Developmental & Comparative Immunology
Volume 31, Issue 1 , 2007, Pages 1-11
http://dx.doi.org/10.1016/j.dci.2006.04.002

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

Montagnani, C. [b], Avarre, J.C.[c], de Lorgeril, J. [a], Quiquand M. [a], Boulo, V. [a], Escoubas J.M.[d*]

[a] Génome, Populations, Interactions, Adaptation (GPIA), UMR5171 (IFREMER, CNRS, UMII) Université de Montpellier II, place Eugène Bataillon, CC80, 34095 Montpellier, France
[b] Present address : Centre Océanologique du Pacifique (IFREMER) - BP 7004, 98719 Taravao, Tahiti, Polynésie Française.
[c] Present address : Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), UMR 113 Campus International de Baillarguet, 34398 Montpellier Cedex 5.
[d] Present address: Écologie Microbienne des Insectes et Interactions Hôte-Pathogène (EMIP), UMR1133 (INRA, UMII) Université de Montpellier II, 2 place E. Bataillon, CC54, 34095 Montpellier, France

***** Corresponding author. Tel.: (33) 4 67 14 47 11; Fax: (33) 4 67 14 46 79.
E-mail address: Jean-Michel.Escoubas@univ-montp2.fr

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 OD_{550} 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, $CaCl_2$ 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 Na_3 citrate $2H_2O$), 8 X Denhardt's (2% BSA, 2% Ficol, 2% PVP in 3x SSPE), 50 mM NaH_2PO_4 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-^{32}P] 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 $CaCl_2$. 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'-TGAGGCAGTACAACTTCCTTCTATT-3') and TimpR (5'-ACCCCTTGAATATGTCTCTCTTCTT-3') respectively. For *e.f.* the forward and reverse primers were EFF (5'-AGTCACCAAGGCTGCACAGAAAG-3') and EFR (5'-TCCGACGTATTTCTTTGCGATGT-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 H_2O 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 $MgCl_2$, 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 radiolabled *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 NaH_2PO_4, 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 UTResource 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 TFSEARCH software (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.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 (eg 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-dependant 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 evolutionary 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.

Acknowledgements

We thank Pr. P. Favrel for providing oyster genomic library. We also thank M. Leroy for technical assistance. This work was supported by the CNRS (Centre National de la Recherche Scientifique), the IFREMER (Institut Français de Recherche pour l'Exploitation de la MER) and the University of Montpellier 2. C. Montagnani and J.-C. Avarre were respectively supported by grants from MENRT and MOREST (national project funded by IFREMER).

References

[1] Lin, C.Q. and Bissell, M.J. Multi-faceted regulation of cell differentiation by extracellular matrix. Faseb J. 1993; 7 (9): 737-43.

[2] Werb, Z. ECM and cell surface proteolysis: regulating cellular ecology. Cell 1997; 91 (4): 439-42.

[3] Nagase, H. and Brew, K. Engineering of tissue inhibitor of metalloproteinases mutants as potential therapeutics. Arthritis Res 2002; 4 (3): 51-61.

[4] Nagase, H. and Woessner, J.F., Jr. Matrix metalloproteinases. J Biol Chem 1999; 274 (31): 21491-4.

[5] Brew, K., Dinakarpandian, D. and Nagase, H. Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta 2000; 1477 (1-2): 267-83.

[6] Westerlund, B. and Korhonen, T.K. Bacterial proteins binding to the mammalian extracellular matrix. Mol Microbiol 1993; 9 (4): 687-94.

[7] Okamoto, T., Akaike, T., Suga, M., Tanase, S., Horie, H., Miyajima, S., Ando, M., Ichinose, Y. and Maeda, H. Activation of human matrix metalloproteinases by various bacterial proteinases. J. Biol. Chem. 1997; 272 (9): 6059-6066.

[8] Miyoshi, S. and Shinoda, S. Microbial metalloproteinases and pathogenesis. Microbes Infect 2000; 2 (1): 91-8.

[9] Renault, T., Le Deuff, R.M., Cochennec, N., Chollet, B. and Maffart, P. Herpes-like viruses associated with high mortality levels in larvae and spat of Pacific oysters, Crassostrea gigas: a comparative study, the thermal effects on virus detection in hatchery-reared larvae, reproduction of the disease in axenic larvae. Vet Res 1995; 26 (5-6): 539-43.

[10] Lambert, C. and Nicolas, J.L. Specific inhibition of chemiluminescent activity by pathogenic Vibrios in hemocytes of two marine bivalves : *Pesten maximus* and *Crassostrea gigas*. J. Invert. Pathbol. 1998; 71: 53-63.

[11] Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gélébart, F., Cordevant, C., Lange, M. and Poulet, S.A. A *Vibrio splendidus* strain is associated with summer mortality of juvenil oyster *Crassostrea gigas* in the bay of Morlaix (north brittany, France). Dise. Aquatic Org. 2001; 46: 139-145.

[12] Gay, M., Berthe, F.C. and Le Roux, F. Screening of Vibrio isolates to develop an experimental infection model in the Pacific oyster Crassostrea gigas. Dis Aquat Organ 2004; 59 (1): 49-56.

[13] Paynter, K.T. The effects of *Perkinsus marinus* infection on physiological processes in the eastern oyster, *Crassostrea virginica*. J. Shellfish Research 1996; 15 (1): 119-125.

[14] Audemard, C., Le, R.F., Barnaud, A., Collins, C., Sautour, B., Sauria, P.G., de, M.X., Coustau, C., Combes, C. and Berthe, F. Needle in a haystack: involvement of the

copepod PARACARTIA grani in the life-cycle of the oyster pathogen Marteilia refringens. Parasitology 2002; 124 (Pt 3): 315-23.

[15] Garreis, K.G., La Peyre, J.F. and Faisal, M. The effect of *Perkinsus marinus* extracellular products and purified proteases on oyster defense parameters *in vitro*. Fish & Shellfish Immuno. 1996; 6: 581-597.

[16] La Peyre, J.F., Yarnall, H.A. and Faisal, M. Contribution of Perkinsus marinus extracellular products in the infection of eastern oysters (*Crassostrea giga*s). J. Invertebr. Pathol. 1996; 68: 312-313.

[17] Munoz, P., Vance, K. and Gomez-Chiarri, M. Protease activity in the plasma of American oysters, Crassostrea virginica, experimentally infected with the protozoan parasite Perkinsus marinus. J Parasitol 2003; 89 (5): 941-51.

[18] Montagnani, C., Le Roux, F., Berthe, F. and Escoubas, J.-M. Cg-TIMP, an inducible tissue inhibitor of metalloproteinase from the Pacific oyster Crassostrea gigas with a potential role in wound healing and defense mechanisms. FEBS Lett 2001; 500 (1-2): 64-70.

[19] Cheng, T.C. Titel. in: Kennedy, V.S., Newell, R.I.E. and Eble, A.F.Kennedy, V.S., Newell, R.I.E. and Eble, A.F.s The eastern oyster *Crassostre virginica*., Maryland Sea Grant College, College Park.1996. 299-333

[20] Barreau-Roumiguière, C., Montagnani, C. and Escoubas, J.-M. Characterization of a Tal/SCL-like transcription factor in the pacific oyster *Crassostrea gigas*. Dev. Comp. Immunol. 2003; 27: 793-800.

[21] Gueguen, Y., Cadoret, J.-P., Flament, D., Barreau-Roumiguière, C., Girardot, A.-L., Garnier, J., Hoareau, A., Bachère, E. and Escoubas, J.-M. Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas*. Gene 2003; 303: 139-145.

[22] Livak, K.J. and Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25 (4): 402-8.

[23] Yu, W.P., Brenner, S. and Venkatesh, B. Duplication, degeneration and subfunctionalization of the nested synapsin-Timp genes in Fugu. Trends Genet 2003; 19 (4): 180-3.

[24] Pohar, N., Godenschwege, T.A. and Buchner, E. Invertebrate tissue inhibitor of metalloproteinase: structure and nested gene organization within the synapsin locus is conserved from Drosophila to human. Genomics 1999; 57 (2): 293-6.

[25] Phillips, B.W., Sharma, R., Leco, P.A. and Edwards, D.R. A sequence-selective single-strand DNA-binding protein regulates basal transcription of the murine tissue inhibitor of metalloproteinases-1 (Timp-1) gene. Journal-of-Biological-Chemistry 1999; 274 (32): 22197-22207.

[26] Hoffmann, J.A., Kafatos, F.C., Janeway, C.A. and Ezekowitz, R.A. Phylogenetic perspectives in innate immunity. Science 1999; 284 (5418): 1313-8.

[27] Sun, Y., Hegamyer, G., Kim, H., Sithanandam, K., Li, H., Watts, R. and Colburn, N.H. Molecular cloning of mouse tissue inhibitor of metalloproteinases-3 and its promoter. Specific lack of expression in neoplastic JB6 cells may reflect altered gene methylation. J Biol Chem 1995; 270 (33): 19312-9.

[28] Dalhammar, G. and Steiner, H. Characterization of inhibitor A, a protease from Bacillus thuringiensis which degrades attacins and cecropins, two classes of antibacterial proteins in insects. Eur J Biochem 1984; 139 (2): 247-52.

[29] Belas, R., Manos, J. and Suvanasuthi, R. Proteus mirabilis ZapA metalloproteinase degrades a broad spectrum of substrates, including antimicrobial peptides. Infect Immun 2004; 72 (9): 5159-67.

[30] Janeway, C.A., Jr. and Medzhitov, R. Innate immune recognition. Annu Rev Immunol 2002; 20: 197-216.
[31] Olafsen, J., Mikkelsen, H., Giaever, H. and Hansen, G. Indigenous Bacteria in Hemolymph and Tissues of Marine Bivalves at Low Temperatures. Appl Environ Microbiol 1993; 59 (6): 1848-1854.
[32] Matzinger, P. The danger model: a renewed sense of self. Science 2002; 296 (5566): 301-5.
[33] Seong, S.Y. and Matzinger, P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. Nat Rev Immunol 2004; 4 (6): 469-78.
[34] Campbell, C.E., Flenniken, A.M., Skup, D. and Williams, B.R. Identification of a serum- and phorbol ester-responsive element in the murine tissue inhibitor of metalloproteinase gene. J Biol Chem 1991; 266 (11): 7199-206.
[35] Bugno, M., Graeve, L., Gatsios, P., Koj, A., Heinrich, P.C., Travis, J. and Kordula, T. Identification of the interleukin-6/oncostatin M response element in the rat tissue inhibitor of metalloproteinases-1 (TIMP-1) promoter. Nucleic Acids Res 1995; 23 (24): 5041-7.
[36] Hall, M.C., Young, D.A., Waters, J.G., Rowan, A.D., Chantry, A., Edwards, D.R. and Clark, I.M. The comparative role of activator protein 1 and Smad factors in the regulation of Timp-1 and MMP-1 gene expression by transforming growth factor-beta 1. J Biol Chem 2003; 278 (12): 10304-13.
[37] Herpin, A., Lelong, C. and Favrel, P. Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. Dev Comp Immunol 2004; 28 (5): 461-85.
[38] Lelong, C., Mathieu, M. and Favrel, P. Structure and expression of mGDF, a new member of the transforming growth factor-beta superfamily in the bivalve mollusc Crassostrea gigas. Eur J Biochem 2000; 267 (13): 3986-93.
[39] Herpin, A., Lelong, C., Becker, T., Rosa, F.M., Favrel, P. and Cunningham, C. Structural and functional evidences for a type 1 TGF-betasensu stricto receptor in the lophotrochozoan Crassostrea gigas suggest conserved molecular mechanisms controlling mesodermal patterning across bilateria. Mech Dev 2005; 122 (5): 695-705.
[40] Montagnani, C., Tirape, A., Boulo, V. and Escoubas, J.-M. The two Cg-timp mRNAs expressed in oyster hemocytes are generated by two gene families and differentially expressed during ontogenesis. Dev. Comp. Immunol. 2005; 29: 831-839.
[41] Baldwin, A.S.J. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu. Rev. Immunol. 1996; 14: 649-83.
[42] Pahl, H.L. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 1999; 18 (49): 6853-66.
[43] Escoubas, J.M., Briant, L., Montagnani, C., Hez, S., Devaux, C. and Roch, P. Oyster IKK-like protein shares structural and functional properties with its mammalian homologues. FEBS Lett 1999; 453 (3): 293-8.
[44] Montagnani, C., Kappler, C., Reichhart, J.M. and Escoubas, J.M. Cg-Rel, the first Rel/NF- κB homologue characterized in a mollusk, the Pacific oyster Crassostrea gigas. FEBS Lett 2004; 561 (1-3): 75.
[45] Tanguy, A., Guo, X. and Ford, S.E. Discovery of genes expressed in response to Perkinsus marinus challenge in Eastern (Crassostrea virginica) and Pacific (C. gigas) oysters. Gene 2004; 338 (1): 121-31.
[46] Kappler, C., Meister, M., Lagueux, M., Gateff, E., Hoffmann, J.A. and Reichhart, J.-M. Insect Immunity. Two 17 bp repeats nesting a κB-related sequence confer

inducibility to the diptericin gene and bind a polypeptide in bacteria-challenged *Drosophila*. EMBO J. 1993; 12: 1561-1568.

[47] Chen, F.E. and Ghosh, G. Regulation of DNA binding by Rel/NF-kappaB transcription factors: structural views. Oncogene 1999; 18 (49): 6845-52.

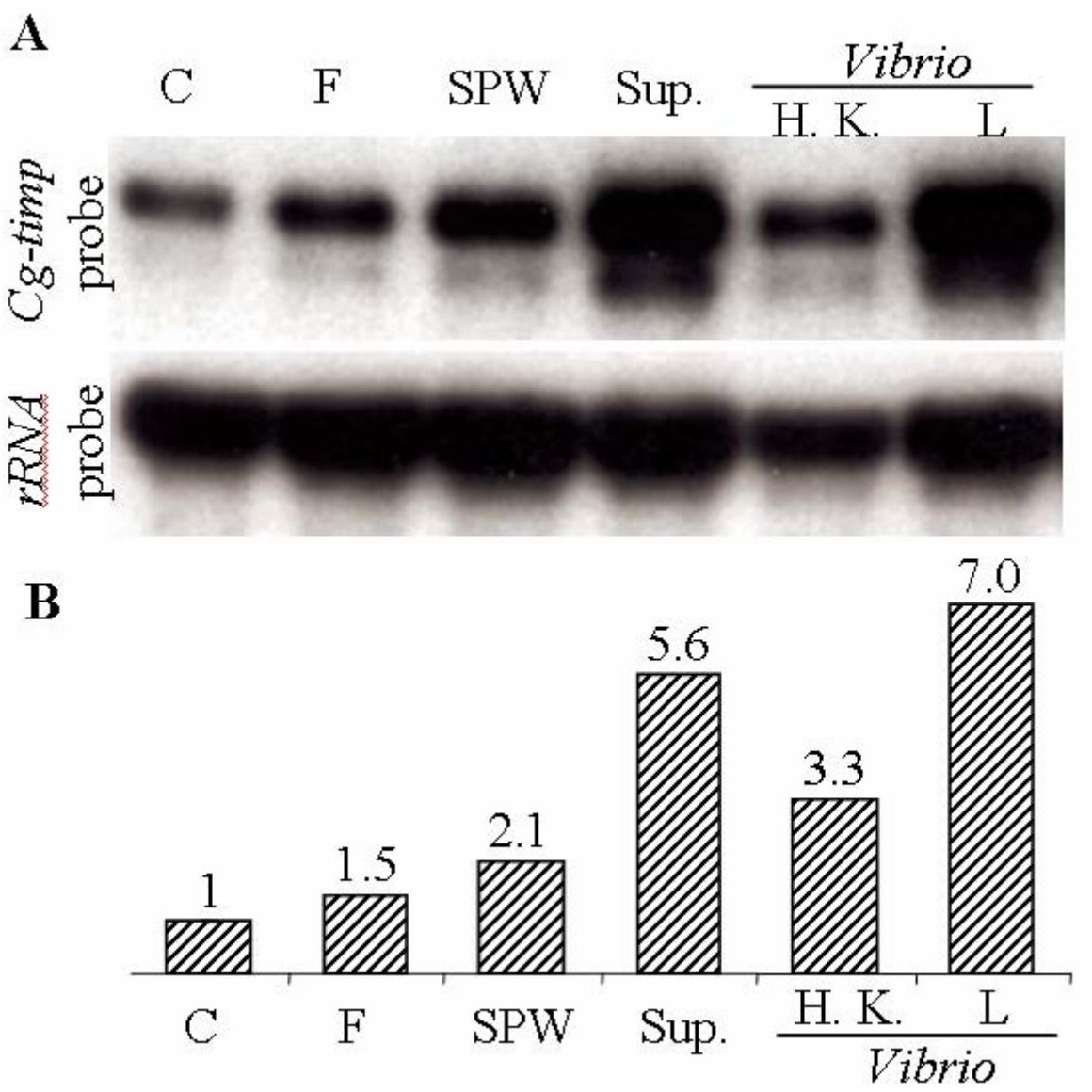


Fig. 1. *Cg-timp* expression was mainly induced by secretory/excretory compounds produced by bacteria. Oysters were injected with saline peptone water (SPW), or supernatants from four *Vibrio* strain cultures (Sup.) or a mixture of four heat-killed (H.K.) or live (L) *Vibrio* strains. Two oyster batches 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 radiolabled 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.

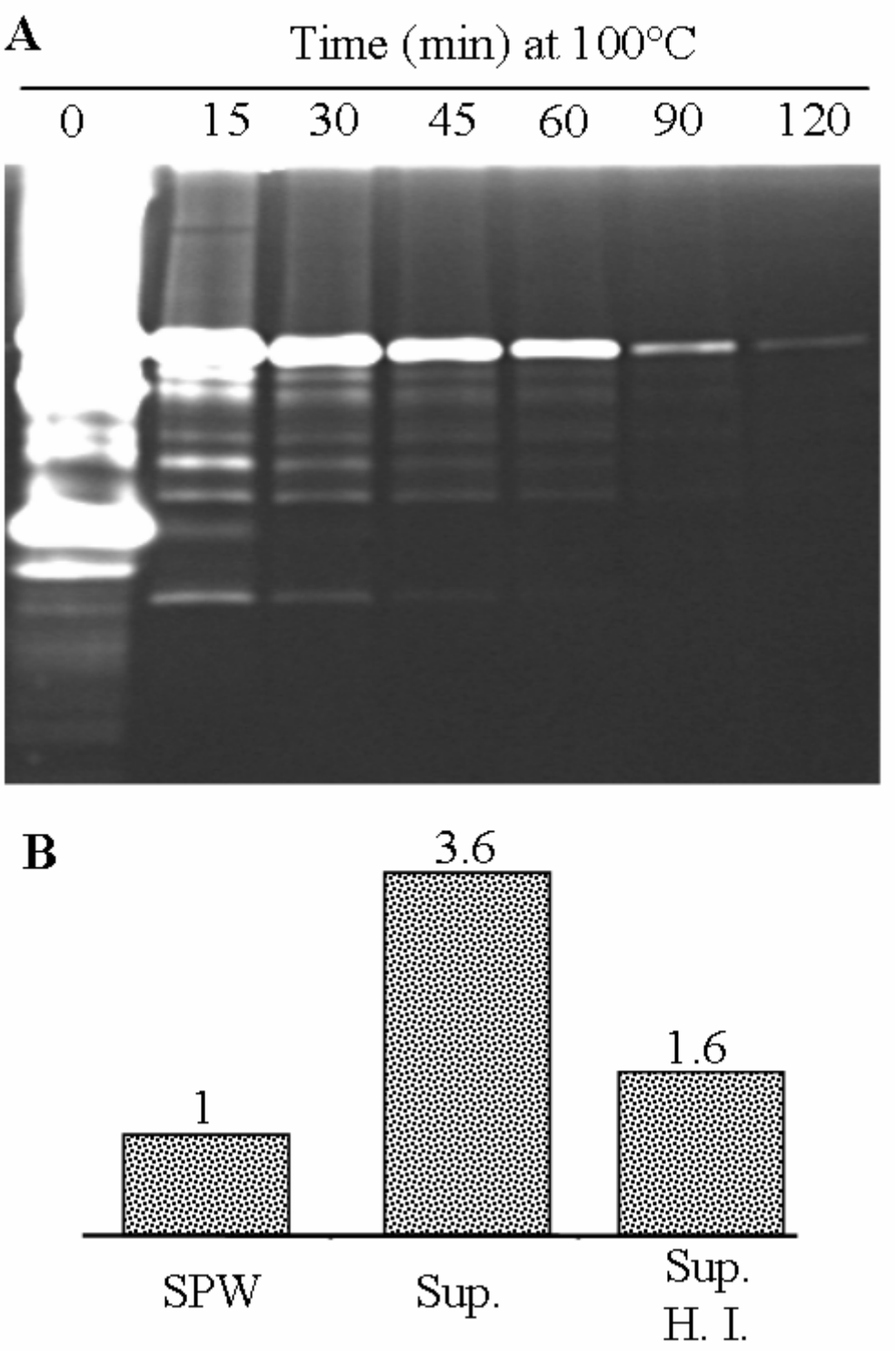


Fig. 2. Protease activities contained in bacteria culture supernatants were involved in *Cg-timp* gene activation. **A)** Supernatants of the four *Vibrio* strain cultures were incubated at 100°C during different periods of time and protease inactivation was analyzed by zymography on gelatin-containing 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.

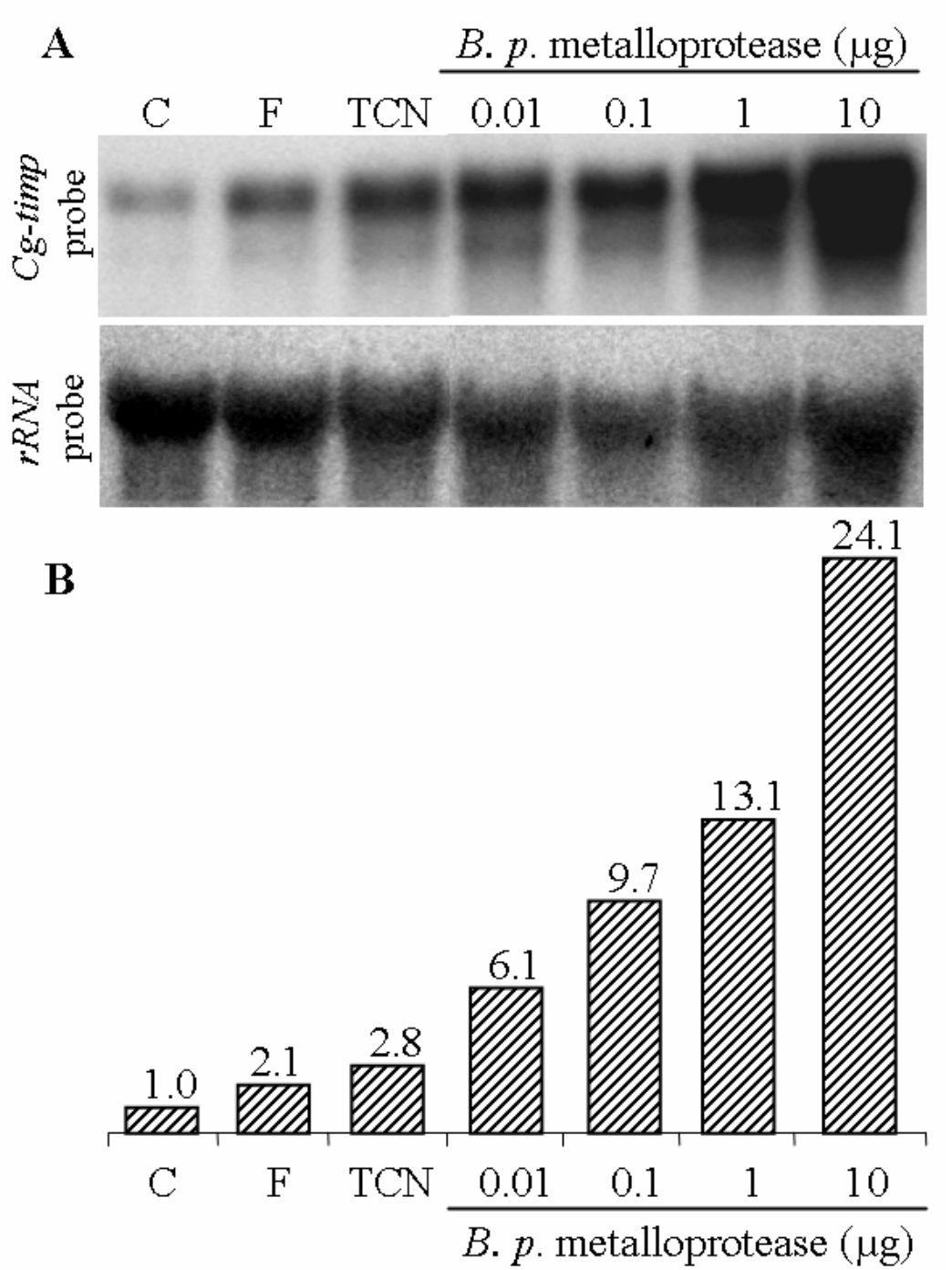
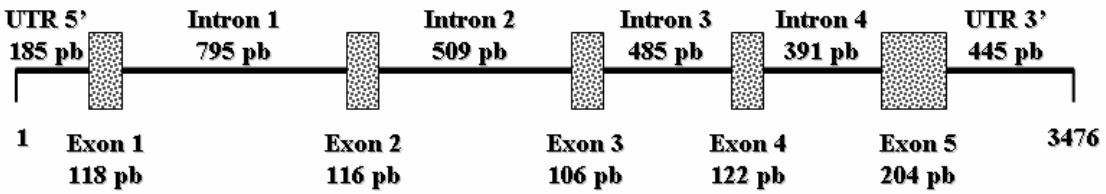


Fig. 3. Dose-dependant induction of *Cg-timp* gene by a metalloproteinase extracted from a Gram$^+$ bacteria. Oysters were challenged with increasing amounts of *Bacillus polymixa* metalloproteinase dissolved in TNC. Three oyster batches were used as controls: the first one corresponds to untreated oysters (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 radiolabled 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.

A



B

```
tgactaagtcacttcatattttaaacatgacattttggtatattctaaaatgacctggaccatgcgatc -1016
  AP1
ctataactattatgtttctaatgctcacccaaattgttgtgctacaagcgagttacagagtaaacagtca  -946
ttgcatatgaaaacaagggttgtgtcctgtttttgtttacgttggttaaatatactggatacagttttca  -876
gcaatttttttctatttgctgtgattgtttgctatccaacaaataaacagtttctagtgtttgtgacatt  -806
taatctttcaaacctgtttttttcctattcagcaaacaatacgtaaacaacaacatagcctgaagtttgtt  -736
tacaaaacaaagaattgtgagctaagtatctcaattataacccaatgtcttagactggaaataccccaa  -666
                                                        κB
tgaaaccatcaaaaatgaataaatgaaatttgaacttgttcagcccttatcgtggccatgttcccacttg  -596
gaactataatgtaaatacaaaataaagaatgatgaggtcaaggtcttagggaaggtcaaagtagatgagt  -526
                                                 κB          AP1
aatgatcttgctatttgttgaatttcttctctccaattttgaaaaaaatgacactggctattatggcgaa  -456
                                                                  κB
gcccagctcgagcactgcagtaggtgaattgctctttctaatacgtttctttgttcaggctagtaacgta  -386
tcgtttttatctagcagcgtgggaataaaaatgttgtagtatatcgaaaagaagatagcttaatatgcac  -316
tctgttttgttagtgcgccgaaaatatgcatacatcaactttctgtatttgtggtattcacaagtgtcta  -246
ctcgtatgcattgctaccacaacaacgctagtaaaaaatatgacgacactcaaacgttgacgtaaattaa  -176
ttcatgtcttcgtcaaaattattacataataacaactgcagttggtattgtttcccttataaattaatat  -106
cgtacgattttgttgttaatctttaagtgttttgagagtgtgagaacggaacccttgagtaggagggccg   -36
                                                                GC-box
tgtatataattattcttgacataagaattccacagtcatactctccgctatatagaacgactcggaccta   +35
  TATA-Box                          |TSS
gtcggtgatgatttagaattaacgtgttcgttccattgctctttggtatccttatctgttggtcgtttcg  +105
aacactctagagggccttagaacacacagtgtgacgttcacaaagttagcttattcgactgccattaccc  +175
ccaggagacg ATG AGG CAG TAC AAC TTC CTT CTT TTC CTG                       +215
           M   R   Q   Y   N   F   L   L   F   L
```

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.

Cg-timp motifs	Position from the TSS	Insect Consensus [a] GGGRN T YYYY	Mammal Consensus [b] GGGRN N YYCC
GGGCT T CGCC	–452 to -461	GGG-T T C-CC	GGG-T T C-CC
GGGAA G GTCA	–547 to -538	GGGAA - -TC-	GGGAA G -TC-
GGGTA T TTCC	–670 to -679	GGG-A T TTCC	GGG-A T TTCC

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] Acording to Kappler and coworkers [46]. [b] Acording to Chen and coworkers [47].