracellular matrix homeostasis, in part via its ability to alter the balance between proteinases and their inhibitors at the level of gene expression. Interestingly, an oyster Jun-like factor (GenBank accession number BQ426504) has been recently isolated and a TGF-β pathway characterized in the oyster [37]. The TGF-β homolog found in C. gigas, (mGDF) displays the characteristic features of the TGF-β superfamily, and it has been suggested that it plays a central role in the biological processes that allow larvae to become competent to metamorphose [38,39]. Concomitantly, we recently showed that Cg-timp transcripts were differentially expressed during oyster ontogenesis, and may thus play a role in tissue remodeling events during development and metamorphosis [40]. It would then be interesting to further investigate the correlation between TGF-β homolog and Cg-timp temporal expression patterns to further assess their interaction and the potential involvement of AP-1 in this pathway. Finally, it is reasonable to expect that the oyster TGF-β/AP-1 pathway would participate in the regulation of Cg-timp gene transcription. This would correlate Cg-timp gene expression with ECM remodeling events occurring during wound healing in adults and metamorphosis in larvae.

The Cg-timp promoter also contains κB-motifs that correspond to DNA binding sites of the Rel/NF-κB family of transcription factors who are structurally related and evolutionarily conserved DNA binding proteins [41]. Rel/NF-κB proteins mediate cellular responses to a wide variety of stimuli by regulating the expression of a large set of genes mainly involved in immune responses [42]. Over the past decade, the Rel/NF-κB signal transduction pathway appeared to be a key component of the immune response owing to its involvement in both the innate immunity in multicellular organisms and adaptive immunity in vertebrates [26]. In our search for genes involved in oyster immunity, we isolated six genes predicted to encode proteins similar to components of the Rel/NF-κB cascade. For two of these genes, we demonstrated that they encode proteins (oIKK and Cg-Rel) that are structurally and functionally homologous to their arthropod and mammalian counterparts [21,43,44]. These results led us to propose that the Rel/NF-κB pathway was also conserved in oyster. In agreement with this, a cDNA encoding a Toll-like receptor was recently isolated in this organism [45]. The presence of three κB-motifs in Cg-timp promoter suggests that Cg-timp gene expression might also be controlled by the oyster Rel/NF-κB pathway.

In conclusion, our data provide the first evidence in a mollusk that products synthesized by bacteria may trigger the expression of a component of the immune response, Cg-TIMP, through a DAMP pathway. Although the presence and high sequence homology of
binding sites for two known transcription factors was evidenced in Cg-timp promoter, their precise role in Cg-timp response to bacterial infection should be addressed by future investigations.

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**References**


Fig. 1. Cg-timp expression was mainly induced by secretory/excretory compounds produced by bacteria. Oysters were injected with saline peptone water (SPW), or supernatants from four Vibrio strain cultures (Sup.) or a mixture of four heat-killed (H.K.) or live (L) Vibrio strains. Two oyster batches were used as control: the first one corresponded to untreated oysters (C), and in the second one, a small notch was carved in the dorsal side of the oyster shell near the adductor muscle (F). Hemocytes were collected, RNAs extracted and analyzed by Northern blot. The presented data correspond to one representative experiment out of three independent replicates. A) Blots were successively hybridized with two radiolabeled probes, Cg-timp and ribosomal (rRNA) for RNA loading control. B) Signals obtained with the two probes were quantified using the Molecular Dynamics Storm system and Cg-timp mRNAs expression was normalized according to the level of rRNA and expressed as a function of the signal obtained for unchallenged oysters.
Fig. 2. Protease activities contained in bacteria culture supernatants were involved in Cg-timp gene activation. A) Supernatants of the four *Vibrio* strain cultures were incubated at 100°C during different periods of time and protease inactivation was analyzed by zymography on gelatin-containing SDS-PAGE. B) Real-time PCR analysis of Cg-timp mRNAs in oysters challenged with culture medium (SPW), untreated supernatant mixture (Sup.) or supernatant mixture heat-inactivated during two hours (Sup. H.I.). The presented data correspond to one representative experiment out of at least three independent replicates.
Fig. 3. Dose-dependant induction of Cg-timp gene by a metalloproteinase extracted from a Gram^+^ bacteria. Oysters were challenged with increasing amounts of Bacillus polymixa metalloproteinase dissolved in TNC. Three oyster batches were used as controls: the first one corresponds to untreated oysters (C); in the second one, a small notch was carved in the dorsal side of the oyster shell near the adductor muscle (F); and the third batch was injected with TNC. Hemocytes were collected, RNAs extracted and analyzed by Northern blot. Data presented correspond to one representative experiment out of three independent replicates. A) Blots were successively hybridized with two radiolabeled probes, Cg-timp and ribosomal (rRNA) for RNA loading control. B) Signals obtained with the two probes were quantified using the Molecular Dynamics Storm system and Cg-timp mRNAs expression was normalized according to the level of rRNA and expressed as a function of the signal obtained for unchallenged oysters.
Fig. 4. Organization of Cg-timp gene and nucleotide sequence of its promoter. A. Schematic representation of Cg-timp gene. Exons are represented by dotted rectangle whereas UTRs (5' and 3') and introns are represented by lines. B. Nucleotide sequence of the upstream region of Cg-timp gene (complete sequence available at GenBank, AY780357). Position of the nucleotides indicated on the right are numbered from the transcription start site (TSS, nt +1). The 5' UTR appears in italics (position +1 to +185) and the beginning of the first exon is in upper case (position +186 to +215). TATA-Box (-33 to -27) and GC-Box (-41 to -36) are boxed; consensus binding sites for transcription factors are in bold and underlined.
Table I
Comparison of κB-like motifs found in Cg-timp promoter with consensus κB-motifs from insects and mammals.

<table>
<thead>
<tr>
<th>Cg-timp motifs</th>
<th>Position from the TSS</th>
<th>Insect Consensus a</th>
<th>Mammal Consensus b</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGGCT T CGCC</td>
<td>−452 to −461</td>
<td>GGG-T T C-CC</td>
<td>GGG-T T C-CC</td>
</tr>
<tr>
<td>GGGAA G GTCA</td>
<td>−547 to −538</td>
<td>GGGAA - -TC-</td>
<td>GGGAA G -TC-</td>
</tr>
<tr>
<td>GGGTA T TTCC</td>
<td>−670 to −679</td>
<td>GGG-A T TTCC</td>
<td>GGG-A T TTCC</td>
</tr>
</tbody>
</table>

R: A/G  N: A/C/G/T  Y: C/T  W: A/T  Nucleotides different from consensus are replaced by (−).

TSS: Transcription start site. a According to Kappler and coworkers [46]. b According to Chen and coworkers [47].