Fish & Shellfish Immunology 30 (2011) 851–858

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Identification, molecular characterization and expression analysis of a mucosal C-type lectin in the eastern oyster, *Crassostrea virginica*

Xing Jing [1], Emmanuelle Pales Espinosa*, Mickael Perrigault, Bassem Allam

School of Marine and Atmospheric Sciences, State University of New York, Stony Brook, NY 11794, USA

ARTICLE INFO

Article history:
Received 22 October 2010
Received in revised form
7 January 2011
Accepted 9 January 2011
Available online 21 January 2011

Keywords:
Bivalve
Lectin
Mucocyte
qPCR
Gene expression

ABSTRACT

Lectins are well known to actively participate in the defense functions of vertebrates and invertebrates where they play an important role in the recognition of foreign particles. They have also been reported to be involved in other processes requiring carbohydrate–lectin interactions such as symbiosis or fertilization. In this study, we report a novel putative C-type lectin (CvML) from the eastern oyster *Crassostrea virginica* and we investigated its involvement in oyster physiology. The cDNA of this lectin is 610 bp long encoding for a 161-residue protein. CvML presents a signal peptide and a single carbohydrate recognition domain (CRD) which contains a YPD motif and two putative conserved sites, WID and DCM, for calcium binding. CvML transcripts were expressed in mucocytes lining the epithelium of the digestive gland and the pallial organs (mantle, gills, and labial palps) but were not detected in other tissues including hemocytes. Its expression was significantly up-regulated following starvation or bacterial bath exposure but not after injection of bacteria into oyster's adductor muscle. These results highlight the potential role of CvML in the interactions between oyster and waterborne microorganisms at the pallial interfaces with possible involvement in physiological functions such as particle capture or mucosal immunity.

1. Introduction

Molecular communication is widespread in the aquatic environment and is commonly used to mediate interactions among organisms including interaction between hosts and symbionts [1] or pathogenic microorganisms [2], mate attraction and mating [3], and predation strategies [4,5]. Among molecules known for non-self recognition and cell-to-cell or cell-to-matrix interactions, lectins are defined as a wide range of carbohydrate binding proteins of non immune origin that are able to agglutinate cells through interaction with carbohydrates associated with cell surface [6,7]. Lectins are widely distributed throughout living organisms including viruses, bacteria, fungi, protista, plants and animals [8], suggesting their involvement in essential physiological functions. Moreover, they present a great structural diversity and a variety of carbohydrate affinity that may reflect their participation in numerous functions in organisms [9]. In metazoans, lectins have been suspected or described to play a role in processes as diverse as self-defense [10–12], parasitism [13,14], symbiosis [1,15,16], reproduction [17], as well as selection of food particles in marine organisms [18,19]. They can assist animals by binding and immobilizing microorganisms through agglutination [18,20] and encapsulation [21,22] or can initiate a cascade of events leading, for example, to host colonization [1] or to limit pathogen infection [23–25].

In bivalves, lectins have been identified in hemolymph/hemocytes and were suggested to be implicated in defense mechanisms or pathogen uptake. For example, the C-type lectin (CfLec-1) recently cloned from the Zhikong scallop, *Chlamys farreri*, is able to aggregate bacteria and inhibit bacterial growth [26]. In addition, the C-type lectin (AiCTL1) identified from the bay scallop *Argopecten irradians* hemocytes is thought to be involved in injury healing and immune responses [27]. Finally, the galectin CvGal identified from *Crassostrea virginica* hemocytes facilitates the recognition of the Alveolate *Perkinsus marinus* and promotes its entry into the hemocytes [11].

In some rare cases, lectins have been suggested to be involved in mechanisms other than defense. For example, the lectin Codakine has been found to be the predominant protein in the gill of the symbiotic clam *Codakia orbicularis* leading Gourdine et al. [28] to propose its involvement in the mediation of symbiosis. In bivalve reproduction, several studies have reported that the binding of sperm to eggs is often mediated by lectin-like molecules. For instance, oyster bindin is similar to the F-lectin family of fucose binding lectins [29] and mussel lysin-M7 resembles the

* Corresponding author. Tel.: +1 631 632 8694; fax: +1 631 632 8915.
E-mail address: Emmanuelle.Palesespinosa@stonybrook.edu (E.P. Espinosa).
[1] Permanent address: Laboratory of Pathology and Immunology of Aquatic Animals, LMMEC, Ocean University of China, Qingdao 266003, PR China.

1050-4648/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2011.01.007

carbohydrate recognition domain of C-type lectins [30]. These molecules have been identified in the acrosomal region of bivalve sperm and are very specific in a given species, helping avoid cross-fertilization [29].

Prior studies have also demonstrated the involvement of lectins in predator–prey interactions. For example, mannose-binding lectins were shown to represent feeding receptors for recognizing preys in the marine dinoflagellate *Oxyrrhis marina* [31] and in the amoeba *Acanthamoeba castellanii* [32]. In bivalve, the presence of lectins have been suspected [33] and recently demonstrated [34] in mucus covering pallial organs (gills, labial palps) in the oyster *C. virginica* [18,19] and the mussel *Mytilus edulis* [35,36]. These lectins have been suspected to be involved in particle capture and sorting in suspension-feeding bivalves [18,19,35,36].

We focused this study on the investigation of lectins associated with the pallial mucus of *C. virginica* in an attempt to identify the physiological functions of oyster lectins, particularly their involvement in mucosal immunity. We screened public EST (Expressed Sequence Tag) databases and used a diverse set of molecular techniques to identify lectin candidates associated with the pallial organs of *C. virginica*. These investigations allowed the identification of a secretory lectin (hereby designated CvML for *C. virginica* mucocyte lectin) that is specifically produced in mucocytes lining the pallial organs (gills, labial palps, and mantle) as well as oyster digestive gland. The full lectin sequence is presented and the transcription level of this molecule in response to different treatments including bacterial challenge and starvation was further investigated. Results highlight the potential involvement of this lectin in particle capture processes and in mucosal immunity.

2. Materials and methods

2.1. Oysters

Adult (80–90 mm in shell length) eastern oysters, *C. virginica*, were obtained from a commercial source (Frank M. Flower and Sons Oyster Company, Oyster Bay, New York, USA). Their external shell surface was scrubbed to remove mud and marine life. Oysters were then randomly subdivided into 3 different groups. The first 2 groups were immediately used for RNA extraction/cDNA amplification or in situ hybridization analysis. The oysters from the last group was carefully v-notched at the dorsal posterior edge of the shell without damaging the mantle and acclimated in the laboratory for 1 week before being used in the challenge experiment (see below).

2.2. Bacterial culture

Vibrio alginolyticus, an opportunistic pathogen of shellfish and finfish, was initially isolated from oyster samples collected from Long Island Sound, New York, and grown on marine agar (Difco BD, USA) for 48 h. Bacterial colonies were collected by washing the plate with filtered artificial seawater (FAS). Resulting bacterial suspension was then centrifuged (800 g, 15 min, 4 °C), washed with FAS and cell concentration was spectrophotometrically adjusted to 1×10^8 bacteria ml^{-1} in FAS.

2.3. RNA extraction

Six oysters were notched at the dorsal posterior edge of the shell and bled from the adductor muscle using a 10-ml syringe fitted with a 18-gauge needle. Hemolymph was pooled and centrifuged at 800 g for 10 min at 4 °C. Supernatant was discarded and total RNA was immediately extracted from hemocyte pellets using TRI Reagent® (MRC, Cincinnati, OH). Additionally, digestive gland, mantle, gills, labial palps, gonad, and muscle from oysters were separately dissected and used for RNA extraction following the same procedure. For each organ, RNA samples were pooled from all six individuals and used for cDNA amplification (section 2.5).

2.4. Homology screening and primers design

Public *C. virginica* EST database available at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) was searched using the sequence of C-type lectin-1 from the Pacific oyster *Crassostrea gigas* [BAF75353, [37]]. This lectin was chosen as a template since it was exclusively found in the epithelia of the digestive gland of *C. gigas* and not in the hemocytes, suggesting a possible role for this molecule in mucosal immunity. Seven EST from *C. virginica* showed very high homology with *C. gigas* lectin-1 (with e-values less than 10^{-48}). The EST CV088799 ($1.e^{-66}$) was selected and specific primers were designed (Table 1).

2.5. cDNA amplification

cDNA was generated from extracted RNA using M-MLV reverse transcriptase (Promega, Madisson, WI) and was used as template with the set of primers listed in Table 1. The PCR reaction was carried out in an Eppendorf Mastercycler (ep gradient S) using GoTaq® DNA Polymerase (Promega, Madisson, WI) for 10 min of initial denaturation, followed by 35 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 1 min), with an additional 10 min primer extension after the final cycle. PCR products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide.

2.6. Rapid amplification of CvML cDNA ends

In order to confirm the exact sequence of the selected protein, the full-length cDNA of CvML was amplified by 3′ and 5′ rapid amplification of cDNA ends (RACE) [38,39] using specific primers listed in Table 1. Briefly, total RNA was extracted from the digestive gland of oysters using methods described above. Reverse transcription for the generation of 5′ cDNA and 3′ cDNA ends were performed with the SuperScript II reverse transcriptase (Invitrogen, USA) using CvML-R and Qt respectively. CvML cDNA ends were

Table 1
Primers used in this study for the amplification of the expressed sequence tag (CvML) identified in *Crassostrea virginica*. The last 4 lines provide sequence information for the primers used in the quantitative Real-Time PCR.

Primer names	Primer Sequences (5′→3′)
RT-PCR primers	
CvML-F (Forward)	ATGACTACATCAAGGAGGGC
CvML-R (Reverse)	CGCAGATGTAGTAGTGTCCG
RACE primers	
Qt	CCAGTGAGCAGAGTGACGAGGA CTCGAGCTCAAGCTTTTTTTTTTTTTTT
Qo	CCAGTGAGCAGAGTGACG
Qi	GAGGACTCGAGCTCAAGC
CvML-R (5′ RACE)	CGCAGATGTAGTAGTGTCCG
CvML-O-F (3′ RACE)	GACTTCAATCAGTGGGGGC
CvML-O-R (5′ RACE)	CGTGGTTACAGTTGGAGTCG
CvML-I-F (3′ RACE)	CTGCATGCTGCAGGTCTAC
CvML-I -R (5′ RACE)	GTAGACCTGCAGCATGCAG
Quantitative real-time RT-PCR primers	
qCvML-F (Forward)	TGTCTGTCTGTCTGTCTGACTGTGG
qCvML-R (Reverse)	AACGGTTACCAGGTAGCTCCTCATC
18S-F (Forward)	CTGGTTAATTCCGATAACGAACGAGACTCTA
18S-R (Reverse)	TGCTCAATCTCGTGTGGCTAAACGCCACTTG

amplified by PCR with Qt/Qo/CvML-O-R (5′ cDNA ends) and Qo/CvML-O-F (3′ cDNA ends). PCR products were thereafter used as template in a second set of amplification using Qi/CvML-I-R (5′ cDNA ends) and Qi/CvML-I-F (3′ cDNA ends) primer combinations. Ultimate PCR products were separated on agarose gel and extracted using Wizard® SV gel and PCR clean up system (Promega, Madison, USA). Purified products were ligated into pGEM-T vector (Promega, Madison, USA) and transformed in the bacteria E-coli DH5α (Invitrogen, Carlsbad, CA). Bacteria were cultured in lysogeny broth medium (with 100 μg L^{-1} ampicillin, final concentration) and plasmids containing insert were extracted and sequenced by extension from both ends using T7 and SP6 universal primers.

2.7. Sequence analysis

The cDNA and deduced amino acid sequences were analyzed by BLAST program (NCBI, http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi) and protein motif features were predicted using the Prosite database (www.expasy.org/prosite/ and http://www.ebi.ac.uk/Tools/ppsearch/), and the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignments were performed using CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/).

2.8. RNA labeling

One cDNA product (186 bp, using primers CvML F and R, Table 1) was ligated into pGEM-T Easy Vector (Promega, Madisson, WI) and used to transform E-coli DH5α bacteria (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. Positive clones were selected on lysogeny broth (LB) agar (with 100 μg L^{-1} ampicillin, final concentration). Vector containing the cDNA insert was extracted from the bacteria using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and insert orientation was determined by sequencing. Purified vector containing the cDNA insert was linearized using the restriction enzyme SpeI and SacII separately (sense and anti-sense) and purified using the DNA purification kit (Promega, Madisson, WI) according to manufacturer's recommendations. Digoxigenin-UTP (DIG) labeled sense and anti-sense RNA was produced from linearized plasmid using the DIG RNA labeling Kit (SP6/T7) (Roche Applied Science, Indianapolis, IN).

2.9. In situ hybridization (ISH)

Oyster tissues were fixed in 10% formalin for 48 h before being dehydrated in an ascending ethanol series, embedded in paraffin blocks and cut in serial sections (5 μm thickness). Four consecutive sections were processed for standard hematoxylin–eosin staining (1 section) or for in situ hybridization (3 sections). The latter sections were deparaffinized in xylene, rehydrated through a descending ethanol series, and equilibrated in diethylpyrocarbonate (DEPC)-treated water. The sections were then digested with Proteinase K in PBS (50 μg ml^{-1}) and permeabilized with Triton X-100 in PBS (0.1%). Sections were then post-fixed in 4% paraformaldehyde in DEPC-treated water, rinsed with PBS containing active DEPC (0.1%) and processed for ISH according to the protocols described by Braissant and Wahli [40]. Two serial sections were separately hybridized with the anti-sense (test) or sense (negative control) probes (600 ng ml^{-1}). No probe was added to the third serial section which was used as another negative control slide. Following incubation (overnight at 60 °C), slides were added with an anti-digoxigenin antibody coupled with alkaline phosphatase (Roche Applied Science, Indianapolis, IN). Positive reactions were revealed using nitroblue tetrazolium (NBT), BCIP (5-bromo-4-chloro-3-indolyl phosphate) and levamisol. Slides were finally counter-stained with fast red solution and sections were dehydrated, mounted with glass coverslips, and observed under a brightfield microscope.

2.10. Effect of bacterial challenge and starvation on CvML transcription

Eighty four oysters were v-notched as described above before being acclimated in the laboratory for a minimum of 1 week (salinity of 28, 15 °C) where they were fed daily (15% dry weight) using DT's Live Marine Phytoplankton (Sycamore, IL) [41]. After 1 week, animals were randomly divided into six equal groups that were submitted to the following treatments. The first group (Group 1) received a *V. alginolyticus* suspension (100 μl containing 1×10^7 bacteria) injected into the adductor muscle. The second group (Group 2) was injected into muscle with 100 μl of filtered artificial seawater and served as a control for Group 1. Following injection, oysters were maintained out of water for 1.5 h before being transferred into their respective 40-L tanks filled with filtered and ultraviolet treated seawater. A third group (Group 3) of oysters was continuously maintained in seawater and served as unaltered control for oysters in Groups 4, 5 and 6. In addition to injection into muscle tissue, we tested the effect of bacterial bath exposure on CvML transcription. Seawater tank containing oysters from the fourth group (Group 4) was added with 50 ml of a *V. alginolyticus* suspension (10^8 cells/ml) to obtain 1.25×10^5 cells/ml final bacterial concentration. Oysters from the fifth group (Group 5) were added with a suspension of mineral particles (Kaolin) at a final concentration of 0.6 $mg.l^{-1}$ [42] Oysters from these 5 groups were kept under constant salinity and temperature conditions (salinity of 28, 15 °C) and were fed daily as described above. The last group (Group 6) was not fed and was intended to evaluate the effect of starvation on CvML transcription. After 6 and 72 h, six oysters from each group were sacrificed; digestive gland, mantle, gills and labial palps were dissected, flash-frozen and conserved at −80 °C until processing for RNA extraction. The expression of CvML transcripts in tissues was measured using Real-time PCR. Total RNA was extracted from tissues and single-strand cDNA was synthesized as described above. One set of gene-specific primers was used to amplify a product of 140 bp and 18S ribosomal RNA was used as housekeeping gene (Table 1, qPCR). Real-time PCR assay was carried out in an Eppendorf RealPlex cycler with 6 μl of 1:15 diluted cDNA. The amplifications were performed in a 20 μl reaction volume containing 1 × Brilliant II SYBR green qPCR Master mix (Stratagene) and 100 nM of each primers. Thermal profile for real-time PCR assay was an initial denaturation step at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 30 s, annealing and extension at 60 °C for 1 min. Each run was followed by a melting curve program for quality control. PCR efficiency (E) was determined for each primer pair (including the housekeeping gene 18S rRNA) by determining the slope of standard curves obtained from serial dilution analysis of cDNA. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to determine the expression level of CvML among tissues [43]. Data obtained from Real-time PCR analysis were subjected to *t*-test (SigmaStat, version 3.1). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Identification of CvML

The complete sequence of the CvML consisted of a 610 bp encoding for a predicted peptide of 161 amino acids (Fig. 1) with a theoretical isoelectric point of 5.56 and an estimated molecular weight of 18.2 kDa (http://au.expasy.org/tools/pi_tool.html).

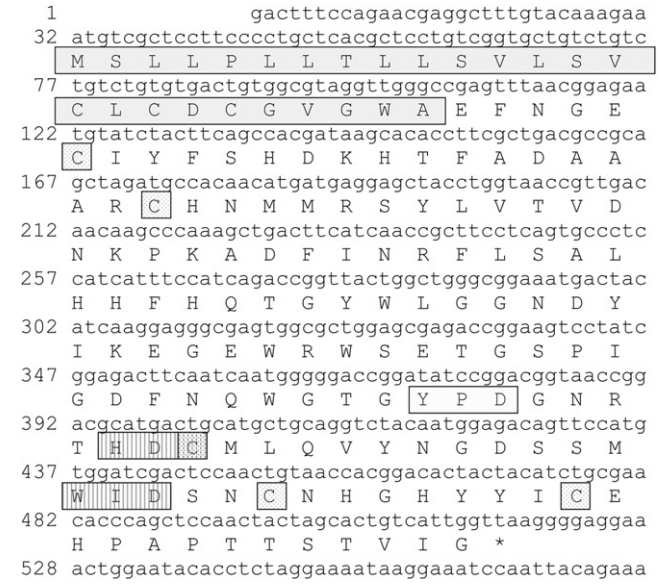

Fig. 1. Nucleotide and deduced amino acid sequences of CvML. The putative signal peptide (gray), the motif of the CRD for ligand binding (white), the cysteine residues (dotted), and the putative calcium binding sites (striped) are boxed. * represents the stop codon.

3.2. Sequence comparison of CvML with other C-type lectins

The sequence analysis of CvML indicated some levels of homology (26–80% amino acid identity, Table 2) with previously described C-type lectins and similarity for specific characteristics of these proteins, including their calcium and carbohydrate binding residues (Table 2 and Fig. 2). In CvML, the CRD (carbohydrate recognition domain) domain consists of 124 residues (Phe^{27}–Glu^{150}, Fig. 1) located in the C-terminal of the protein (www.expasy.org/prosite/). CvML displays several characteristics of C-type lectins, including a matching pattern for C-type 1 lectins (from Cys^{124} to Cys^{149}), four consensus cysteine residues (Cys^{48}, Cys^{124}, Cys^{141}, Cys^{149}) and one optional cysteine (Cys^{31}) that are expected to form disulfide bonds [12]. Like most of the C-type lectins, CvML also presents a conserved WID residue (Trp^{136}, Ile^{137}, Asp^{138}) that is considered to be the principal site for calcium binding [44,45]. The pattern HDC (His^{122}, Asp^{123}, Cys^{124}) is also present within the sequence and is suspected to represent a secondary calcium binding site. Additionally, CvML shows a YPD motif (Tyr^{116}, Pro^{117}, Asp^{118}) determinant for sugar specificity, also found in the *C. gigas* C-type lectin-1. The first 25 amino acid residues from the N-terminus are mostly uncharged, hydrophobic and show homology to signal peptides known to be present in secreted proteins (www.expasy.org/prosite/). One N-glycosylation site (Asn^{119}, Arg^{120}, Thr^{121}) and one EGF-like domain (epidermal growth factor, from 18 to 31) was detected in the CvML sequence (http://www.ebi.ac.uk/Tools/ppsearch/index.html).

3.3. Localization of CvML transcripts in oyster tissue

Positive PCR signals were detected in all analyzed tissues except hemocytes and adductor muscle (Fig. 3). Only very faint signals were detected in the gonad likely as a result of contamination with epithelial tissue associated with pallial surfaces and/or digestive gland. CvML mRNA was detected by in situ hybridization (antisense probe) in specific cells within the epithelial layer of the digestive tract, as well as pallial organs: mantle, gills, and labial palps (Fig. 4). In situ hybridization signals were absent from, hemocytes, muscle and gonad, confirming PCR trends (Fig. 3). The examination of serial sections processed for in situ hybridization or stained with hematoxylin–eosin allowed a more precise localization of production sites and indicated that CvML transcripts are present in the mucocytes lining the epithelium of the digestive tubules and the pallial organs. In labial palps, the CvML transcripts are mainly localized in the palp troughs (concave) area as well as in the posterior area of the labial palp crests (Fig. 4).

3.4. CvML-mRNA expression after starvation and bacterial challenge

Variation of CvML expression in oyster gills, labial palps, mantle and digestive gland was investigated in response to four treatments (bacterial injection into the circulatory system, bacterial bath exposure, starvation and bath exposure to inert mineral particles). Six hours after each treatment, no significant changes were observed in the different tissues compared to their corresponding controls (Fig. 5A), although a weak increase in CvML transcripts level was observed in pallial organs (e.g. labial palps and/or mantle) after the addition of kaolin and bacteria in seawater (bath exposure). Similarly, the injection of bacteria in the circulatory system slightly increased CvML expression in gills.

After 72 h, transcripts level of CvML was similar in unaltered oysters and in oysters added with kaolin for all tested organs except for labial palps where a 17-fold increase in expression level was measured (Fig. 5B, *t*-test, $p = 0.01$). Seventy-two hours of starvation induced a significant increase in CvML expression in labial palps (91,404-fold, *t*-test, $p < 0.001$), mantle (211-fold, *t*-test, $p = 0.001$) and gills (120-fold, *t*-test, $p = 0.002$) of *C. virginica* compared to fed controls (Fig. 5B). Similarly, bacterial bath exposure caused a significant increase in the expression of CvML in labial palps (147,904-fold, *t*-test, $p < 0.001$), mantle (279-fold, *t*-test, $p = 0.003$) and gills (112-fold, *t*-test, $p < 0.001$) but not in the digestive gland

Table 2
Lectins presenting similarities with CvML based on BLAST comparisons.

Protein ID	Species	Fragment size	E-values (E), Identity (I) with amino acid (%)	Ligands	Sugar binding site	References
CvML	*Crassostrea virginica* (oyster), several tissues	161 aa	NA	Undefined	YPD	This study
BAF75353	*Crassostrea gigas* (oyster), digestive gland	158 aa	E = 6e−74, I = 115/143 (80%)	Undefined	YPD	Yamaura et al. (2008)
ABZ89710	*Argopecten irradians* (scallop), hemocytes	176 aa	E = 2e−28, I = 59/158 (37%)	Undefined	QPD	Zhu et al. (2008)
ACI69741	*Salmo salar* (salmon), undefined	168 aa	E = 1e−17, I = 37/131 (28%)	Undefined	EPS	Unpublished (GeneBank)
ABB71672	*Chlamys farreri* (scallop), all body	130 aa	E = 5e−16, I = 43/159 (27%)	Mannose	EPD	Zheng et al. (2007)
ACO36046	*Pinctada fucata* (oyster), undefined	168 aa	E = 1e−16, I = 44/172 (25%)	Undefined	QPD	Unpublished (GeneBank)
EAT36508	*Aedes aegypti* (mosquito), undefined	154 aa	E = 1e−13, I = 45/151 (29%)	Undefined	EPS	Unpublished (GeneBank)
BAB47156	*Anguilla japonica* (eel), skin mucus	166 aa	E = 7e−13, I = 45/154 (29%)	Lactose	EPN	Tasumi et al. (2002)
MeML	*Mytilus edulis* (mussel), pallial organs, intestine	152 aa	E = 3e−11, I = 41/157 (26%)	Undefined	QPS	Pales Espinosa et al. (2010)
AAX19697	*Codakia orbicularis* (clam), gill extract	148 aa	E = 4e−09, I = 36/135 (26%)	Mannose	EPN	Gourdine et al. (2007)

```
C.virginica        ----------MSLLPLLTLLSVLS----VCLCDCGVGWAEFNGECIYFSHDKHTFADAAA  46
C.gigas            ----------MSVLPLLTLLSVLS----VCLCDCGVGWAELNGECIYFSHDKHTWSDART  46
A.irradians        RHEANTVTLKMAASWILFTSVAFSGF-FLVLSTCPSGWIEFNQECFLFGASKKDWNDAEA  59
A.japonica         -----MVSFPMSNLIFVAVLSGLTLVSEQCADTCPEGWKGFNGCCYKHFDLLKNWREAEF  55
C.orbicularis      ----------MKILVAVFLVLVVVG---TAAAGCPDGWTQFLDLCYIYQSAKASWASAQS  47
A.aegypti          ----------MFGATSISLKIVLI----VLFYQLCCTHAQFN---FHIASEATNWFEASE  43

C.virginica        RCHNMMRSYLVTVDNKPKADFINRFLSAL--HHFHQTGYWLGGNDYIKEGEWRWSETGSP  104
C.gigas            KCHNMNRSYLVTIDNQPKADYINKFLSTL--HHFRQVGYWLGGNDYIVEGQWRWSETGTG  104
A.irradians        DCR-RHSSYLSTDDNAEKHSFLKTYLNIF--HSWKLGHFWLGGNDLAVENSWRWFESGHA  116
A.japonica         YCM-IRGGHLASVHSNVEYQFLR-ELNKA--SDPQDSMFWIGLTDIRKEGTWVWSDG--S  109
C.orbicularis      SCQ-ALGGILAEPDTACENEVLIHMCREN--GDAGSFGPWLGGQKVG--GAWQWSSSG-A  101
A.aegypti          YCH-RMSMRLAVVNSEAKHNAVVNAAMATGLHHSGYFGVWLGATDLAQTGIFTWRETG-K  101

C.virginica        IGDFNQWGTGYPDGNRT-HDCMLQVYNGDS-SMWIDSNCNHGHYYICEHPAPTTS- TVIG 161
C.gigas            LGDFTQWGPGYPDGNRT-HDCMLQVFNGET-SMWIDSNCAHPHYYICEHQAPTVS- T--  158
A.irradians        IGPATFWDVGQPDGNNSANCMSFYMNADNN-LVWRDDRCTARYNYICEQNATASTPMPVVG 176
A.japonica         AVDFTTWNPGQPDDWQGNEDCVHANVPEQ--KNWNDVDCSTPYRFICALRSNAAGK----  163
C.orbicularis      AFDYLRWGPNEPNNSGGNEDCLHYN----W-LSWNDLRCHYQASYLCQRAAE--------  148
A.aegypti          RLEFTRWAPGEPSEYGENCVMMAYWPSQGFHWTWNDAYCSSKYYAICELRYCI-------  154
```

Fig. 2. Multiple sequence alignment (ClustalW) of CvML (*Crassostrea virginica*) with similar C-Type lectins from *Crassostrea gigas* (BAF75353), *Argopecten irradians* (ABZ89710), *Anguilla japonica* (BAB47156), *Codakia orbicularis* (AAX19697) and *Aedes aegypti* (EAT36508). Amino acid residues that are 100% conserved are noted with an *, and similar amino acids are presented with one (.) or two (:) dots. The motif of the CRD for ligand binding (white) and the putative calcium binding sites (gray) are boxed.

(6.5-fold, *t*-test, $p = 0.3$) of *C. virginica* compared to unaltered controls (Fig. 5B). Seventy-two hours after injection of bacteria in the adductor muscle, no significant increase in CvML expression was detected in the digestive gland (50-fold, *t*-test, $p > 0.05$), labial palps (37-fold, *t*-test, $p > 0.05$), mantle (4-fold, *t*-test, $p > 0.05$), and gills (3-fold, *t*-test, $p > 0.05$) of *C. virginica* compared to oysters injected with seawater (Fig. 5B).

4. Discussion

Most lectins described in marine organisms are either closely associated to hemocytes or hemolymph or are suspected or found to be involved in self-defense. In the present study, we identified a secretory C-type lectin specifically produced in mucocytes associated with the epithelial layer covering the digestive gland and the pallial organs of the eastern oyster *C. virginica*. This new lectin was tentatively named CvML (*C. virginica* Mucocyte Lectin). We further demonstrated increase in CvML transcript levels in response to starvation and presence of bacteria in the pallial cavity but not to bacterial injection into the circulatory system. Based on these evidences, we suggest that CvML could be involved in the capture of particles (microalgae or pathogen), therefore contributing to oyster mucosal immunity.

CvML was detected in mucocytes lining the digestive gland and pallial organs of *C. virginica*, including mantle, gills, and labial palps but not in internal organs and tissues such as gonads or hemocytes. The secretory nature of this lectin was strongly suggested by the presence of a peptide signal at the N-terminus part of the protein [46,47] and is consistent with its location in mucocytes, which secrete mucus covering digestive tract and pallial organs. Current results do not allow us to confirm that CvML is in fact released in oyster mucus although this is likely the case. For instance, mucosal lectins have been described in specialized epithelial cells in several fish species [12,48–50] before being secreted into mucus. Skin mucus is one of the most important defensive barriers in fish [51] and therefore, it is not surprising that mucus lectins have been suggested or found to act as important defense molecules against pathogens [12,52]. Mucosal lectins have also been described in several invertebrates [15,18,53–55] but their origin and function were not always clearly established. Bulgheresi et al. [15] have however shown that *Laxus oneistus*, a marine nematode, produces a C-type lectin located in the posterior glandular sensory organs underlying the animal cuticle. This lectin is secreted onto the cuticle surface along with mucus and was suggested to mediate the colonization of the worm by their bacterial symbionts.

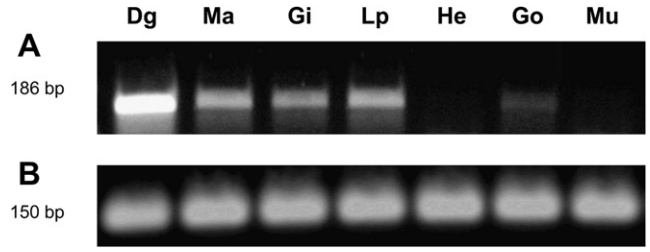

Fig. 3. Expression analysis of CvML transcripts by RT-PCR ($n = 1$ pool of 8 oysters). Identical amounts of total RNA from digestive gland (Dg), mantle (Ma), gills (Gi), labial palps (Lp), hemocytes (He), gonad (Go), and muscle (Mu) were reverse transcribed into cDNA. PCR amplifications were performed using CvML specific primers (A, see Table 1). The expression of the housekeeping gene (18S) in each sample is presented in (B).

Further analysis revealed that CvML sequence presents several similarities with C-type lectins identified in the digestive gland of the oyster *C. gigas*, in the hemocytes of the scallop *A. irradians*, and in the skin mucus of anguilliformes such as the Japanese eel *Anguilla japonica* (Table 2 and Fig. 2). They all share similar patterns and have comparable size ranges (130–180 amino acids). In addition, the CRD (carbohydrate recognition domain) sequence of CvML includes two putative sites, WID and HDC, for calcium binding [28,56] as well as a triplet (X-Pro-Y) known to be involved in carbohydrate binding [57,58]. For example, the QPD motif (Gln, Pro, Asp) has a high affinity for galactose and resembling residues especially when a W (Trp) is located in the vicinity of the motif [57,58]. In CvML, this triplet is YPD (Tyr, Pro, Asp), which is also present in C. gigas C-type lectin (Table 2 and Fig. 2) and in a *Drosophila persimilis* C-type lectin (NCBI, 194101872). To the best of our knowledge, the carbohydrate specificity of the YPD motif has not been described yet, but motifs close to QPD are known, however, to bind a variety of sugars with weak affinity [59]. These observations suggest that CvML might have a wide recognition

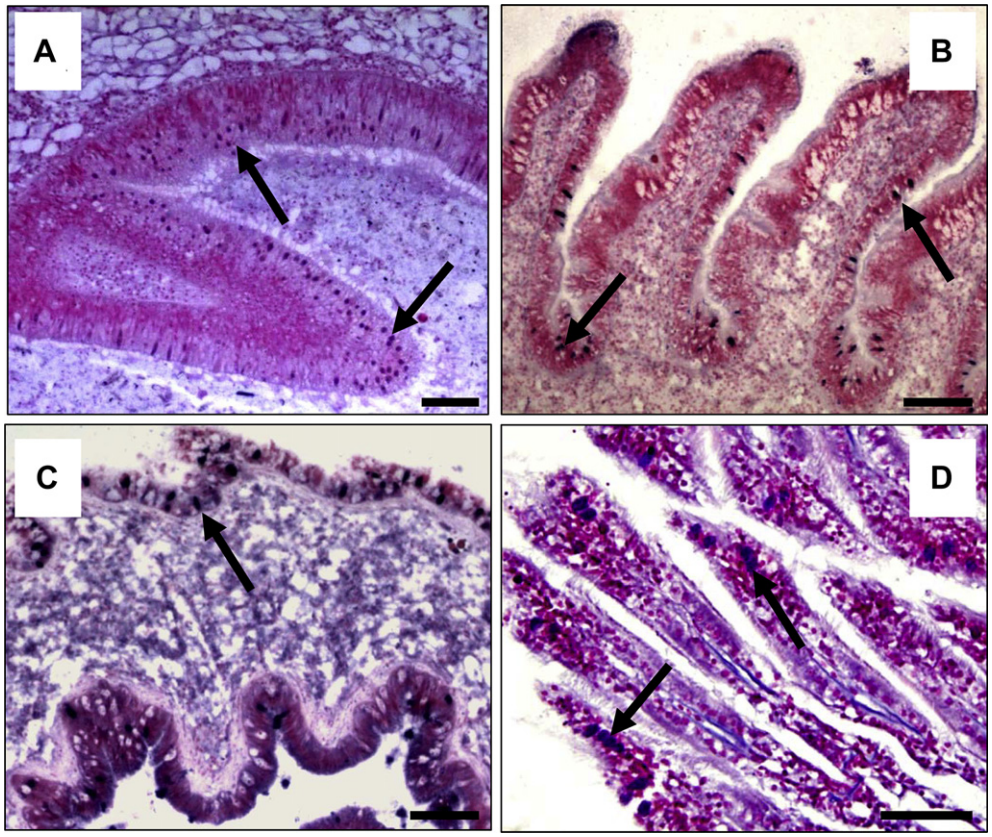

Fig. 4. In situ hybridization localization of CvML in the epithelia of digestive tract (A), labial palps (B), mantle (C), and gills (D) of *Crassostrea virginica*. The arrows indicate positive cells for CvML transcripts. Scale bar = 50 μm.

range allowing mucus to interact and bind carbohydrates covering the cell surface of a diverse group of microorganisms.

Further experiments were conducted to establish the function of CvML in oyster based on the limited information on the function of pallial mucus in bivalve immunity. As a matter of fact, our recent investigations in bivalves demonstrated that mucus covering pallial organs (gills, palps) contains lectins that are involved in the mechanism of food particle selection [18,19,36]. It is well known that suspension-feeding bivalves (including oysters) are able to sort particles using their gills and/or labial palps to enhance the nutritive value of consumed particles by ingesting preferentially particles of interest while undesirable ones (such as detritus or mineral particles) are rejected in pseudofeces before ingestion [60,61]. Several factors are known to control this mechanism, but we recently demonstrated that biochemical recognition, via lectins present in pallial mucus, mediates particle selection as well [18,19,36]. Based on these prior results, we hypothesized that starvation and the increase of the mineral load (and consequently the dilution of nutritive particles) could affect CvML gene transcription level. Results of real-time PCR indicated a clear induction of the lectin in pallial tissues after 3 days of starvation. Moreover, the addition of mineral particles (kaolin) in seawater also caused a significant increase in CvML transcript levels in labial palps. Upregulation of CvML in gills and, above all, in labial palps of starved oysters may reflect an attempted increase in the efficiency of capture of nutritive particles, further supporting the involvement of this lectin in particle selection mechanism. Our recent studies on a similar mucosal lectin identified in *M. edulis* (MeML) demonstrated that 5 days starvation induced a significant increase in lectin expression in gills and labial palps [35]. These findings strongly support the role of these 2 bivalve mucosal lectins in particle capture/selection.

Invertebrate lectins have been reported to be involved in various immune responses, including opsonisation [62–64], antibacterial activity [65,66], prophenoloxidase activation [24,67], and encapsulation and melanization [68,69]. In addition, pathogen challenge (including bacteria and protists) is known to induce an overexpression of lectin genes in various bivalve species [27,70–72]. In our study, however, injection of the opportunistic pathogen *V. alginolyticus* did not induce significant changes in lectin expression in any tested organ and only a weak increase of CvML was detected in oyster gills 6 h post-inoculation. Nevertheless, our results showed significant upregulation of CvML following bath exposure to the same bacteria. These results suggest that CvML can be regulated in response to external stimuli perceived at the pallial interfaces. Bacteria injected into circulation and their products are unlikely to reach CvML-producing mucocytes and for this reason may not regulate CvML expression in spite of the important stress caused by bacterial injection and the likely changes in several hemolymph parameters. On the other hand, bath exposure to bacteria appears to provide the signaling needed to initiate CvML regulation. A prior study by Allam et al. [73] showed significant increase in lysozyme activity in the extrapallial fluid (fluid located between the mantle and shell) but not in hemolymph from clams one day after inoculation of *Vibrio tapetis* into the mantle (shell) cavity fluid. These authors concluded that local signaling at the pallial surfaces can initiate local (as opposed to systemic) regulation of specific defense factors. Our results also support this scenario and highlight the role of pallial surfaces as a second defense line beyond the shell. Therefore, mucus covering pallial organs appears

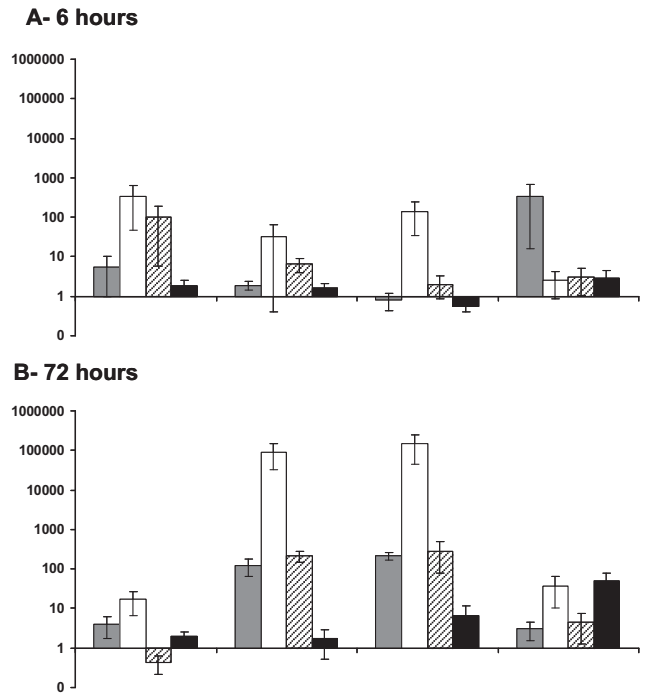

Fig. 5. Expression of CvML in gills, labial palps, mantle and digestive gland after exposure to mineral particles (kaolin), starvation, bacterial bath exposure and injection of bacteria into circulation determined by quantitative real-time PCR. Expression levels were determined at 6 h (A) and 72 h (B) and normalized to 18S RNA. Expression values in each experimental group were then normalized to their corresponding controls (represented by the x-axis) and results are presented as relative expression (mean ± SD, $n = 6$ oysters/treatment). For each organ, * indicates significantly higher expression (t-test, $p < 0.05$) in treated oysters compared to their corresponding controls.

to play a central role in the interactions with waterborne particles whether these particles are nutritious [18,35] or noxious (this study). With that regard, CvML could act passively by immobilizing waterborne microorganisms in mucus, facilitating their elimination through ingestion and subsequent digestion or rejection in pseudofeces. Dual functions of mucosal lectins have been previously proposed in the coral *Acropora millepora*. In this species, the millectin (a mannose–lectin binding) is suspected to be implicated in both symbiosis and defense mechanism since this molecule is able to bind coral symbionts and pathogens [53].

CvML is a novel putative C-type lectin identified in the eastern oyster, *C. virginica*. More specifically, this lectin was detected in the mucocytes lining the epithelium of the digestive gland and the pallial organs, which are in direct contact with seawater and are used by oysters to process waterborne particles. The expression of this lectin was strongly regulated after starvation and bacterial bath exposure. Taken together, these findings support the involvement of CvML in particle capture (and/or selection) and in oyster mucosal immunity. Further studies are needed to determine the specific role of this lectin in particle (food, pathogens, etc.) processing and its possible participation in other physiological functions. Finally, CvML regulation in oysters in different life stages and physiological conditions should be evaluated.

Acknowledgments

We would like to thank F. M. Flower and Sons Oyster Company, Oyster Bay, NY, USA for providing adult and juvenile oysters and Dr. J.P. Gourdine for offering valuable advices. This work was funded in part by a grant from the National Science Foundation to EPE and BA (IOS-0718453) and by China Scholarship Council (CSC) scholarship (to XJ).

References

[1] Nyholm SV, McFall-Ngai MJ. The winnowing: establishing the squid–*Vibrio* symbiosis. Nat Rev Microbiol 2004;2:632–42.
[2] Sperandio V, Torres AG, Jarvis B, Nataro JP, Kaper JB. Bacteria-host communication: the language of hormones. Proc Natl Acad Sci U S A 2003;100:8951–6.
[3] Cummins SF, Degnan BM, Nagle GT. Characterization of *Aplysia* Alb-1, a candidate water-borne protein pheromone released during egg laying. Peptides 2008;29:152–61.
[4] Ferrer RP, Zimmer RK. The scent of danger: arginine as an olfactory cue of reduced predation risk. J Exp Biol 2007;210:1768–75.
[5] Griffiths CL, Richardson CA. Chemically induced predator avoidance behaviour in the burrowing bivalve *Macoma balthica*. J Exp Mar Biol Ecol 2006;331:91–8.
[6] Sharon N, Lis H. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology 2004;14:53R–62R.
[7] Vasta GR, Ahmed H. Animal lectins: a functional view. Boca Raton: CRC Press; 2008.
[8] Vasta GR. Roles of galectins in infection. Nat Rev Microbiol 2009;7:424–38.
[9] Naganuma T, Ogawa T, Hirabayashi J, Kasai K, Kamiya H, Muramoto K. Isolation, characterization and molecular evolution of a novel pearl shelf lectin from a marine bivalve, *Pteria penguin*. Mol Divers 2006;10:607–18.
[10] Kenjo A, Takahashi M, Matsushita M, Endo Y, Nakata M, Mizuochi T, et al. Cloning and characterization of novel ficolins from the solitary ascidian, *Halocynthia roretzi*. J Biol Chem 2001;276:19959–65.
[11] Tasumi S, Vasta GR. A galectin of unique domain organization from hemocytes of the eastern oyster (*Crassostrea virginica*) is a receptor for the protistan parasite *Perkinsus marinus*. J Immunol 2007;179:3086–98.
[12] Tasumi S, Ohira T, Kawazoe I, Suetake H, Suzuki Y, Aida K. Primary structure and characteristics of a lectin from skin mucus of the Japanese eel *Anguilla japonica*. J Biol Chem 2002;277:27305–11.
[13] Hager KM, Carruthers VB. MARveling at parasite invasion. Trends Parasitol 2008;24:51–4.
[14] Rabinovich GA, Gruppi A. Galectins as immunoregulators during infectious processes: from microbial invasion to the resolution of the disease. Parasite Immunol 2005;27:103–14.
[15] Bulgheresi S, Schabussova I, Chen T, Mullin NP, Maizels RM, Ott JA. A new C-type lectin similar to the human immunoreceptor DC-SIGN mediates symbiont acquisition by a marine nematode. Appl Environ Microb 2006;72:2950–6.
[16] Wood-Charlson EM, Hollingsworth LL, Krupp DA, Weis VM. Lectin/glycan interactions play a role in recognition in a coral/dinoflagellate symbiosis. Cell Microbiol 2006;8:1985–93.
[17] Springer SA, Moy GW, Friend DS, Swanson WJ, Vacquier VD. Oyster sperm bindin is a combinatorial fucose lectin with remarkable intra-species diversity. Int J Dev Biol 2008;52:759–68.
[18] Pales Espinosa E, Perrigault M, Ward JE, Shumway SE, Allam B. Lectins associated with the feeding organs of the oyster, *Crassostrea virginica*, can mediate particle selection. Biol Bull 2009;217:130–41.
[19] Pales Espinosa E, Perrigault M, Ward JE, Shumway SE, Allam B. Microalgal cell surface carbohydrates as recognition sites for particle sorting in suspension-feeding bivalves. Biol Bull 2010;218:75–86.
[20] Fisher WS, Dinuzzo AR. Agglutination of bacteria and erythrocytes by serum from 6 species of marine mollusks. J Invertebr Pathol 1991;57:380–94.
[21] Koizumi N, Imai Y, Morozumi A, Imamura M, Kadotani T, Yaoi K, et al. Lipopolysaccharide-binding protein of *Bombyx mori* participates in a hemocyte-mediated defense reaction against gram-negative bacteria. J Insect Physiol 1999;45:853–9.
[22] Yu XQ, Kanost MR. Immulectin-2, a pattern recognition receptor that stimulates hemocyte encapsulation and melanization in the tobacco hornworm, *Manduca sexta*. Dev Comp Immunol 2004;28:891–900.
[23] Holmskov U, Thiel S, Jensenius JC. Collectins and ficolins: humoral lectins of the innate immune defense. Annu Rev Immunol 2003;21:547–78.
[24] Yu XQ, Gan H, Kanost MR. Immulectin, an inducible C-type lectin from an insect, *Manduca sexta*, stimulates activation of plasma prophenol oxidase. Insect Biochem Mol Biol 1999;29:585–97.
[25] Suzuki T, Takagi T, Furukohri T, Kawamura K, Nakauchi M. A calcium-dependent galactose-binding lectin from the tunicate *Polyandrocarpa misakiensis*. Isolation, characterization and amino acid sequence. J Biol Chem 1990;265:1274–81.
[26] Wang H, Song LS, Li CH, Zhao JM, Zhang H, Ni DJ, et al. Cloning and characterization of a novel C-type lectin from zhikong scallop *Chlamys farreri*. Mol Immunol 2007;44:722–31.
[27] Zhu L, Song LS, Xu W, Qian PY. Molecular cloning and immune responsive expression of a novel C-type lectin gene from bay scallop *Argopecten irradians*. Fish Shellfish Immunol 2008;25:231–8.
[28] Gourdine JP, Markiv A, Smith-Ravin J. The three-dimensional structure of codakine and related marine C-type lectins. Fish Shellfish Immunol 2007;23:831–9.
[29] Moy GW, Springer SA, Adams SL, Swanson WJ, Vacquier VD. Extraordinary intraspecific diversity in oyster sperm bindin. Proc Natl Acad Sci U S A 2008;105:1993–8.

[30] Springer SA, Crespi BJ. Adaptive gamete-recognition divergence in a hybridizing *Mytilus* population. Evolution 2007;61:772–83.
[31] Wootton EC, Zubkov MV, Jones DH, Jones RH, Martel CM, Thornton CA, et al. Biochemical prey recognition by planktonic protozoa. Environ Microbiol 2007;9:216–22.
[32] Allen PG, Dawidowicz EA. Phagocytosis in *Acanthamoeba* .1. A mannose receptor is responsible for the binding and phagocytosis of yeast. J Cell Physiol 1990;145:508–13.
[33] Fisher WS. Occurrence of agglutinins in the pallial cavity mucus of oysters. J Exp Mar Biol Ecol 1992;162:1–13.
[34] Pales Espinosa E, Perrigault M, Shumway SE, Ward JE, Wikfors G, Allam B. The sweet relationship between microalgae and *Crassostrea virginica*: implication of carbohydrate and lectin interactions in particle selection in suspension feeding bivalves. J Shellfish Res 2008;27:1006–7.
[35] Pales Espinosa E, Perrigault M, Allam B. Identification and molecular characterization of a mucosal lectin (MeML) from the blue mussel *Mytilus edulis* and its potential role in particle capture. Comp Biochem Physiol A-Mol Integr Physiol 2010;156:495–501.
[36] Pales Espinosa E, Hassan D, Ward JE, Shumway SE, Allam B. Role of epicellular molecules in the selection of particles by the blue mussel, *Mytilus edulis*. Biol Bull 2011;219:50–60.
[37] Yamaura K, Takahashi KG, Suzuki T. Identification and tissue expression analysis of C-type lectin and galectin in the pacific oyster, *Crassostrea gigas*. Comp Biochem Physiol B 2008;149:168–75.
[38] Scotto-Lavino E, Du GW, Frohman MA. 5′ end cDNA amplification using classic RACE. Nat Protoc 2006;1:2555–62.
[39] Scotto-Lavino E, Du GW, Frohman MA. 3′ End cDNA amplification using classic RACE. Nat Protoc 2006;1:2742–5.
[40] Braissant O, Wahli W. A simplified in situ hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. Biochemica 1998;1:10–6.
[41] Pales Espinosa E, Allam B. Comparative growth and survival of juvenile hard clams, *Mercenaria mercenaria*, fed commercially available diets. Zoo Biol 2006;25:513–25.
[42] Cognie B, Barille L. Does bivalve mucus favour the growth of their main food source, microalgae? Oceanol Acta 1999;22:441–50.
[43] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-delta delta C) method. Methods 2001;25:402–8.
[44] Drickamer K. Evolution of Ca_2^+-dependent animal lectins. Prog Nucleic Acid Res Mol Biol 1993;45:207–32.
[45] Drickamer K. 2 distinct classes of carbohydrate-recognition domains in animal lectins. J Biol Chem 1988;263:9557–60.
[46] Blobel G, Dobberstein B. Transfer of proteins across membranes .1. Presence of proteolytically processed and unprocessed nascent immunoglobulin light-chains on membrane-bound ribosomes of *Murine myeloma*. J Cell Biol 1975;67:835–51.
[47] Hegde RS, Bernstein HD. The surprising complexity of signal sequences. Trends Biochem Sci 2006;31:563–71.
[48] Tsutsui S, Iwamoto K, Nakamura O, Watanabe T. Yeast-binding C-type lectin with opsonic activity from conger eel (*Conger myriaster*) skin mucus. Mol Immunol 2007;44:691–702.
[49] Suzuki Y, Kaneko T. Demonstration of the mucous hemagglutinin in the club cells of eel skin. Dev Comp Immunol 1986;10:509–18.
[50] Tsutsui S, Yamaguchi M, Hirasawa A, Nakamura O, Watanabe T. Common skate (*Raja kenojei*) secretes pentraxin into the cutaneous secretion: the first skin mucus lectin in cartilaginous fish. J Biochem 2009;146:295–306.
[51] Okamoto M, Tsutsui S, Tasumi S, Suetake H, Kikuchi K, Suzuki Y. Tandem repeat l-rhamnose-binding lectin from the skin mucus of ponyfish, *Leiognathus nuchalis*. Biochem Biophys Res Commun 2005;333:463–9.
[52] Suzuki Y, Tasumi S, Tsutsui S, Okamoto M, Suetake H. Molecular diversity of skin mucus lectins in fish. Comp Biochem Physiol B 2003;136:723–30.
[53] Kvennefors ECE, Leggat W, Hoegh-Guldberg O, Degnan BM, Barnes AC. An ancient and variable mannose-binding lectin from the coral *Acropora millepora* binds both pathogens and symbionts. Dev Comp Immunol 2008; 32:1582–92.
[54] Furuta E, Takagi T, Yamaguchi K, Shimozawa A. Incilaria mucus agglutinated human erythrocytes. J Exp Zool 1995;271:340–7.
[55] Fountain DW, Campbell BA. A lectin isolate from mucus of *Helix aspersa*. Comp Biochem Physiol B 1984;77:419–25.
[56] Mullin NP, Hitchen PG, Taylor ME. Mechanism of Ca_2^+ and monosaccharide binding to a C-type carbohydrate-recognition domain of the macrophage mannose receptor. J Biol Chem 1997;272:5668–81.
[57] Kolatkar AR, Weis WI. Structural basis of galactose recognition by C-type animal lectins. J Biol Chem 1996;271:6679–85.
[58] Drickamer K. Engineering galactose-binding activity into a C-type mannose-binding protein. Nature 1992;360:183–6.
[59] Childs RA, Wright JR, Ross GF, Yuen CT, Lawson AM, Chai WG, et al. Specificity of lung surfactant protein Sp-A for both the carbohydrate and the lipid moieties of certain neutral glycolipids. J Biol Chem 1992;267:9972–9.
[60] Ward JE, Levinton JS, Shumway SE, Cucci T. Particle sorting in bivalves: in vivo determination of the pallial organs of selection. Mar Biol 1998;131:283–92.
[61] Defossez JM, Daguzan J. About preferential ingestion of organic matter by bivalves. J Mollus Stud 1996;62:394–7.
[62] Luo T, Yang HJ, Li F, Zhang XB, Xu X. Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon*. Dev Comp Immunol 2006;30:607–17.
[63] Kondo M, Matsuyama H, Yano T. The opsonic effect of lectin on phagocytosis by hemocytes of kuruma prawn, *Penaeus japonicus*. Fish Pathol 1992;27: 217–22.
[64] Kim YM, Park KI, Choi KS, Alvarez RA, Cummings RD, Cho M. Lectin from the manila clam *Ruditapes philippinarum* is induced upon infection with the protozoan parasite *Perkinsus olseni*. J Biol Chem 2006;281:26854–64.
[65] Schroder HC, Ushijima H, Krasko A, Gamulin V, Thakur NL, Diehl-Seifert B, et al. Emergence and disappearance of an immune molecule, an antimicrobial lectin, in basal Metazoa – a tachylectin-related protein in the sponge *Suberites domuncula*. J Biol Chem 2003;278:32810–7.
[66] Hatakeyama T, Suenaga T, Eto S, Niidome T, Aoyagi H. Antibacterial activity of peptides derived from the C-terminal region of a hemolytic lectin, CEL-III, from the marine invertebrate *Cucumaria echinata*. J Biochem 2004;135: 65–70.
[67] Chen CL, Durrant HJ, Newton RP, Ratcliffe NA. A study of novel lectins and their involvement in the activation of the prophenoloxidase system in *Blaberus discoidalis*. Biochem J 1995;310:23–31.
[68] Ling EJ, Yu XQ. Cellular encapsulation and melanization are enhanced by immulectins, pattern recognition receptors from the tobacco hornworm *Manduca sexta*. Dev Comp Immunol 2006;30:289–99.
[69] Ma THT, Benzie JAH, He JG, Chan SM. PmLT, a C-type lectin specific to hepatopancreas is involved in the innate defense of the shrimp *Penaeus monodon*. J Invertebr Pathol 2008;99:332–41.
[70] Kim JY, Kim YM, Cho SK, Choi KS, Cho M. Noble tandem-repeat galectin of Manila clam *Ruditapes philippinarum* is induced upon infection with the protozoan parasite *Perkinsus olseni*. Dev Comp Immunol 2008;32:1131–41.
[71] Kang YS, Kim YM, Park KI, Cho SK, Choi KS, Cho M. Analysis of EST and lectin expressions in hemocytes of Manila clams (*Ruditapes philippinarum*) (Bivalvia: Mollusca) infected with *Perkinsus olseni*. Dev Comp Immunol 2006;30: 1119–31.
[72] Perrigault M, Tanguy A, Allam B. Identification and expression of differentially expressed genes in the hard clam, *Mercenaria mercenaria*, in response to quahog parasite unknown (QPX). BMC Genomics 2009;10:17.
[73] Allam B, Paillard C, Auffret M. Alterations in hemolymph and extrapallial fluid parameters in the Manila clam, *Ruditapes philippinarum*, challenged with the pathogen *Vibrio tapetis*. J Invertebr Pathol 2000;76:63–9.