Journal of Proteomics 132 (2016) 63–76

Contents lists available at ScienceDirect

Journal of Proteomics

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

Proteomic characterization of mucosal secretions in the eastern oyster, *Crassostrea virginica*

Emmanuelle Pales Espinosa [a,*], Antonius Koller [b], Bassem Allam [a]

[a] School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY 11794-5000, United States
[b] Proteomics Center, Stony Brook University Medical Center, Stony Brook, NY 11794-8691, United States

ARTICLE INFO

Article history:
Received 25 September 2015
Received in revised form 3 November 2015
Accepted 17 November 2015
Available online xxxx

Keywords:
Bivalve
Mucus
Pallial cavity
Proteomic
Mass spectrometry

ABSTRACT

The soft body surface of marine invertebrates is covered by a layer of mucus, a slippery gel secreted by mucocytes lining epithelia. The functions of this gel are diverse including locomotion, cleansing, food particles processing and defense against physicochemical injuries and infectious agents. In oysters, mucus covering pallial organs has been demonstrated to have a major importance in the processing of food particles and in the interactions with waterborne pathogens. Given the limited information available on mucus in bivalves and the apparent wide spectra of activity of bioactive molecules present in this matrix, the characterization of these mucosal secretions has become a research priority. In this study, mucus was separately collected from the mantle, gills and labial palps of the eastern oyster (*Crassostrea virginica*) and analyzed by liquid chromatography and tandem mass spectrometry. Results showed the presence of a wide variety of molecules involved in host–microbe interactions, including putative adhesion molecules (e.g. c-type lectins) confirming that transcripts previously identified in epithelial cells are translated into proteins secreted in mucus. Mucus composition was different among samples collected from different organs. These results generate a reference map for *C. virginica* pallial mucus to better characterize the various physiological functions of mucosal secretions.

1. Introduction

The body surface of marine invertebrates is often covered by a layer of mucus, a slippery gel secreted by mucocytes lining epithelia [1–4]. It is made of mucins, water, electrolytes, epithelial and blood cells and a wide range of bioactive molecules produced by these and mucus-secreting cells [5]. The consistency, viscosity and elasticity of mucus are generally attributed to polymers within the gel and to the physical entanglement of these polymers with other molecules [6–8]. These polymers are mucoproteins associated with carbohydrates, such as high molecular weight mucins and mucin-like glycoproteins [9] that are heavily glycosylated (up to 90% of carbohydrate) and present short carbohydrate chains [10–12] whose charges are slightly negative. In addition to large mucin type glycoproteins, mucus matrices have been found to also contain repetitive highly sulfated polysaccharides [13] and a wide variety of bioactive molecules [14–16]. Based on the biochemical diversity of molecules contained in vertebrate and invertebrate mucus, it is not surprising that these gels are involved in various functions as speculated by Auld [17] as early as 1920.

The major role of mucus in invertebrates is to serve as a protector of epithelial cells against physicochemical injuries [18–21] or infectious agents [22,23]. In addition, mucus is also used in many other activities including locomotion [24,25], adhesion [26], cleansing of body surface [27], and nutrition [28,29]. If the roles of mucus in invertebrates are particularly studied [2,30], information on its composition is limited, probably due to its complexity, seasonal variation [31] and the limitation of analytical methods. In particular, the exact nature of glycoconjugate matrices and associated carbohydrates (mucin-type glycoproteins and polysaccharides, see review by [2]) are poorly known [13]. Despite these limitations, some studies have identified or suspected the presence in invertebrate mucus of lysozymes [1,32,33], terpenoids [34], antimicrobial peptides [22], antioxidants [14], proteases [1,35], agglutinins [36] and lectins [15,37,38].

In the eastern oyster, *Crassostrea virginica*, mucus is abundantly secreted and contributes to the processing of waterborne particles. As all suspension-feeding bivalves, *C. virginica* use their gill to pump water into their pallial (i.e. shell) cavity to capture, process and transport food particles [39–41]. Particles captured on gills are transferred to a pair of sorting organs surrounding the mouth called the labial palps, and from there are either rejected as pseudofeces through a specialized area on the mantle, or directed to the mouth and ingested [42]. In addition, *C. virginica* as many other bivalves, was found to be able to differentiate between nutritious and detrital particles (see the review by [29]). Interestingly, mucus is involved in all of these steps. Particles directed as pseudofeces are embedded in mucus and rejected back to the environment as masses of mucoid substances entangling live unwanted cells, debris and abiotic material of low nutritional

* Corresponding author.
E-mail address: Emmanuelle.PalesEspinosa@stonybrook.edu (E. Pales Espinosa).

http://dx.doi.org/10.1016/j.jprot.2015.11.018
1874-3919/

value. Those directed to the mouth are ingested in a cohesive mucus string [43]. In addition, mucus has been suspected to mediate particle selection [44]. Recently, lectins have been identified in mucus covering *C. virginica* feeding organs [15,45] and were found to interact with carbohydrates associated with microalgae cell surface and to mediate food particle sorting [15,45–47].

Bivalve mucus has also been found to promote or inhibit the growth of diverse microorganisms. For example, the mucus contained in the biodeposits (i.e. pseudofeces and feces) of the oyster *Crassostrea gigas* was shown to stimulate microalgae growth [48]. Similarly, Allam et al. [49] showed that while mucus collected from oyster pallial organs (mantle in particular) enhanced the proliferation of *Perkinsus marinus* (a lethal parasite of the eastern oyster *C. virginica*), mucus collected from the digestive gland was inhibitory. Interestingly, pallial mucus of the noncompatible host *C. gigas* (Pacific oyster) was strongly inhibitory suggesting that *P. marinus* host specificity may begin in the mucus. The *in vivo* virulence of *P. marinus* was also significantly enhanced when the parasite was exposed to pallial mucus from *C. virginica* [50]. Mortality was significantly higher (up to 10 fold) in oysters injected with parasite cultures supplemented with pallial mucus as compared to oysters injected with parasite cells supplemented with digestive mucus or unsupplemented cultures.

Given the limited information available on the biochemical composition of pallial mucus in bivalves and the apparent wide spectra of activity of molecules present in this matrix, the identification of the proteomic makeup of these secretions has become a research priority. In this study, mucus was collected from the principal pallial organs (i.e. mantle, gills and labial palps) of *C. virginica* and analyzed by tandem mass spectrometry to create a proteome reference map for the eastern oyster pallial mucus. Results were analyzed with a particular focus on molecules involved in adhesion and interaction with waterborne microbes. To our knowledge, this work represents the first comprehensive proteomic analysis of mucus in bivalves (see [51] for review).

2. Material and methods

2.1. Mucus collection

Adults *C. virginica* (85–90 mm in length, n = 9) were obtained from a commercial source (Frank M. Flower and Sons Oyster Company, Oyster Bay, New York, USA) in April 2012 (temperature = 7 °C, salinity = 28). Animals were carefully opened and tissues were abundantly rinsed with artificial seawater (ASW28, salinity of 28). Mucus from pallial organs (i.e. gills, mantle and labial palps) was separately collected following the general procedures described by Pales Espinosa et al. [15]. Briefly, mucus was carefully collected using small sterile pieces of cotton-balls. Cotton-balls were then immersed in 5 to 10 ml of ice-cold ASW. Tubes containing cotton-balls were placed at 4 °C for 1 h on a rotating shaker. The resulting fluids (i.e. 27 samples) were centrifuged (3000 *g*, 30 min, 4 °C), filter sterilized (0.22 μm syringe filters) and maintained at 4 °C until use, typically within the following hour. A 25 μl aliquot of each fluid was used to determine protein concentrations with a Pierce BCA protein assay reagent kit (Pierce, Rockford, Illinois, USA) as per manufacturer's recommendations. Fluids were then diluted with ASW28 to a protein concentration of 2 $mg \cdot ml^{-1}$. Fluids from each pallial organ (i.e. gills, mantle or labial palps) were then pooled (equal volume) in order to obtain 3 pools made from 3 oysters each.

2.2. Electrophoresis

Plasma and extrapallial fluid of *C. virginica* contain a major protein designated dominin (Itoh et al., 2011), and our preliminary analyses showed relatively high abundance of dominin in pallial mucus as well. A pre-separation step on gel was therefore implemented to improve the resolution of our proteomic analysis and favor the detection of low abundance proteins. Mucus samples (25 μl) were mixed with 25 μl of 2× denaturing sample buffer, heated to 100 °C for 10 min and separated (20 μg per well) on a precast 12% Tris–Glycine gel (Jule Biotechnologies, Inc., Milford, CT). After electrophoresis, gels were stained using standard Comassie blue protocol. Each gel lane was excised into 12 equal slices, de-stained, reduced, alkylated and digested with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, USA) as described by Shevchenko et al. [52] with minor modifications. Special care was taken to prevent keratin contamination. Samples from different organs (i.e. gills, mantle or labial palps) were run on separate gels in order to avoid contamination and replicates were run on the same gel. Results were similar within each of the 3 groups.

2.3. Mass spectrometry and data analysis

The resulting concentrated peptide extract was diluted into a solution of 2% acetonitrile (ACN), 0.1% formic acid (FA) (buffer A) for analysis. Ten microliters of the peptide mixture were analyzed by automated microcapillary LC/MS-MS. Fused-silica capillaries (100 μm inner diameter (i.d.)) were pulled using a P-2000 CO2 laser puller (Sutter Instruments, Novato, CA, USA) to a 5 μm i.d. tip and packed with 10 cm of 5 μm Magic C18 material (Agilent Technologies, Santa Clara, CA, USA) using a pressure bomb. Ten microliters of the resulting 20 μl of concentrate were pressure-loaded onto a 10 cm 100 μm i.d. fused-silica capillary packed with 3 μm Magic C18 reverse phase (RP) particles (Michrome, USA) which have been pulled to a 5 μm i.d. tip using a P-2000 CO2 laser puller (Sutter Instruments). This column was then installed in-line with a Dionex 3000 HPLC pump running at 300 $nL\ min^{-1}$. Peptides were loaded with an auto-sampler directly onto the column and were eluted from the column by applying a 30 min gradient from 5% buffer B to 40% buffer B (98% ACN, 0.1% FA). The gradient was switched from 40% to 80% buffer B over 5 min and held constant for 3 min. Finally, the gradient was changed from 80% buffer B to 100% buffer A over 0.1 min, and then held constant at 100% buffer A for 15 min longer. The application of a 1.8 kV distal voltage was used to electro-spray the eluting peptides directly into an LTQ XL ion trap mass spectrometer equipped with a nano-liquid chromatography electrospray ionization source. Full MS spectra were recorded on the peptides over 400–2000 m/z, followed by five MS/MS fragmentation events on the five most intense ions. MS scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Finnigan, San Jose, CA, USA). MS/MS spectra were extracted from the RAW data file with ReAdW.exe (http://sourceforge.net/projects/sashimi). The resulting mzXML file contained all of the data for all MS/MS spectra and could subsequently be read by the analysis software.

All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version CYCLONE 2013.02.01.1) set up to search a database (98,316 entries) created by combining published protein sequences from *C. virginica* and *C. gigas* on NCBI and Uniprot/Swissprot (26,612 proteins), longest open reading frames (ORFs) of expressed sequence tags (ESTs) databases from NCBI and marinegenomics.org created with DNA2pep [53] (22,518 proteins) and common contaminants (28 proteins). In addition, a Decoy database (all proteins in reverse order) was also added from this database with compass [54]. This database was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 1.8 Da. Carbamidomethyl of cysteine was specified in X! Tandem as a fixed modification. Glu- > pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln- > pyro-Glu of the n-terminus, deamidated of asparagine and glutamine, oxidation of methionine and tryptophan and dioxidation of methionine and tryptophan were specified in X! Tandem as variable modifications.

Scaffold (version Scaffold_4.4.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 5.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were

assigned by the Protein Prophet algorithm [55]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. The sequences of the proteins identified in pallial mucus were then uploaded into the Blast2GO application [56] to be blasted.

2.4. Statistical analysis

Statistical analysis compared protein composition in mucus collected from the various organs. Some of the identified proteins had one or more missing values (see Supplementary data 2) and were excluded from statistical comparisons (see Supplementary data 3, FDR = NA). Data were not normalized because the distribution of the proteins was uniform across all samples. An empirical Bayesian moderated linear model was fitted to the data in conjunction with an ANOVA F-test assessing the null hypothesis that there is no difference among all groups. The variance of each test at a specific protein was estimated by a Bayesian prior distribution on all proteins. R package "limma" was used to implement the algorithm. The generated p-values for each protein were then adjusted and controlled by FDR via R package "fdrtool". By setting FDR level at 0.1, a candidate list of 205 significant proteins was obtained.

3. Results and discussion

The pallial organs in bivalves are directly exposed to surrounding water and epithelia covering these organs have to be isolated and protected from numerous hazards. Mucus plays this major role, acting as the first barrier of defense against physical, chemical and biological aggressions. Despite the essential role of bivalve mucus, very little is known about its composition. To our knowledge, this work represents the first comprehensive proteomic analysis of pallial mucus in bivalves. Results showed the presence in pallial mucus of a rich repertoire of proteins involved in immune defense and host homeostasis. The results also identified significant differences in the composition of mucus derived from various pallial organs.

3.1. Proteins present in oyster pallial mucus

In this study, 1514 proteins matching *C. gigas* and *C. virginica* predicted proteins were identified (Supplementary data 1) in pallial mucus. For downstream analysis, only proteins for which at least 2 unique peptides were identified, that were present in two out of three replicates and that presented a Log(e) value <−9 were considered. These selection criteria yielded 902 selected proteins that were grouped into 14 categories (Fig. 1) based on their annotation (NCBI database) and a complementary search using Blast2go (GO terms, Enzyme Codes, IPR).

These 902 molecules included proteins from intracellular origin such as actin, elongation factor or ribosomal proteins. The presence of intracellular proteins in pallial mucus is not surprising since these mucosal secretions are known to contain hemocytes and exfoliated epithelial cells [51,57–60]. Interestingly, proteins known to be present in oyster plasma were also detected in pallial mucus, such as the *C. virginica* galectin (CvGal: ABG75998, [61]), suggesting that plasma components transudate into mucus as previously reported in vertebrates [62]. In addition, the 902 identified proteins include 24 proteins matching the GO term "extracellular" and most of these display additional GO functions related to immunity or defense against pathogens (Table 1). Another 100 proteins present a signal peptide (Signal P) suggesting a secretory route.

Proteins of particular interest in host–pathogen or predator–prey relationships were grouped into 4 major categories including: extracellular matrix and glycosylation; immune recognition; immune activation and cell signaling; while effector molecules were divided into 2 main functions: elimination of pathogens and repair of damaged molecules (Supplementary data 2). A more detailed description of these categories is given below.

3.1.1. Extracellular matrix and glycosylation

As mentioned previously, mucin-like glycoproteins form the matrix of mucus (see the review by [51]). Functions and features of these gels are highly correlated with the nature and the proportion of these backbone molecules [7]. Five proteins associated with extracellular matrix and glycosylation were identified in oyster pallial mucus (Supplementary data 2). The occurrence of these proteins in mucus may have been

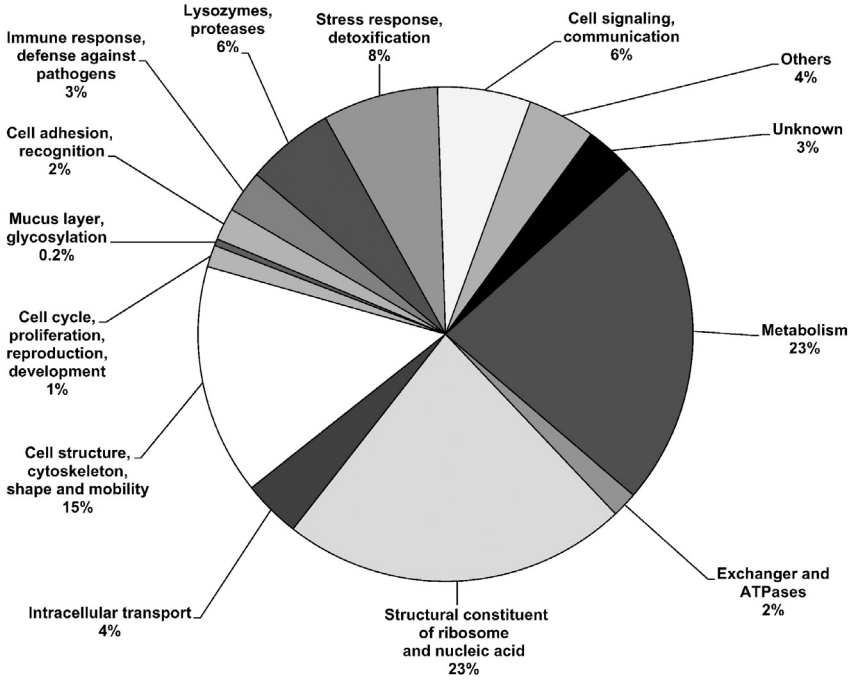

Fig. 1. Classification of the proteins identified in pallial mucus of *C. virginica*.

Table 1
Proteins with the GO (Gene Ontology) term "extracellular" identified in oyster mucus.

Code	Protein identification	GenBank	eValue	GO terms
MGID91410	alkaline phosphatase	EKC39918	3.99E-37	C:extracellular space; F:alkaline phosphatase activity; C:anchored to membrane; P:cellular response to organic cyclic compound, glucocorticoid stimulus, antibiotic
K1QDX7_CRAGI	SCO-spondin	EKC32153	0	P:cellular process; F:cell adhesion; C:extracellular matrix structural constituent
MGID153402	x-box binding	EKC29415	2.14E-105	P:cell-matrix adhesion; F:calcium ion binding; C:extracellular region
MGID152531	macrophage mannose receptor 1	EKC26386	7.54E-64	F:binding; F:calcium ion binding; C:extracellular region; P:pathogenesis
MGID90144	serine protease inhibitor cvsi-2	B9A8D7	1.01E-41	P:negative regulation of peptidase activity; C:extracellular region; F:serine-type endopeptidase inhibitor activity
MGID90360	kyphoscoliosis peptidase	EKC37229	3.35E-99	P:cell-matrix adhesion; F:calcium ion binding; C:extracellular region
gi|78675527	lysozyme 1	P83673	3.24E-116	C:extracellular region; P:defense response to Gram-positive bacterium; P:cytolysis; F:lysozyme activity; P:defense response to Gram-negative bacterium
gi|152812971	prohormone convertase 1	NP_001131134	1.18E-46	P:peptide hormone processing; C:extracellular space; F:protein binding; F:serine-type endopeptidase activity; C:secretory granule; C:Golgi apparatus
gi|152818513	IgGFc-binding protein	EKC36905	1.48E-77	P:extracellular
gi|152815434	x-box binding protein	AEF33390	2.34E-68	C:extracellular matrix
MGID95042	glucose-6-phosphate isomerase	EKC19730	2.39E-143	F:cytokine activity; C:extracellular space; F:glucose-6-phosphate isomerase activity; C:cytoplasm; C:nucleus; C:plasma membrane
K1RGQ0_CRAGI	ras-related c3 botulinum toxin substrate	EKC40565	0	P:nerve growth factor receptor signaling pathway; P:cell-matrix adhesion; F:GTP-dependent protein binding; P:inflammatory response
MGID91034	n-acetylmuramoyl-l-alanine amidase	EKC26199	3.29E-34	C:extracellular region; P:defense response to bacterium; F:N-acetylmuramoyl-L-alanine amidase activity; P:peptidoglycan catabolic process
K1QAH0_CRAGI	thaumatin-like protein	EKC25850	1.37E-125	F: response to other organism; C: extracellular region
K1QHR5_CRAGI	thioester-containing protein	EKC28380	0	P:negative regulation of endopeptidase activity; C:extracellular space; C:extracellular region; F:endopeptidase inhibitor activity
K1RWY1_CRAGI	thioester-containing protein	EKC39431	0	C:extracellular space; F:endopeptidase inhibitor activity; P:negative regulation of endopeptidase activity
K1QAH8_CRAGI	thioester-containing protein-c	EKC33672	0	C:extracellular space; F:endopeptidase inhibitor activity; P:negative regulation of endopeptidase activity
gi|152814077	thioester-containing protein-d	EKC33672	3.86E-86	C:extracellular space; F:endopeptidase inhibitor activity; P:negative regulation of endopeptidase activity
K1PVK1_CRAGI	thioester-containing protein-d	EKC28382	0	P:negative regulation of endopeptidase activity; C:extracellular space; C:extracellular region; F:endopeptidase inhibitor activity
K1QI93_CRAGI	thioester-containing protein-g	EKC33503	0	C:extracellular region; F:endopeptidase inhibitor activity
MGID93755	thioester-containing protein-g	EKC33672	1.06E-53	C:extracellular region; F:endopeptidase inhibitor activity
K1QDV2_CRAGI	neuroendocrine convertase 1	EKC26985	0	C:secretory granule; P:response to glucose stimulus and lipopolysaccharide; F:serine-type endopeptidase activity; F:insulin binding; C:extracellular space
K1QET2_CRAGI	coatomer subunit alpha	EKC29564	0	P:intra-Golgi vesicle-mediated transport; P:retrograde vesicle-mediated transport, Golgi to ER; C:extracellular region; C:COPI vesicle coat; F:hormone activity
gi|152814483	cdc42-like protein	AEF33422	4.46E-75	F:GTP binding; P:regulation of protein heterodimerization activity; P:positive regulation of peptidyl-serine phosphorylation; C:secretory granule

underestimated as an artifact of our sample processing procedures which may reduce sticky proteins that can adhere to the sample filtration devices. The genome of *C. gigas* [63] does not contain sequences coding for mucin but does contain sequences coding for a halomucin (EKC38926), which is homologous to the mammalian mucins (Sublimi-Saponetti et al., 2011), and a SCO-spondin (EKC32153). Spondin is characterized by 2 mucin-2 protein repeats and presents high similarity with the mucin-5 AC-like (*Aplysia californica*, XP_005092239). Both halomucin (only identified in preliminary analysis, data not shown) and SCO-spondin (this study) were identified with low abundance. SCO-spondin is relatively poor in threonine (9%), proline (7.5%) and serine (6%) as opposed to mucin-like molecules from other organisms where the proportions of these amino acids can vary from 20 to 60% [13,64]. Interestingly, mucus covering gastropod limpet is also poor in threonine and serine and its composition was suggested to be based on polymers other than mucin molecules [26]. Additionally, a preliminary glycosyl profiling performed on the same mucus samples revealed that the O-linked glycans in these mucopolysaccharides are mostly composed of mannose residues in terminal reducing position (data not shown) and not the standard GalNAc residues described in mucin found in vertebrates [65]. These two results (i.e. polymer with low percentage in threonine, proline or serine coupled with non-standard glycosyl composition) suggest the presence in oyster pallial mucus of mucopolysaccharides other than the classical mucin, in agreement with results reported earlier in limpets [26].

Pallial mucus also contained an IgGFc-binding protein (EKC36905), which is thought to be involved in the maintenance of the mucosal structure as a gel-like matrix. In fact, this protein is known to strongly interact with mucin 2 [66] and was suggested to play a role in mucosal immunological defenses [67]. The glycoprotein glucosyltransferase 1 (EKC37861) was also identified and maybe involved in the re-glycosylation of misfolded glycoproteins [68]. Finally, two X-box binding glycoproteins with conserved ependymin domain were found.

3.1.2. Immune recognition

The aptitude of an organism to distinguish between self and non-self is vital in innate immunity because it allows the initiation of the destruction of foreign organisms or damaged cells. Invertebrates have developed a complex system of receptors designated as PRR (pattern recognition receptors) made of dissolved or cell surface-associated proteins that are able to identify specific molecular patterns expressed by either microorganisms or damaged host cells. Several groups of PRRs have been identified in mollusks [51,69–71], but information on recognition receptors in molluscan mucosa (i.e., epithelial cells or mucus) is extremely scarce. In this proteomic analysis, a total of 21 proteins were homologous to molecules involved in immune recognition (Supplementary data 2) even though the boundary between "recognition" and "effectors" is sometimes unclear and a unique protein can be involved in multiple functions. This is the case of two peptidoglycan hydrolases (N-acetylmuramoyl-L-alanine amidases, EK26199) that are

similar to the CgPGRP-S3 (BAG31899, from digestive diverticula), the CgPGRP-S1S (BAG31896, from mantle and gills), and CgPGRP-S1L (BAG31897, from gills) expressed in mucosal tissues of *C. gigas* [72]. PGRPs, and most specifically the amidases subgroup, are involved in the recognition, binding and degradation of bacterial cell-wall glycopeptides in many species from insects to mammals. Additionally, one of these proteins is also characterized by an "extracellular region" GO term indicating its humoral nature and supporting its presence in mucosal secretions.

The analysis further revealed the presence of 10 proteins containing either a galactose-binding or C-type lectin domain. Lectins are a large and diverse group of sugar-binding proteins that specifically and reversibly bind to glycans including those covering living cells [73,74]. Their functions are diverse and they have been described to be involved in host–microbe interactions in the framework of parasitic [61,75,76], or mutualistic [37,77,78] associations. Some of these lectins are known or suspected to be present in hemolymph or associated with hemocyte membranes (e.g. the galectin2 = CvGal: ABG75998, detected on hemocyte membrane, Tasumi and Vasta [61]; the tandem-repeat galectin (EKC40501) highly similar to the galectin found on *Ostrea edulis* hemocytes, Morga et al., [79]; the macrophage mannose receptor (EKC26386), similar to proteins associated with the surface of invertebrate phagocytic cells, Franc et al. [80]). The presence of these hemocyte-associated lectins in mucus can be explained by plasma transudation and the presence of transmigrating hemocytes associated with mucus [57–59,81].

In contrast, other lectins are known or suspected to be produced by epithelial or mucosal cells [45,46,82–84] and have been proposed to play a major role during interactions between mucosal tissues and surrounding microbes. In this study, we identified in pallial mucus a protein named CvML (for *C. virginica* mucocyte lectin) we previously showed to be specifically transcribed in mucocyte lining the pallial organs [15,45,47]. This study, provided evidence that the transcripts of CvML are translated into active proteins released into the mucus matrix. In addition, c-type mannose receptors (c-type lectin family, EKC30902) were abundantly detected in oyster mucus. Further analysis indicated an increase of the transcripts of these 2 lectins in pallial organs after starvation (data not shown). Overall, lectins present in bivalve pallial mucus have been shown to agglutinate infectious microbes and microalgae and have been suggested to play a role in the capture and the sorting of food particles and in mucosal immunity [15,45,46,82,85]. As stated previously, the functions of lectins are diverse, including not only recognition but also opsonization and destruction of pathogens [86–88] and can be consider as effectors as well.

Several thioester-containing proteins (TEPs) were also found in pallial mucus, some of which were abundantly represented (Supplementary data 2). The complex TEPs family is made of three major groups described as immune related proteins: the complement components group, the A2M group, and the group formed by invertebrate TEPs and cell surface TEP (e.g. CD109; [89]). A rapid amplification of cDNA ends (RACE, data not shown) coupled with a phylogenetic analysis of the TEPs found in this study allowed the identification and classification of three of these proteins (EKC28380, EKC33672, EKC39431) as invertebrates TEPs. As a matter of fact, they all share high similarities with the BgTEP (*Biomphalaria glabrata*, [89]), CfTEP (*Chlamys farreri*, [90]) and EtTEP (*Euphaedusa tau*). The hallmark of invertebrate TEPs is the conserved thioester motif (GCGEQ) flanked by 2 proline residues, which underlines the unique property of these proteins to bind covalently to the target substrates (see [91] for additional characteristics). In the snail *B. glabrata*, the BgTEP was found to be closely associated to the complex formed between a fibrinogen-related protein (FREP, produced by the host) and a mucin (produced by the pathogen *Schistosoma mansoni*), supporting the involvement of invertebrate TEP in the recognition and elimination of parasites [89]. In the mosquito *Anopheles gambiae*, the TEP1 was shown to play a crucial role as opsonin, enhancing the phagocytosis and killing of microbes [92]. The tissue distribution of TEPs is wide and probably linked to the function of each specific molecule. For example the TEP1 from *A. gambiae*, was secreted by hemocytes and was shown to mature in the plasma [91]. In contrast, CfTEP transcripts in the scallop *Chlamys farreri*, were detected in hepatopancreas and gonad but not in other organs even after bacterial challenge [90].

Finally, a scavenger receptor cysteine-rich protein and a "deleted in malignant brain tumors 1 protein" (EKC40701), were identified in mucus. They are scavenger receptors (SRs), a wide family of distinct molecules that mediate phagocytosis by recognizing a variety of ligands, including ligand from pathogens but also damaged molecules from the host [93]. As a consequence, these molecules are thought to be involved in apoptosis and inflammation processes [94]. Little information is available on invertebrate scavenger receptors. For example, in the starfish *Asterina pectinifera*, Ap*SRCR1* was identified in both adult and larvae and was suggested to play a role in the recognition and elimination of bacteria [95]. In the scallop *Chlamys farreri*, the truncated recombinant CfSR (GQ260639) was found to bind various pathogens associated molecular patterns, including lipopolysaccharide, peptidoglycan, mannan and zymosan [96]. The two SRs found in this study are similar to CfSR (3e-71) and maybe suspected to have similar properties.

3.1.3. Immune activation and cell signaling

In mollusks, a complex network of signaling pathways control the host defense system. Many pathogen recognition receptors are involved in the activation of signaling pathways, which, in turn, induce a cascade of reactions and immune responses to, *in fine*, eliminate pathogens [51]. Some of these pathways in mollusks are well known (NFkB, MAPK, JAK-STAT, Toll-like receptor pathways) and detailed in the reviews by Loker et al. [97] and Song et al. [71]. Because of their involvement in multiple processes [98,99], members of these pathways are widely distributed in various types of cells/tissues, and there is little information on their role in regulating specific immune effectors in mollusks and even less so for mucosal immune factors.

A total of 57 proteins found in pallial mucus matched molecules involved in signaling pathways and cell communication (Supplementary data 2). For example several kinases (e.g. Map kinases, receptor for activated C-kinase), phosphatases, G proteins, including small GTPases, ras-related protein rab, GTPase Rho, and guanine nucleotide-binding protein involved in signal transduction were detected. Similarly, several cell surface receptors (e.g. 14-3-3 proteins) involved in signal transduction have also being identified. The 14-3-3 proteins are a family of molecules with the ability to bind and regulate diverse signaling proteins. For example, they were previously detected in the secretomes of *Leishmania donovani* (intracellular pathogen) and are thought to prolong the lifespan of infected cells [100]. Similarly, 14-3-3 proteins were found to counteract cell death in response to multiple stresses in vertebrates [101]. They are frequently found in mucus [33,102,103] and may interact with other proteins and with environmental microbes.

This study also allowed the identification of a caspase 7 (CASP7), known to cleave proteins inside the cell triggering the apoptotic process. This cascade is regulated by different modulators such as apoptosis inhibitor 5 (EKC39907) and apoptosis inducing factor (EKC20321). Other proteins identified in mucus (although more scarcely) are also thought to be involved in apoptotic processes, including the programmed cell death 6 (EKC18377). Similarly, Rho proteins have been found to be involved in multiple cellular functions, including cell proliferation, gene expression and apoptosis, affecting defense mechanism. Rho GDP-dissociation inhibitor 1 (RhoGDI), found abundantly in pallial mucus, regulates Rho proteins and consequently is involved in defense against pathogens. For example, RhoGDI was found to modulate the superoxide-generating NADPH oxidase system in phagocytes [104]. Interestingly, RhoGDI was found in salmon mucus, especially after lice infection [105].

3.1.4. Effector molecules

Due to the composition of its matrix (mostly made of glycoproteins or long chain of polysaccharides), mucus is a perfect carrier for bioactive molecules. The matrix provides a stable medium in which bioactive molecules are embedded, preventing their dispersion in water [18]. Bivalves possess a wide range of active molecules, many of which have been shown to be associated with mucosal tissues and secretions (see multiple examples in the review by [51]). Because the matrix is the first rampart against invaders, it is not surprising to find it enriched in proteins involved in immunity or defense against pathogens. Some of these effectors play an active role in eliminating pathogens (e.g. antimicrobial peptides, lysozyme) and others, nonspecific factors (e.g. HSPs), are dedicated to the protection of the host against divers stresses, including damage caused by pathogens.

3.1.4.1. Microbe neutralization. Antimicrobial peptides (AMPs) are a major component of the innate immune defense system in marine invertebrates. They are very often small molecules showing efficient, immediate and rapid antimicrobial response to invading microorganisms [106]. Among the AMPs, defensins represent an important family, abundant and ubiquitous in both vertebrates and invertebrates [107]. Because of their small size, amino acid composition and cationic charge, defensins are able to bind to the microbial cell membrane and cause multiple and irreversible damages [108]. These peptides are mainly synthesized in blood cells [109–111] and in epithelial cells [111–113] and are consequently found in mucus [114] where they can serve as a first barrier of defense. In this study, the American oyster defensin (AOD; P85008) previously purified from *C. virginica* gills [115] has been detected in moderate levels. This AMP displays a strong activity against Gram-positive and -negative bacteria including *Staphylococcus aureus* and *Vibrio parahemolyticus* [115].

In addition to AMPs, a total of 23 proteases were identified in this study, including aspartic, cysteine, metallo and serine peptidases. The detection of protease activity in mucus of marine organisms is well known [116–119] and a previous study by Brun et al. [35] showed that mucus covering the gills of eastern oysters contains at least 3 proteases identified as a putative acid protease (96 kDa), a zinc metalloprotease (64 kDa), and a serine protease (33 kDa) using zymogram methods. In the current study, three proteases were particularly abundant in the pallial mucus (e.g. meprin EKC43126, dipeptidase EKC34306 and aminopeptidase EKC41968). The function of proteases in invertebrate mucus is not clearly established but it is likely that some of these (trypsin-like or cathepsins) contribute to host immunity as previously described in fish [120].

In parallel, eleven proteases inhibitors were also detected in oyster mucus. Eight among these harbor the GO term "extracellular" suggesting that they are secreted (e.g. humoral). These findings are not surprising since protease inhibitors are common components of fish [103] and human mucus [121,122]. Among the most abundant protease inhibitors, the cystatin b-like protein (ADI33157) is classified as an inhibitor of the papain-like cysteine peptidases from the C1 family. The serine protease inhibitor CvSI-2 (B9A8D7) was also found in oyster mucus (scarce). CvSI-2 was previously purified by Xue et al. [123] from oyster plasma and was found to inhibit a serine protease (perkinsin) from the *C. virginica* pathogen, *P. marinus*. Our findings suggest that mucus contributes to the oyster defense activity against this devastating parasite.

In addition to proteases, several glycoside hydrolases were also found in pallial mucus. Thus, lysozymes, a group of enzymes that cleave glycosidic bonds on peptidoglycan present on bacterial cell wall [124,125], have been unanimously described as a major effector in invertebrate immunity [126–129], including *C. virginica* [32,130]. They have been found associated with bivalve blood [130] and digestive system [128,132], and were detected in secretions from different animal taxa (e.g. saliva, milk, and mucus, [103,122,133]). Recently, one of the 3 lysozymes inventoried in *C. virginica* (i.e. lysozyme 1) was purified from oyster plasma by Xue et al., [131] and was found to inhibit the growth of several Gram-negative and -positive bacteria. The same lysozyme (P83673) was identified in this study highlighting the important role of oyster pallial mucus as a first line of defense against microbial invaders.

Additionally, the thaumatin-like proteins (TLPs, EKC25850) were also identified in oyster pallial mucus. They belong to the glycoside hydrolase family 64 (GH64-TLP-SF) able to cleave long-chain polysaccharide beta-1,3-glucans, typically found in fungal cell wall [134]. In fact, TLPs are known to be involved in defense in many plants, where their production is stimulated in the event of pathogen attack [135]. Among invertebrates, TLPs have been found in beetles [136], mussels [137] and clams [138] and may have antifungal activity.

Among proteins that may have an active effect on pathogens, 3 molecules containing C1q domain (C1qDC) and tumor necrosis factor domain (TNF) were identified in oyster pallial mucus (EKC25476, EKC37564, EKC41040). C1qDC proteins belong to a large group of immune recognition proteins found in many organisms (vertebrates and invertebrates) including bivalves [139]. They play a fundamental role in the complement pathway that mediates antibody response in vertebrates. They were also defined as a "major connecting link between innate and acquired immunity" by Kishore et al. [140] because C1q are involved in multiple immunological processes including host defense, inflammation and apoptosis [140]. Recently, hundreds of C1q domain-containing protein genes have been identified in both mussel [139] and oyster [141] using genomic surveys but no functional assays were performed to clearly identify their function, suggested to be very broad. Proteins of the complement system are commonly found in vertebrate mucosal secretions and have been identified in mucus of mouse colon [142], in human cervical mucus [122] or fish mucus [105].

Cytokines (IL, TNF, IFN) are another group of proteins known to be central in mollusk immunity. They are known to stimulate hemocyte motility, to increase phagocytosis and to induce cellular signaling molecules such as NOS [143]. In this study, a macrophage migration inhibitory factor (ADU19847) was identified and suggested to be a proinflammatory cytokine with the ability to induce various immunomodulatory and proinflammatory responses in vertebrates and invertebrates [144–146]. Transcripts of MIF have been shown to be expressed and regulated in response to microbial stimuli in different organs including pallial or digestive tissues in mussels [147] and oysters [148].

In parallel, two cyclophilins were also identified in oyster mucus. These proteins are suspected to play a primary role in immune defense [149–151]. For instance, vertebrate cyclophilins regulate immunosuppression by inhibiting calcineurin and preventing the transcription of interleukin [152]. Interestingly, cyclophilins have been identified in fish mucus [103] suggesting their involvement in mucosal immunity across different taxa.

On the other hand, two ferritins were detected in pallial mucus (EKC42967 and EKC30759; Table 1). These iron-binding proteins are well known to be involved in host–pathogen interactions and are often upregulated in animal hosts following exposure to microbial pathogens therefore impeding pathogen access to limited iron resources [153].

To complete the repertoire of tools used by bivalves to actively fight pathogens, several antioxidant proteins were identified in mucus, including superoxide dismutases (SODs), catalases, peroxiredoxins and thioredoxins. Reactive oxygen species (ROS) play important roles in cell signaling and homeostasis but if produced in too high quantity, they can become harmful to the cell (see the review by [154]). In order to prevent oxidative stress, antioxidant molecules are produced to control the action of ROS and maintain the oxidative system balanced. SODs catalyze the dismutation of ROS to hydrogen peroxide that could then be scavenged by catalases or peroxidases. In addition, several proteins act as antioxidants by providing electrons to catalases or peroxidases (e.g. thioredoxins). These different proteins have already been

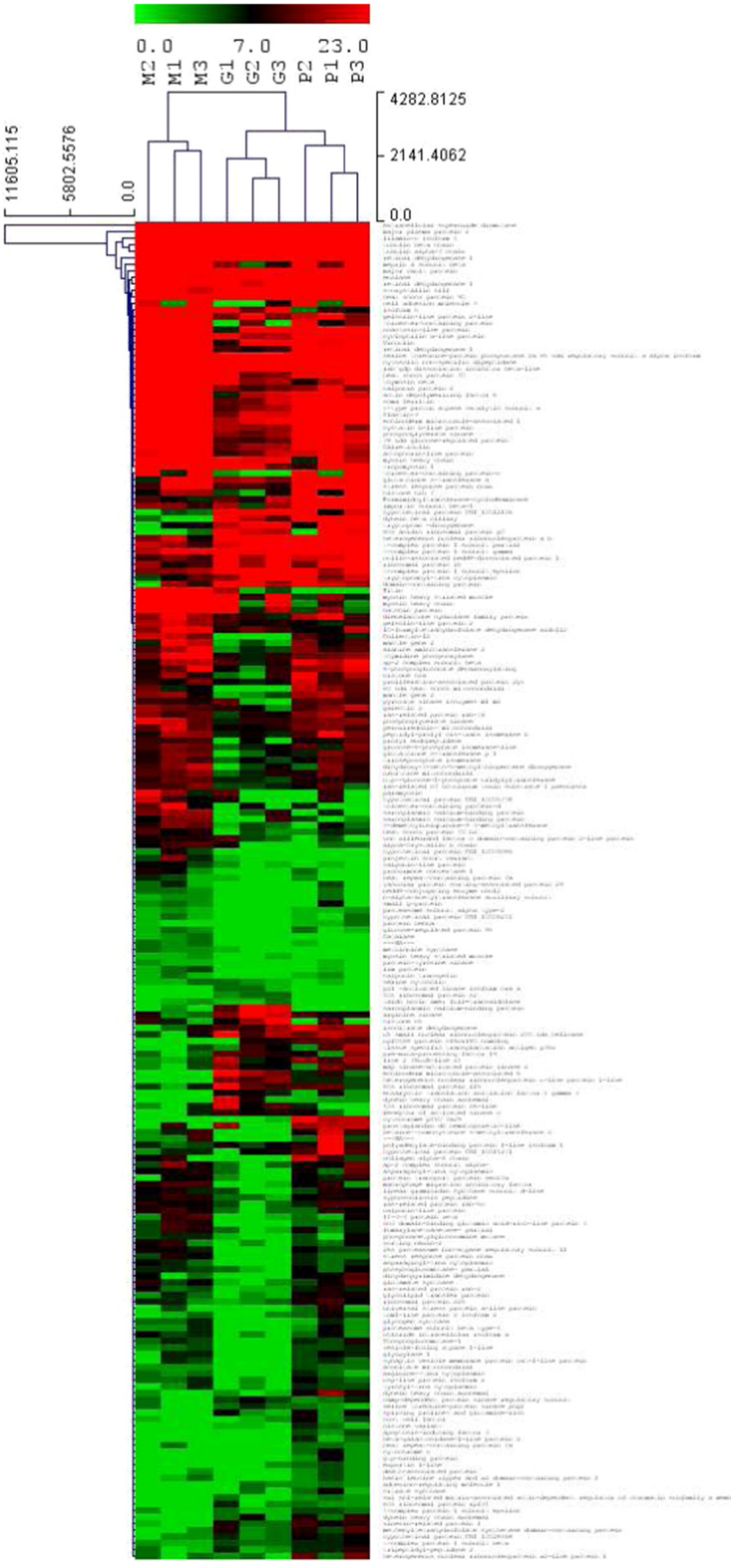

Fig. 2. Heat map of the 205 differentially abundant proteins in mucus derived from mantle (M), gills (G) and labial palps (P). See Table 2 for the complete list.

Table 2
List of the 205 differentially abundant proteins in mucus samples derived from gills, mantle and labial palps (ANOVA-F test, FDR < 0.1). Proteins were grouped according to their average spectral counts into proteins with high abundance in mucus from gills (group 1), mantle (group 2) and labial palps (group 3). In addition, proteins with low abundance in mucus covering gills (group 4), mantle (group 5) and labial palps (group 6) were also grouped.

Code	Protein identification	Categories	Average spectral counts		
			Gills	Mantle	Labial palps
Group 1					
K1QHI6_CRAGI	dynein heavy chain axonemal	Cell structure	10.7	0.0	2.7
gi|31901720	arginine kinase	Metabolism	19.7	5.3	9.0
gi|31905414	histone h5	Nucleic acid	19.0	6.3	4.3
MGID93276	40s ribosomal protein s6-like	Nucleic acid	13.3	3.0	2.0
gi|31907958	40s ribosomal protein s2	Nucleic acid	3.7	0.0	0.3
gi|152818521	sarcoplasmic calcium-binding protein	Other	20.7	1.0	0.0
MGID94855	receptor of activated kinase c	Signaling	9.3	2.0	4.0
K1RBU9_CRAGI	p21 -activated kinase isoform cra_a	Signaling	3.3	0.0	0.0
Group 2					
MGID94782	tropomyosin 1	Cell structure	43.7	75.3	26.7
MGID94604	thymosin beta	Cell structure	24.0	42.7	19.7
MGID89735	gelsolin-like protein 2-like	Cell structure	19.0	76.7	20.0
gi|152819229	calponin protein 2	Cell structure	13.7	50.3	23.3
K1PE57_CRAGI	gelsolin-like protein 2	Cell structure	9.3	18.7	8.3
K1RVK9_CRAGI	Titin	Cell structure	8.3	33.3	0.0
K1QTC1_CRAGI	paramyosin	Cell structure	4.3	20.3	3.3
MGID94620	calponin-like protein	Cell structure	1.7	6.3	0.7
gi|152817577	myosin heavy striated muscle	Cell structure	1.0	2.3	0.0
K1R5L7_CRAGI	projectin short variant	Cell structure	0.7	8.7	0.0
MGID92282	lim protein	Cell structure	0.0	3.7	0.7
MGID95165	cystatin b-like protein	Immunity	17.0	39.0	23.0
K1PVK1_CRAGI	thioester-containing protein-d	Immunity	2.3	18.3	2.0
K1QCC1_CRAGI	phosphoglycerate kinase	Metabolism	15.3	38.3	23.7
K1QHH8_CRAGI	10-formyltetrahydrofolate dehydrogenase aldh1l2	Metabolism	11.3	22.7	11.0
gi|152818829	3-demethylubiquinone-9 3-methyltransferase	Metabolism	3.7	12.3	3.7
K1P5E0_CRAGI	methionine synthase	Metabolism	0.7	3.3	0.0
K1QAF2_CRAGI	protein-tyrosine kinase	Metabolism	0.0	2.3	0.7
K1R8V5_CRAGI	nedd8-conjugating enzyme ubc12	Nucleic acid	0.0	3.7	1.3
gi|31903750	extracellular superoxide dismutase	Other	1262.7	2371.3	1311.3
gi|383549954	major plasma protein 2	Other	305.3	722.3	451.0
K1PY28_CRAGI	sarcoplasmic calcium-binding protein	Other	4.3	16.0	2.0
K1Q5G7_CRAGI	sarcoplasmic calcium-binding protein	Other	3.7	12.3	2.7
K1PJE6_CRAGI	heat repeat-containing protein 7a	Other	0.7	4.3	1.0
gi|152812971	prohormone convertase 1	Proteolysis	0.0	6.3	1.3
K1RGQ0_CRAGI	ras-related c3 botulinum toxin substrate 1 precursor	Signaling	5.3	12.7	8.0
K1PJB0_CRAGI	heat shock protein 70 b2	Stress response	3.0	9.3	4.7
gi|152818372	alpha-crystallin b chain	Stress response	2.7	10.0	2.7
K1QTD6_CRAGI	Catalase	Stress response	0.0	3.0	0.0
K1PGH0_CRAGI	vacuolar protein sorting-associated protein 29	Transport	0.0	4.0	1.7
MGID91462	hypothetical protein CGI_10005738	Unknown	3.0	12.7	3.3
gi|152815468	hypothetical protein CGI_10009086	Unknown	0.0	5.7	0.0
gi|152815546	NA	Unknown	0.0	3.7	0.7
Group 3					
MGID89412	cytochrome c	Atpases activity	0.0	0.0	4.7
K1R473_CRAGI	tubulin alpha-3 chain	Cell structure	315.7	266.0	434.7
K1PX83_CRAGI	dynein heavy chain axonemal	Cell structure	2.0	1.0	11.0
MGID153392	t-complex protein 1 subunit epsilon	Cell structure	2.7	1.3	4.7
K1QFI3_CRAGI	apoptosis-inducing factor 3	Immunity	0.0	0.0	4.3
MGID90310	glutathione s-transferase a	Metabolism	16.7	22.3	49.0
K1R5S8_CRAGI	Formimidoyltransferase-cyclodeaminase	Metabolism	13.7	8.3	33.3
MGID90452	cytochrome p450 3a29	Metabolism	0.7	0.0	21.3
MGID89361	prostaglandin d2 hematopoietic-like	Metabolism	5.3	3.3	18.3
MGID89535	citrate synthase	Metabolism	1.3	0.0	4.3
MGID93849	beta-galactosidase-1-like protein 2	Metabolism	0.3	0.7	4.0
K1RJH5_CRAGI	polyadenylate-binding protein 1-like isoform 1	Nucleic acid	6.7	5.0	15.0
K1QXS6_CRAGI	heterogeneous nuclear ribonucleoprotein a2-like protein 1	Nucleic acid	2.3	2.7	10.3
MGID94702	ribosomal protein s25	Nucleic acid	3.0	5.0	8.7
K1PYA2_CRAGI	host cell factor	Nucleic acid	1.7	0.7	5.3
MGID92706	histone variant	Nucleic acid	1.3	0.0	4.0
K1S1X3_CRAGI	swi snf-related matrix-associated actin-dependent regulator of chromatin	Nucleic acid	1.3	0.0	3.3
K1S6V7_CRAGI	serine threonine-protein phosphatase 2a 65 kda regulatory	Other	28.7	30.7	48.0
K1PW81_CRAGI	tripeptidyl-peptidase 2	Proteolysis	3.7	2.0	7.3
MGID151292	meprin a subunit beta	Proteolysis	10.0	34.3	176.7
gi|152817800	universal stress protein a-like protein	Stress response	0.0	1.7	5.0
K1R2V1_CRAGI	importin subunit beta-1	Transport	13.3	14.0	28.3
K1QLK8_CRAGI	gtp-binding protein	Transport	0.0	0.0	4.3
K1QFK8_CRAGI	tom1-like protein 2 isoform 2	Transport	1.0	2.0	5.7
K1RK33_CRAGI	exportin 1-like	Transport	1.0	0.0	4.0
MGID93433	hypothetical protein CGI_10014271	Unknown	5.0	5.0	18.3

Table 2 (*continued*)

Code	Protein identification	Categories	Average spectral counts		
			Gills	Mantle	Labial palps
Group 4					
K1P339_CRAGI	vinculin	Adhesion	20.0	56.7	42.3
MGID93190	cell adhesion molecule 3	Adhesion	3.0	30.0	71.7
K1Q9V3_CRAGI	v-type proton atpase catalytic subunit a	ATPases activity	15.3	29.7	31.0
MGID91109	chloride intracellular isoform a	ATPases activity	1.0	6.0	4.7
K1P7K8_CRAGI	vesicle-fusing atpase 1-like	ATPases activity	0.3	4.0	5.3
K1RHP3_CRAGI	proliferation-associated protein 2 g4	Cell cycle	7.0	10.7	15.3
K1PW06_CRAGI	filamin-c isoform 4	Cell structure	202.7	393.3	335.3
gi|152818089	coactosin-like protein	Cell structure	20.0	89.3	54.0
MGID94771	actin depolymerizing factor 6	Cell structure	15.0	39.3	33.3
K1R2D6_CRAGI	plastin-3	Cell structure	19.0	36.3	32.7
K1PWP8_CRAGI	echinoderm microtubule-associated 1	Cell structure	21.0	39.3	29.0
MGID91714	actophorin-like protein	Cell structure	15.0	29.3	23.7
K1PFT9_CRAGI	mantle gene 2	Cell structure	3.0	17.7	11.3
gi|152814323	mantle gene 2	Cell structure	3.3	13.3	16.0
K1R1X5_CRAGI	calponin-like protein	Cell structure	2.7	9.3	7.0
K1QGR8_CRAGI	asparaginyl-trna cytoplasmic	Cell structure	1.3	8.0	8.3
MGID149859	collagen alpha-6 chain	Cell structure	0.0	5.7	12.0
gi|152814255	calponin transgelin	Cell structure	0.3	2.7	1.7
MGID92334	Collectin-12	Immunity	0.0	18.3	8.7
MGID94396	cyclophilin a-like protein	Immunity	20.3	69.0	58.7
K1PXS8_CRAGI	Calreticulin	Immunity	16.0	31.3	23.7
K1QHR5_CRAGI	thioester-containing protein	Immunity	4.7	58.7	25.7
K1QAH8_CRAGI	thioester-containing protein-c	Immunity	1.0	18.3	29.3
gi|110559484	galectin 2	Immunity	6.7	14.3	15.0
MGID89676	retinal dehydrogenase 1	Metabolism	65.3	240.0	226.0
K1QX37_CRAGI	enolase	Metabolism	58.3	141.3	108.0
K1R266_CRAGI	retinal dehydrogenase 1	Metabolism	31.3	129.0	113.7
K1QVG5_CRAGI	retinal dehydrogenase 1	Metabolism	11.0	55.0	51.3
gi|152817929	thymidine phosphorylase	Metabolism	8.3	20.7	14.7
MGID151439	dienelactone hydrolase family protein	Metabolism	9.0	19.7	18.7
gi|152812685	phosphoglycerate kinase	Metabolism	8.7	18.7	14.7
K1RGF4_CRAGI	alanine aminotransferase 2	Metabolism	4.7	17.7	13.0
K1RH70_CRAGI	6-phosphogluconate decarboxylating	Metabolism	6.7	15.7	18.3
gi|152812909	dihydroxy-3-keto-5-methylthiopentene dioxygenase	Metabolism	4.3	15.3	9.3
K1Q5P7_CRAGI	peptidyl-prolyl cis-trans isomerase b	Metabolism	6.7	14.3	16.3
K1Q1I3_CRAGI	ornithine mitochondrial	Metabolism	6.3	13.7	11.0
K1PTI6_CRAGI	glucose-6-phosphate isomerase-like	Metabolism	5.7	13.0	11.0
gi|152813365	glutathione s-transferase p 1	Metabolism	3.7	12.7	11.0
K1Q1D1_CRAGI	utp–glucose-1-phosphate uridylyltransferase	Metabolism	5.7	12.0	9.3
K1PJ59_CRAGI	triosephosphate isomerase	Metabolism	5.3	11.7	10.7
gi|31909030	pyruvate kinase isozymes m1 m2	Metabolism	5.0	10.0	12.0
gi|152815203	fumarylacetoacetase- partial	Metabolism	2.0	8.3	5.7
K1Q6B8_CRAGI	linear gramicidin synthase subunit d-like	Metabolism	1.7	7.3	7.3
K1QJR6_CRAGI	glutamate synthase	Metabolism	1.7	7.0	8.7
gi|152819162	betaine–homocysteine s-methyltransferase 2	Metabolism	0.0	6.7	13.3
K1RRS8_CRAGI	phosphoglucomutase-partial	Metabolism	2.0	5.7	6.0
K1QVE8_CRAGI	phosphoacetylglucosamine mutase	Metabolism	0.7	5.7	4.3
K1PQD4_CRAGI	Phosphoglucomutase-1	Metabolism	0.3	5.0	6.7
K1QSC1_CRAGI	glycogen synthase	Metabolism	0.0	4.7	3.7
gi|152813248	glycolipid transfer protein	Metabolism	1.7	4.3	8.3
K1R647_CRAGI	glyoxylase 1	Metabolism	0.0	4.0	6.0
K1PZ93_CRAGI	dihydropyrimidine dehydrogenase	Metabolism	0.0	3.7	8.7
gi|156571895	aconitate mitochondrial	Metabolism	0.0	3.0	4.3
MGID93979	hypothetical protein CGI_10006272	Metabolism	0.0	2.7	2.0
K1QYV6_CRAGI	n-alpha-acetyltransferase auxiliary subunit	Metabolism	0.0	2.7	1.3
K1QHH0_CRAGI	protein henna	Metabolism	0.3	1.0	2.7
K1Q9Q6_CRAGI	synaptic vesicle membrane protein vat-1-like protein	Metabolism	0.0	3.7	5.0
K1PUQ5_CRAGI	histone h2b 7	Nucleic acid	5.3	14.0	23.3
K1P421_CRAGI	histone h2a	Nucleic acid	5.3	12.0	17.3
MGID94880	asparaginyl-trna cytoplasmic	Nucleic acid	0.0	5.7	5.3
K1QYI9_CRAGI	arginine–trna cytoplasmic	Nucleic acid	0.7	3.7	4.0
K1PRB5_CRAGI	tyrosyl-trna cytoplasmic	Nucleic acid	1.0	3.0	5.3
gi|31907916	basic leucine zipper and w2 domain-containing protein 1	Nucleic acid	0.3	2.3	3.7
K1P8D9_CRAGI	sh3 domain-binding glutamic acid-rich-like protein 3	Other	0.0	8.3	4.7
K1R9A6_CRAGI	heat repeat-containing protein 7a	Other	0.0	1.3	3.7
MGID89256	cytosolic non-specific dipeptidase	Proteolysis	26.7	45.0	37.3
K1R2E8_CRAGI	prolyl endopeptidase	Proteolysis	2.3	11.3	10.3
gi|152813756	kyphoscoliosis peptidase	Proteolysis	3.3	8.7	8.0
K1PNP4_CRAGI	26 s proteasome non-atpase regulatory subunit 11	Proteolysis	0.0	7.3	5.7
K1QBG8_CRAGI	proteasome subunit beta type-4	Proteolysis	0.0	4.0	4.3
MGID92433	proteasome subunit alpha type-2	Proteolysis	0.0	2.3	2.3
gi|152814883	macrophage migration inhibitory factor	Signaling	0.7	10.3	6.7
K1PHM8_CRAGI	14-3-3 protein zeta	Signaling	0.7	8.3	4.7

(*continued on next page*)

Table 2 (*continued*)

Code	Protein identification	Categories	Average spectral counts		
			Gills	Mantle	Labial palps
Group 4					
MGID90077	death-associated protein	Signaling	0.0	1.0	3.7
K1QIR8_CRAGI	78 kda glucose-regulated protein	Signaling	12.3	30.3	23.7
K1PZ08_CRAGI	ras-related protein rab-7a	Signaling	7.3	13.3	14.3
K1R1Q8_CRAGI	ras-related protein rab-5c	Signaling	3.0	8.3	9.7
K1QBM3_CRAGI	ras-related protein rab-2	Signaling	1.3	5.7	7.0
MGID93056	small g-protein	Signaling	0.0	3.3	2.3
gi|31901344	heat shock protein 70	Stress response	21.0	43.3	35.3
MGID94745	stress response protein nhax	Stress response	11.0	25.7	29.3
gi|152818317	peroxiredoxin-mitochondrial	Stress response	8.0	15.3	13.3
K1Q5G6_CRAGI	60 kda heat shock mitochondrial	Stress response	0.0	9.0	15.7
MGID90502	stress response protein nhax	Stress response	0.0	7.0	4.7
gi|152817965	usp-like protein isoform 2	Stress response	1.0	4.7	5.3
MGID90395	glucose-regulated protein 94	Stress response	0.0	3.0	2.0
MGID149779	soma ferritin	Transport	16.0	32.3	35.3
K1S151_CRAGI	rab gdp dissociation inhibitor beta-like	Transport	29.3	48.0	37.7
K1QQ16_CRAGI	ap-2 complex subunit beta	Transport	9.0	15.3	21.7
K1QAB1_CRAGI	ap-2 complex subunit alpha-	Transport	2.7	9.3	9.7
K1Q9D7_CRAGI	sorting nexin-2	Transport	1.7	7.0	5.3
K1R983_CRAGI	protein transport protein sec23a	Transport	1.0	5.0	7.7
MGID95063	NA	Unknown	0.0	5.7	13.0
Group 5					
K1R7V7_CRAGI	tubulin beta chain	Cell structure	345.7	229.7	357.0
K1RLC5_CRAGI	t-complex protein 1 subunit epsilon	Cell structure	30.3	16.0	31.0
K1PAG1_CRAGI	dynein beta ciliary	Cell structure	28.0	1.3	36.0
K1R466_CRAGI	t-complex protein 1 subunit gamma	Cell structure	28.0	21.0	32.0
K1S4Q2_CRAGI	t-complex protein 1 subunit partial	Cell structure	25.7	16.0	29.7
K1QFE3_CRAGI	echinoderm microtubule-associated 6	Cell structure	18.3	0.0	6.7
K1QUC7_CRAGI	kinesin-related protein 1	Cell structure	5.7	0.7	9.7
K1QK11_CRAGI	dynein heavy chain axonemal	Cell structure	4.3	0.0	7.7
gi|152817032	t-complex protein 1 subunit beta	Cell structure	4.3	2.3	6.3
gi|31908168	s-crystallin sl11	Metabolism	71.7	32.7	73.0
MGID94750	tryptophan -dioxygenase	Metabolism	36.3	5.0	23.0
K1RZE2_CRAGI	isocitrate dehydrogenase	Metabolism	13.0	4.7	18.7
K1PK93_CRAGI	tissue specific transplantation antigen p35b	Metabolism	7.0	1.7	11.0
MGID89963	heterogeneous nuclear ribonucleoprotein a b	Nucleic acid	34.0	14.7	30.7
MGID94684	tryptophanyl-trna cytoplasmic	Nucleic acid	32.0	8.3	20.7
MGID151282	ribosomal protein l5	Nucleic acid	31.7	12.7	23.3
K1PK85_CRAGI	cullin-associated nedd8-dissociated protein 1	Nucleic acid	25.3	12.3	21.7
MGID150883	60s acidic ribosomal protein p0	Nucleic acid	20.7	1.3	18.0
K1QBH0_CRAGI	u5 small nuclear ribonucleoprotein 200 kda helicase	Nucleic acid	19.7	4.3	16.3
MGID150847	60s ribosomal protein l15	Nucleic acid	15.7	4.3	10.0
K1PMT6_CRAGI	heterogeneous nuclear ribonucleoprotein u-like protein 1-like	Nucleic acid	14.3	0.0	5.7
K1QRG9_CRAGI	pre-mrna-processing factor 19	Nucleic acid	11.0	2.7	8.7
K1PV49_CRAGI	like 2 (RuvB-like 2)	Nucleic acid	10.7	3.3	14.7
K1RCW5_CRAGI	eukaryotic translation initiation factor 4 gamma 3	Nucleic acid	7.7	0.3	4.3
K1QRL6_CRAGI	methenyltetrahydrofolate synthetase domain-containing protein	Nucleic acid	5.3	1.0	8.7
K1PNY5_CRAGI	splicing proline- and glutamine-rich	Nucleic acid	4.0	0.0	6.0
MGID153354	60s ribosomal protein rpl34	Nucleic acid	3.0	0.0	3.3
K1PH24_CRAGI	domain-containing protein	Other	24.7	9.3	20.3
K1R2T7_CRAGI	upf0468 protein c16orf80 homolog	Other	6.0	0.0	12.3
K1QCL6_CRAGI	adhesion-regulating molecule 1	Other	2.3	0.0	3.3
K1QJE1_CRAGI	map kinase-activated protein kinase 2	Signaling	10.3	2.7	9.7
K1RXA0_CRAGI	camp-dependent protein kinase regulatory subunit	Signaling	5.7	1.3	4.3
MGID94589	serine threonine-protein kinase phg2	Signaling	4.7	1.3	3.0
K1PNQ5_CRAGI	heat shock protein 90	Stress response	97.7	70.7	84.7
K1QQR1_CRAGI	major vault protein	Transport	171.0	89.0	130.3
gi|152812846	hypothetical protein CGI_10012106	Unknown	30.7	3.0	30.7
K1S2Y0_CRAGI	hypothetical protein CGI_10028468	Unknown	4.3	2.0	7.0
Group 6					
K1QRU8_CRAGI	myosin heavy chain	Cell structure	44.0	77.7	18.7
K1R1B3_CRAGI	myosin heavy striated muscle	Cell structure	25.3	47.0	9.0
K1RZ99_CRAGI	isoform h (filamin)	Cell structure	31.7	81.0	7.7
K1RSS3_CRAGI	myosin heavy chain	Cell structure	23.3	44.7	5.7
gi|152817786	catchin protein	Cell structure	25.0	34.0	8.3
MGID89228	von willebrand factor c domain-containing protein 2-like protein	Adhesion	7.0	8.7	1.7
gi|152817463	serine cytosolic	Metabolism	3.0	2.7	0.7
K1QVK0_CRAGI	transaldolase	Metabolism	2.7	2.0	0.7

reported in vertebrate mucus [103,122] and may mitigate oxidative stress and contribute to the protection of mucosal tissues against intrinsic or extrinsic oxidative stress.

3.1.4.2. Nonspecific response to stress. Besides the numerous active effectors identified in this study, other classes of proteins have the ability to protect bivalves against diverse stresses, including pathogens. They are

often nonspecific but serve to reduce cellular stress, prevent protein degradation and mediate the correct folding of proteins [155]. These included several heat shock proteins (HSPs, e.g. AAO41703) (some of which were very abundant in pallial mucus), chaperonin subunits, Dnaj subunits, alpha crystallin, several universal stress protein-like, glucose-regulated proteins and proteasome subunits, and a calreticulin. These molecules are commonly found in mucus produced by vertebrates [103,156] and in pallial organs or epithelial cells lining pallial organ in bivalves [138,157].

3.2. Organ-specific proteins

Using an ANOVA-F test (FDR < 0.1), the abundance of 205 proteins was found to be significantly different between the three sample types (i.e. mucus derived from mantle, labial palps or gills, Fig. 2; Supplementary data 3). The average spectral counts for each of the differentially expressed proteins are given in Table 2. In addition, this table also segregates proteins with higher abundance only in mucus from gills (group 1), mantle (group 2) or labial palps (group 3), as well as proteins with low abundance only in mucus covering gills (group 4), mantle (group 5) and labial palps (group 6).

Mucus covering gills is characterized by 67% of proteins considered scarce (averaging less than 10 spectral counts) and only 5% of proteins were described as abundant (between 50 to 2371 average spectral counts, Table 2). Proteins that were highest (Group 1, n = 8) or second highest (Groups 5 and 6) in gills are associated with the GO terms "cell structure" (i.e. tubulin, filamin, myosin), "metabolism", "nucleic acid" or "signaling". Few proteins, however, that were well represented in gill mucus are involved in additional functions such as the chaperon HSP90 (group 5, EKC25687, protein folding after possible damage), the major vault protein (group 5, EKC39307, molecular transport) and the von Willebrand factor c domain-containing protein 2-like protein (group 6, EKC25636, adhesion).

Fifty nine percent of differentially abundant proteins were considered scarce in mucus covering the mantle while 10% of these were described as abundant (Table 2). Proteins displaying the highest abundance in mantle (Group 2) are classified by the GO terms "cell structure", "metabolism" and "other". This last set included the hypothetical extracellular superoxide dismutase (BAF30874; also named dominin, [158]) and the major plasma protein 2 (AFH41574; also known as segon, [159]). These two major proteins were found to be upregulated in oysters following shell damage suggesting that they are involved in shell mineralization [160]. In addition, proteins related to the GO terms "proteolysis" (i.e. prohormone convertase), "stress response" (i.e. heat shock protein 70 b2, catalase) and "immunity" (i.e. cystatin b-like protein, thioester-containing protein-d) were more abundant in mucus from mantle as compared to gills and labial palps (Table 2). Furthermore, proteins for which mantle mucus was relatively enriched (Groups 4 and 6) included molecules related to the GO terms "cell structure", "metabolism", "proteolysis" (i.e. cytosolic non-specific dipeptidase, prolyl endopeptidase), "stress response" (i.e. HSP90, HSP70), "adhesion" (i.e. cell adhesion molecule 3, vinculin, TEP) and "transport" (i.e. soma ferritin). Group 4 also displayed a variety of proteins with functions related to "immunity" (i.e. collectin-12, cyclophilin a-like protein, cystatin b-like protein, TEP, galectin 2) and "signaling" (i.e. macrophage migration inhibitory factor, 14-3-3 protein zeta, ras related proteins).

Lowly abundant and scarce proteins represented 37 and 56% of differentially expressed proteins from mucus covering labial palps, respectively (Table 2). Proteins displaying the highest abundance in labial palps (Group 3) are mostly associated with the GO terms "nucleic acid", "metabolism" or "transport". Additionally, the meprin a subunit beta (EKC43126), involved in proteolysis was found abundantly in the mucus covering labial palps. Other proteins relatively enriched in labial palps mucus (groups 4 and 5) included molecules involved in "stress response" (i.e. HSP90, HSP70), "signaling", "adhesion" (i.e. vinculin, cell adhesion molecule 3) and "immunity". Among these last 2 groups, a collectin-12 (EKC20791) a galectin 2 (ABG75998), two TEPs (EKC28380, EKC33672), a cyclophilin a-like protein (AEJ08750), a calreticulin (EKC23904), a macrophage migration inhibitory factor (ADU19847), a 14-3-3 protein zeta (EKC18419), a death-associated protein (EKC38229), and a 78 kda glucose-regulated protein (EKC33663) were identified.

Based on these results, gill mucus appears to be the less specific, containing few distinctive proteins and sharing most proteins with the 2 other mucus types (Fig. 3). In contrast, labial palps and mantle mucus contained more distinctive proteins, in particular those related to immunity and recognition. Higher abundance of these proteins in mucus derived from mantle and labial palps may reflect a higher specialization of these organs in functions involving host–microbe interactions, such as defense against invaders.

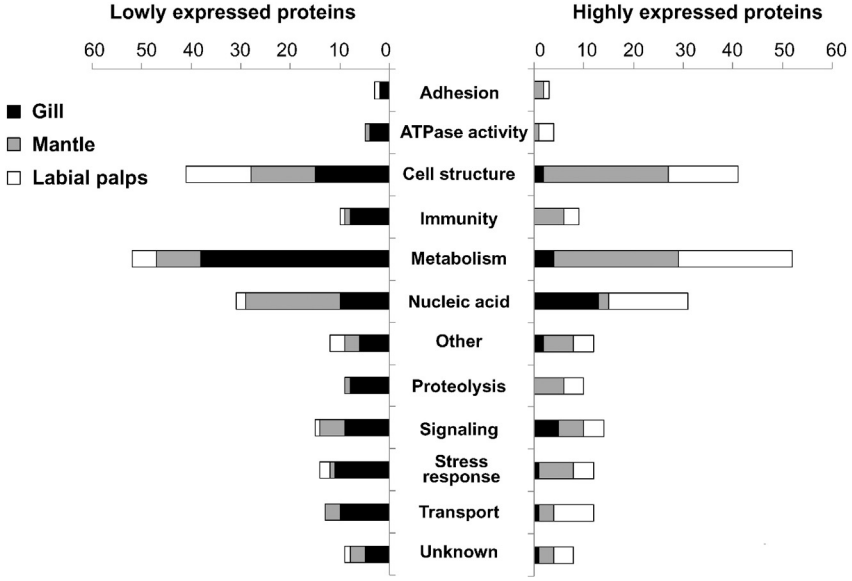

Fig. 3. Number of proteins present in mucus covering oyster pallial organs (gill, mantle and labial palps). See Table 2 for the complete list.

3.3. Conclusions

This work not only provides a strong base for further proteomic studies of mucus in mollusks but also highlights the fundamental role of these secretions in bivalve health and interactions with microbes. It is true that the rheological properties of mucus help in body lubrication, in the transport of particles during feeding and cleansing processes or against body desiccation. Mucus does act as a physical barrier against environmental stresses but it is also an active biological weapon. In this analysis, we demonstrated the presence in oyster mucus of numerous proteins already known or suspected to be central in bivalve immunity including immune recognition proteins and a wide range of immune effectors. This battery of tools acts as a first rampart against waterborne microbes. Among the burning questions that still need to be answered is how bivalves regulate mucosal immune factors in response to environmental or pathologic stress? And how these factors interact with various microbial symbionts (e.g. mutualistic, opportunistic or obligate pathogens) to maintain homeostasis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.11.018.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

Authors thank Dr. Ruofeng Wen for providing help with statistical analysis. We also would like to acknowledge financial support provided by the National Science Foundation (projects IOS 1050596 and IOS 1146920).

References

[1] C. Canicatti, G. Dancona, Biological protective substances in *Marthasterias glacialis* (Asteroidea) epidermal secretion, J. Zool. 222 (1990) 445–454.
[2] M.S. Davies, S.J. Hawkins, Mucus from marine molluscs, in: J.H.S. Blaxter, A.J. Southward, P.A. Tyler (Eds.), Advances in Marine Biology, 34, Academic Press Ltd-Elsevier Science Ltd, London 1998, pp. 1–71.
[3] P. Meikle, G.N. Richards, D. Yellowlees, Structural investigations on the mucus from six species of coral, Mar. Biol. 99 (1988) 187–193.
[4] V. Storch, U. Welsch, The ultrastructure of epidermal mucous cells in marine invertebrates (Nemertini, Polychaeta, Prosobranchia, Opisthobranchia), Mar. Biol. 13 (1972) 167–175.
[5] H. Schachter, D. Williams, Biosynthesis of mucus glycoproteins, in: E. Chantler, J. Elder, M. Elstein (Eds.), Mucus in Health and Disease—II, Springer, US 1982, pp. 3–28.
[6] J. Audie, A. Janin, N. Porchet, M. Copin, B. Gosselin, J. Aubert, Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by in situ hybridization, J. Histochem. Cytochem. 41 (1993) 1479–1485.
[7] M.C. Rose, W.A. Voter, C.F. Brown, B. Kaufman, Structural features of human tracheobronchial mucus glycoprotein, Biochem. J. 222 (1984) 371–377.
[8] A.M. Smith, The structure and function of adhesive gels from invertebrates, Integr. Comp. Biol. 42 (2002) 1164–1171.
[9] M. Kesimer, J. Sheehan, Mass spectrometric analysis of mucin core proteins, in: M.A. McGuckin (Ed.), Thornton DJ, Editors, Humana Press, Mucins 2012, pp. 67–79.
[10] A. Allen, Structure and function in gastric mucus, in: M. Elstein, D. Parke (Eds.), Mucus in Health and Disease, Adv. Exp. Med. Biol, Plenum Press, New York 1977, pp. 283–299.
[11] Y. Andersch-Björkman, K.A. Thomsson, J.M.H. Larsson, E. Ekerhovd, G.C. Hansson, Large scale identification of proteins, mucins, and their O-glycosylation in the endocervical mucus during the menstrual cycle, Mol. Cell. Proteomics 6 (2007) 708–716.
[12] E. Hafez, Functional anatomy of mucus-secreting cells, Mucus in Health and Disease, Springer 1977, pp. 19–38.
[13] B. Coddeville, E. Maes, C. Ferrier-Pages, Y. Guerardel, Glycan profiling of gel forming mucus layer from the scleractinian symbiotic coral *Oculina arbuscula*, Biomacromolecules 12 (2011) 2064–2073.
[14] T.B. Moraes, J.L. Ribas Ferreira, C.E. da Rosa, J.Z. Sandrini, A.P. Votto, G.S. Trindade, et al., Antioxidant properties of the mucus secreted by *Laeonereis acuta* (Polychaeta, Nereididae): a defense against environmental pro-oxidants? Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 142 (2006) 293–300.
[15] E. Pales Espinosa, M. Perrigault, J.E. Ward, S.E. Shumway, B. Allam, Lectins associated with the feeding organs of the oyster, *Crassostrea virginica*, can mediate particle selection, Biol. Bull. 217 (2009) 130–141.
[16] L.A. Rollins-Smith, J.D. King, P.F. Nielsen, A. Sonnevend, J.M. Conlon, An antimicrobial peptide from the skin secretions of the mountain chicken frog *Leptodactylus fallax* (Anura: Leptodactylidae), Regul. Pept. 124 (2005) 173–178.
[17] A.G. Auld, The function of mucus, Br. Med. J. (1920) 681.
[18] M.W. Denny, Invertebrate mucous secretions: functional alternatives to vertebrate paradigms, in: E. Chantler, N.A. Ratcliffe (Eds.), Symposia of the Society for Experimental Biology, XLIII Mucus and related topics, Cambridge The Company of Biologists Limited 1989, pp. 337–366.
[19] K.R. Reel, F.A. Fuhrman, An acetylcholine antagonist from the mucous secretion of the dorid nudibrach, *Doriopsilla albopunctata*, Comp. Biochem. Physiol. C Comp. Pharmacol. 68 (1981) 49–53.
[20] P.W.C. Sze, S.Y. Lee, The potential role of mucus in the depuration of copper from the mussels *Perna viridis* (L.) and *Septifer virgatus* (Wiegmann), Mar. Pollut. Bull. 31 (1995) 390–393.
[21] T.G. Wolcott, Physiological ecology and intertidal zonation in limpets (Acmaea): a critical look at "limiting factors", Biol. Bull. 145 (1973) 389–422.
[22] S.M.M. Iguchi, T. Aikawa, J.J. Matsumoto, Antibacterial activity of snail mucus mucin, Comp. Biochem. Physiol. A Physiol. 72 (1982) 571–574.
[23] K.B. Ritchie, Regulation of microbial populations by coral surface mucus and mucus-associated bacteria, Mar. Ecol. Prog. Ser. 322 (2006) 1–14.
[24] M.S. Davies, P. Beckwith, Role of mucus trails and trail-following in the behaviour and nutrition of the periwinkle *Littorina littorea*, Mar. Ecol. Prog. Ser. 179 (1999) 247–257.
[25] M. Denny, The role of gastropod pedal mucus in locomotion, Nature 285 (1980) 160–161.
[26] A.M. Smith, T.J. Quick, R.S. Peter, Differences in the composition of adhesive and non-adhesive mucus from the limpet lottia limatula, Biol. Bull. 196 (1999) 34–44.
[27] C.D. Bavington, R. Lever, B. Mulloy, M.M. Grundy, C.P. Page, N.V. Richardson, et al., Anti-adhesive glycoproteins in echinoderm mucus secretions, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 139 (2004) 607–617.
[28] J.E. Duerden, The role of mucous in corals, Q. J. Microsc. 49 (1906) 591–614.
[29] J.E. Ward, S.E. Shumway, Separating the grain from the chaff: particle selection in suspension- and deposit-feeding bivalves, J. Exp. Mar. Biol. Ecol. 300 (2004) 83–130.
[30] B. Brown, J. Bythell, Perspectives on mucus secretion in reef corals, Mar. Ecol. Prog. Ser. 296 (2005) 291–309.
[31] M.S. Davies, H.D. Jones, S.J. Hawkins, Seasonal variation in the composition of pedal mucus from *Patella vulgata* L, J. Exp. Mar. Biol. Ecol. 144 (1990) 101–112.
[32] J.E. McDade, M.R. Tripp, Lysozyme in oyster mantle mucus, J. Invertebr. Pathol. 9 (1967) 581–582.
[33] E. Hennebert, B. Leroy, R. Wattiez, P. Ladurner, An integrated transcriptomic and proteomic analysis of sea star epidermal secretions identifies proteins involved in defense and adhesion, J. Proteomics 128 (2015) 83–91.
[34] T. Miyamoto, K. Yamada, N. Ikeda, T. Komori, R. Higuchi, Bioactive terpenoids from *Octocorallia*, I. bioactive diterpenoids: litophynols A and B from the mucus of the soft coral *Litophyton* sp, J. Nat. Prod. 57 (1994) 1212–1219.
[35] N.T. Brun, N.W. Ross, A.D. Boghen, Changes in the electrophoretic profiles of gill mucus proteases of the Eastern oyster *Crassostrea virginica* in response to infection by the turbellarian *Urastoma cyprinae*, J. Invertebr. Pathol. 75 (2000) 163–170.
[36] W.S. Fisher, Occurrence of agglutinins in the pallial cavity mucus of oysters, J. Exp. Mar. Biol. Ecol. 162 (1992) 1–13.
[37] S. Bulgheresi, I. Schabussova, T. Chen, N.P. Mullin, R.M. Maizels, J.A. Ott, A new C-type lectin similar to the human immunoreceptor DC-SIGN mediates symbiont acquisition by a marine nematode, Appl. Environ. Microbiol. 72 (2006) 2950–2956.
[38] E. Pales Espinosa, X. Jing, M. Perrigault, B. Allam, Mucosal c-type lectins in *Crassostrea virginia* and *Mytilus edulis*: potential involvement in particle capture and mucosal immunity, J. Shellfish Res. 30 (2011) 503.
[39] P.G. Beninger, S.C. Dufour, J. Bourque, Particle processing mechanisms of the eulamellibranch bivalves *Spisula solidissima* and *Mya arenaria*, Mar. Ecol. Prog. Ser. 150 (1997) 157–169.
[40] P.G. Beninger, A. Veniot, The oyster proves the rule: mechanisms of pseudofeces transport and rejection on the mantle of *Crassostrea virginica* and *C. gigas*, Mar. Ecol. Prog. Ser. 190 (1999) 179–188.
[41] J.E. Ward, B.A. Macdonald, R.J. Thompson, P.G. Beninger, Mechanisms of suspension-feeding in bivalves — resolution of current controversies by means of endoscopy, Limnol. Oceanogr. 38 (1993) 265–272.
[42] P. Beninger, S. Dufour, Mucocyte distribution and relationship to particle transport on the pseudolamellibranch gill of *Crassostrea virginica* (Bivalvia: Ostreidae), Mar. Ecol. Prog. Ser. 137 (1996) 133–138.
[43] J.E. Ward, R.I. Newell, R.J. Thompson, B.A. MacDonald, In vivo studies of suspension-feeding processes in the eastern oyster, *Crassostrea virginica* (Gmelin), Biol. Bull. 186 (1994) 221–240.
[44] R.I.E. Newell, S.J. Jordan, Preferential ingestion of organic material by the American oyster *Crassostrea virginica*, Mar. Ecol. Prog. Ser. 13 (1983) 47–53.
[45] X. Jing, E. Pales Espinosa, M. Perrigault, B. Allam, Identification, molecular characterization and expression analysis of a mucosal C-type lectin in the eastern oyster, *Crassostrea virginica*, Fish Shellfish Immunol. 30 (2011) 851–858.
[46] E. Pales Espinosa, D. Hassan, J.E. Ward, S.E. Shumway, B. Allam, Role of epicellular molecules in the selection of particles by the blue mussel, Mytilus edulis, Biol. Bull. 219 (2010) 50–60.
[47] E. Pales Espinosa, M. Perrigault, J.E. Ward, S.E. Shumway, B. Allam, Microalgal cell surface carbohydrates as recognition sites for particle sorting in suspension-feeding bivalves, Biol. Bull. 218 (2010) 75–86.

[48] B. Cognie, L. Barille, Does bivalve mucus favour the growth of their main food source, microalgae? Oceanol. Acta. 22 (1999) 441–450.
[49] B. Allam, W.E. Carden, J.E. Ward, G. Ralph, S. Winnicki, E.E. Pales, Early host–pathogen interactions in marine bivalves: evidence that the alveolate parasite *Perkinsus marinus* infects through the oyster mantle during rejection of pseudofeces, J. Invertebr. Pathol. 113 (2013) 26–34.
[50] E. Pales Espinosa, S.M. Winnicki, B. Allam, Early host–pathogen interactions in marine bivalves: pallial mucus of *Crassostrea virginica* modulates the growth and virulence of its pathogen *Perkinsus marinus*, Dis. Aquat. Org. 104 (2013) 237–247.
[51] B. Allam, E. Pales Espinosa, Mucosal immunity in mollusks, in: B. Beck, E. Peatman (Eds.), Mucosal Health in Aquaculture, Academic Press 2015, pp. 325–370.
[52] A. Shevchenko, O.N. Jensen, A.V. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, et al., Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels, Proc. Natl. Acad. Sci. 93 (1996) 14440–14445.
[53] R. Wernersson, Virtual ribosome—a comprehensive DNA translation tool with support for integration of sequence feature annotation, Nucleic Acids Res. 34 (2006) W385-W8.
[54] C.D. Wenger, D.H. Phanstiel, M. Lee, D.J. Bailey, J.J. Coon, COMPASS: a suite of pre- and post-search proteomics software tools for OMSSA, Proteomics 11 (2011) 1064–1074.
[55] A.I. Nesvizhskii, A. Keller, E. Kolker, R. Aebersold, A statistical model for identifying proteins by tandem mass spectrometry, Anal. Chem. 75 (2003) 4646–4658.
[56] A. Conesa, S. Götz, J.M. García-Gómez, J. Terol, M. Talón, M. Robles, Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics 21 (2005) 3674–3676.
[57] B. Allam, Role of Extrapallial Fluids in Bivalve Immunity: the Case of Brown Ring Disease in the Clam *Ruditapes Philippinarum*, University of Western Brittany, Brest, France, 1998.
[58] S.I. Takatsuki, On the nature and functions of the amoebocytes of *Ostrea edulis*, Q. J. Microsc. Sci. 76 (1934) 379–431.
[59] C.M. Yonge, J. Mar. Biol. Assoc. U. K. (New Ser.) 14 (1926) 295–386.
[60] B. MacDonald, J. Ward, C. McKenzie, Exfoliation of epithelial cells from the palliai organs of the sea scallop, *Placopecten magellanicus*, J. Exp. Mar. Biol. Ecol. 191 (1995) 151–165.
[61] S. Tasumi, G.R. Vasta, 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. 179 (2007) 3086–3098.
[62] B. Meckelein, D. Externest, M.A. Schmidt, A. Frey, Contribution of serum immunoglobulin transudate to the antibody immune status of murine intestinal secretions: influence of different sampling procedures, Clin. Diagn. Lab. Immunol. 10 (2003) 831–834.
[63] G.F. Zhang, X.D. Fang, X.M. Guo, L. Li, R.B. Luo, F. Xu, et al., The oyster genome reveals stress adaptation and complexity of shell formation, Nature 490 (2012) 49–54.
[64] F. Fogg, D. Hutton, K. Jumel, J. Pearson, S. Harding, A. Allen, Characterization of pig colonic mucins, Biochem. J. 316 (1996) 937–942.
[65] I. Brockhausen, H. Schachter, P.O. Stanley, GalNAc glycans, in: A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, et al., (Eds.), Essentials of Glycobiology, Cold Spring Harbor, New York 2009, p. 11527.
[66] M.E. Johansson, J.M.H. Larsson, G.C. Hansson, The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host–microbial interactions, Proc. Natl. Acad. Sci. 108 (2011) 4659–4665.
[67] K. Kobayashi, H. Ogata, M. Morikawa, S. Iijima, N. Harada, T. Yoshida, et al., Distribution and partial characterisation of IgG Fc binding protein in various mucin producing cells and body fluids, Gut 51 (2002) 169–176.
[68] B.R. Pearse, T. Tamura, J.C. Sunryd, G.A. Grabowski, R.J. Kaufman, D.N. Hebert, The role of UDP-Glc: glycoprotein glucosyltransferase 1 in the maturation of an obligate substrate prosaposin, J. Cell Biol. 189 (2010) 829–841.
[69] L.A. Ford, Host defense mechanisms of cephalopods, Annu. Rev. Fish Dis. 2 (1992) 25–41.
[70] E.S. Loker, Gastropod immunobiology, Invertebrate immunity, Springer 2010, pp. 17–43.
[71] L. Song, L. Wang, L. Qiu, H. Zhang, Bivalve immunity, Invertebrate Immunity, Springer 2010, pp. 44–65.
[72] N. Itoh, K.G. Takahashi, Distribution of multiple peptidoglycan recognition proteins in the tissues of Pacific oyster, *Crassostrea gigas*, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 150 (2008) 409–417.
[73] N. Sharon, H. Lis, History of lectins: from hemagglutinins to biological recognition molecules, Glycobiology 14 (2004) 53R–62R.
[74] G. Vasta, Roles of galectins in infection, Nat. Rev. Microbiol. 7 (2009) 424–438.
[75] K.M. Hager, V.B. Carruthers, MARveling at parasite invasion, Trends Parasitol. 24 (2008) 51–54.
[76] J. Stevens, O. Blixt, J.C. Paulson, I.A. Wilson, Glycan microarray technologies: tools to survey host specificity of influenza viruses, Nat. Rev. Microbiol. 4 (2006) 857–864.
[77] J.P. Gourdine, E.J. Smith-Ravin, Analysis of a cDNA-derived sequence of a novel mannose-binding lectin, codakine, from the tropical clam *Codakia orbicularis*, Fish Shellfish Immunol. 22 (2007) 498–509.
[78] E.M. Wood-Charlson, L.L. Hollingsworth, D.A. Krupp, V.M. Weis, Lectin/glycan interactions play a role in recognition in a coral/dinoflagellate symbiosis, Cell. Microbiol. 8 (2006) 1985–1993.
[79] B. Morga, I. Arzul, N. Faury, A. Segarra, B. Chollet, T. Renault, Molecular responses of *Ostrea edulis* haemocytes to an in vitro infection with *Bonamia ostreae*, Dev. Comp. Immunol. 35 (2011) 323–333.
[80] N.C. Franc, J.-L. Dimarcq, M. Lagueux, J. Hoffmann, R.A.B. Ezekowitz, Croquemort, a novel drosophila hemocyte/macrophage receptor that recognizes apoptotic cells, Immunity 4 (1996) 431–443.
[81] J. Lau, L. Sussman, S. Katalai, E. Pales Espinosa, B. Allam, Characterization of hemocytes from different body fluids of the eastern oyster *Crassostrea virginica*, https://www.was.org/meetings/ShowAbstract.aspx?Id=290702013.
[82] E. Pales Espinosa, M. Perrigault, B. Allam, 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. 156 (2010) 495–501.
[83] S. Tasumi, T. Ohira, I. Kawazoe, H. Suetake, Y. Suzuki, K. Aida, Primary structure and characteristics of a lectin from skin mucus of the Japanese eel *Anguilla japonica*, J. Biol. Chem. 277 (2002) 27305–27311.
[84] J. Nio, Y. Kon, T. Iwanaga, Differential cellular expression of galectin family mRNAs in the epithelial cells of the mouse digestive tract, J. Histochem. Cytochem. 53 (2005) 1323–1334.
[85] E. Pales Espinosa, B. Allam, Food quality and season affect gene expression of the mucosal lectin MeML and particle sorting in the blue mussel *Mytilus edulis*, Mar. Biol. 160 (2013) 1441–1450.
[86] M. Huang, L. Wang, J. Yang, H. Zhang, L. Wang, L. Song, A four-CRD C-type lectin from *Chlamys farreri* mediating nonself-recognition with broader spectrum and opsonization, Dev. Comp. Immunol. 39 (2013) 363–369.
[87] S. Tunkijjanukij, H.V. Mikkelsen, J.A. Olafsen, A heterogeneous sialic acid-binding lectin with affinity for bacterial LPS from horse mussel (*Modiolus modiolus*) hemolymph, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 117 (1997) 273–286.
[88] J. Yang, L. Wang, H. Zhang, L. Qiu, H. Wang, L. Song, C-type lectin in *Chlamys farreri* (CfLec-1) mediating immune recognition and opsonization, PLoS ONE 6 (2011), e17089.
[89] Y. Mone, B. Gourbal, D. Duval, L. Du Pasquier, S. Kieffer-Jaquinod, G. Mitta, A large repertoire of parasite epitopes matched by a large repertoire of host immune receptors in an invertebrate host/parasite model, PLoS Negl. Trop. Dis. 4 (2010) 18.
[90] H. Zhang, L. Song, C. Li, J. Zhao, H. Wang, Q. Gao, et al., Molecular cloning and characterization of a thioester-containing protein from Zhikong scallop *Chlamys farreri*, Mol. Immunol. 44 (2007) 3492–3500.
[91] E.A. Levashina, L.F. Moita, S. Blandin, G. Vriend, M. Lagueux, F.C. Kafatos, Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*, Cell 104 (2001) 709–718.
[92] S.A. Blandin, E. Marois, E.A. Levashina, Antimalarial responses in anopheles gambiae: from a complement-like protein to a complement-like pathway, Cell Host Microbe 3 (2008) 364–374.
[93] S. Mukhopadhyay, S. Gordon, The role of scavenger receptors in pathogen recognition and innate immunity, Immunobiology 209 (2004) 39–49.
[94] L.J.W. van der Laan, E.A. Döpp, R. Haworth, T. Pikkarainen, M. Kangas, O. Elomaa, et al., Regulation and functional involvement of macrophage scavenger receptor MARCO in clearance of bacteria *in vivo*, J. Immunol. 162 (1999) 939–947.
[95] R. Furukawa, M. Matsumoto, H. Kaneko, Characterization of a scavenger receptor cysteine-rich-domain-containing protein of the starfish, *Asterina pectinifera*: ApSRCR1 acts as an opsonin in the larval and adult innate immune systems, Dev. Comp. Immunol. 36 (2012) 51–61.
[96] L. Liu, J. Yang, L. Qiu, L. Wang, H. Zhang, M. Wang, et al., A novel scavenger receptor-cysteine-rich (SRCR) domain containing scavenger receptor identified from mollusk mediated PAMP recognition and binding, Dev. Comp. Immunol. 35 (2011) 227–239.
[97] E.S. Loker, C.M. Adema, S.M. Zhang, T.B. Kepler, Invertebrate immune systems–not homogeneous, not simple, not well understood, Immunol. Rev. 198 (2004) 10–24.
[98] J.S.C. Arthur, S.C. Ley, Mitogen-activated protein kinases in innate immunity, Nat. Rev. Immunol. 13 (2013) 679–692.
[99] M. Rincón, D. Conze, L. Weiss, N.L. Diehl, K.A. Fortner, D. Yang, et al., Do T cells care about the mitogen-activated protein kinase signalling pathways? Immunol. Cell Biol. 78 (2000) 166–175.
[100] J.M. Silverman, S. Chan, D. Robinson, D. Dwyer, D. Nandan, L. Foster, et al., Proteomic analysis of the secretome of *Leishmania donovani*, Genome Biol. 9 (2008) R35.
[101] C. Clapp, L. Portt, C. Khoury, S. Sheibani, G. Norman, P. Ebner, et al., 14–3-3 Protects against stress-induced apoptosis, Cell Death Dis. 3 (2012).
[102] D.G. Bocchinfuso, P. Taylor, E. Ross, A. Ignatchenko, V. Ignatchenko, T. Kislinger, et al., Proteomic profiling of the *Planarian Schmidtea mediterranea* and its mucous reveals similarities with human secretions and those predicted for parasitic flatworms, Mol. Cell. Proteomics 11 (2012) 681–691.
[103] B. Rajan, J.M. Fernandes, C.M. Caipang, V. Kiron, J.H. Rombout, M.F. Brinchmann, Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules, Fish Shellfish Immunol. 31 (2011) 224–231.
[104] T. Mizuno, K. Kaibuchi, S. Ando, T. Musha, K. Hiraoka, K. Takaishi, et al., Regulation of the superoxide-generating NADPH oxidase by a small GTP-binding protein and its stimulatory and inhibitory GDP/GTP exchange proteins, J. Biol. Chem. 267 (1992) 10215–10218.
[105] F. Provan, L. Jensen, K. Uleberg, E. Larssen, T. Rajalahti, J. Mullins, et al., Proteomic analysis of epidermal mucus from sea lice-infected Atlantic salmon, *Salmo salar* L, J. Fish Dis. 36 (2013) 311–321.
[106] J.A. Tincu, S.W. Taylor, Antimicrobial peptides from marine invertebrates, Antimicrob. Agents Chemother. 48 (2004) 3645–3654.
[107] R.D. Rosa, A. Santini, J. Fievet, P. Bulet, D. Destoumieux-Garzón, E. Bachère, Big defensins, a diverse family of antimicrobial peptides that follows different patterns of expression in hemocytes of the oyster *Crassostrea gigas*, PLoS ONE 6 (2011), e25594.

[108] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (2005) 238–250.
[109] M. Gonzalez, Y. Gueguen, G. Desserre, J. de Lorgeril, B. Romestand, E. Bachère, Molecular characterization of two isoforms of defensin from hemocytes of the oyster *Crassostrea gigas*, Dev. Comp. Immunol. 31 (2007) 332–339.
[110] Y. Gueguen, A. Herpin, A. Aumelas, J. Garnier, J. Fievet, J.-M. Escoubas, et al., Characterization of a defensin from the oyster *Crassostrea gigas*: recombinant production, folding, solution structure, antimicrobial activities, and gene expression, J. Biol. Chem. 281 (2006) 313–323.
[111] R.I. Lehrer, Primate defensins, Nat. Rev. Microbiol. 2 (2004) 727–738.
[112] D.L. Diamond, J.R. Kimball, S. Krisanaprakornkit, T. Ganz, B.A. Dale, Detection of β-defensins secreted by human oral epithelial cells, J. Immunol. Methods 256 (2001) 65–76.
[113] D.A. O'Neil, E.M. Porter, D. Elewaut, G.M. Anderson, L. Eckmann, T. Ganz, et al., Expression and regulation of the human β-defensins hBD-1 and hBD-2 in intestinal epithelium, J. Immunol. 163 (1999) 6718–6724.
[114] A.M. Cole, S. Tahk, A. Oren, D. Yoshioka, Y.-H. Kim, A. Park, et al., Determinants of *Staphylococcus aureus* nasal carriage, Clin. Diagn. Lab. Immunol. 8 (2001) 1064–1069.
[115] J.-K. Seo, J.M. Crawford, K.L. Stone, E.J. Noga, Purification of a novel arthropod defensin from the American oyster, *Crassostrea virginica*, Biochem. Biophys. Res. Commun. 338 (2005) 1998–2004.
[116] F.M.Y. Fung, S. Tachibana, L.M. Chou, J.L. Ding, Cytotoxic and anticancer agents in mucus of *Galaxea fascicularis*: purification and characterization, J. Mar. Biotechnol. 5 (1997) 50–57.
[117] K. Hjelmeland, M. Christie, J. Raa, Skin mucus protease from rainbow-trout, *Salmo gairdneri* Richardson, and its biological significance, J. Fish Biol. 23 (1983) 13–22.
[118] C.L. Rogers-Lowery, R.V. Dimock, Changes in activity and composition of the proteases of fish skin mucus during development of acquired resistance to glochidia larvae of freshwater mussels, Integr. Comp. Biol. 45 (2005) 1185.
[119] C.M.C. Salles, P. Gagliano, S.A.T. Leitao, J.B. Salles, H.L.M. Guedes, V.P.F. Cassano, et al., Identification and characterization of proteases from skin mucus of tambacu, a Neotropical hybrid fish, Fish Physiol. Biochem. 33 (2007) 173–179.
[120] F. Aranishi, Lysis of pathogenic bacteria by epidermal cathepsins L and B in the Japanese eel, Fish Physiol. Biochem. 20 (1999) 37–41.
[121] H. Fritz, Human mucus proteinase inhibitor (human MPI). Human seminal inhibitor I (HUSI-I), antileukoprotease (ALP), secretory leukocyte protease inhibitor (SLPI), Biol. Chem. Hoppe Seyler (369 Suppl.) (1988) 79–82.
[122] G. Panicker, Y. Ye, D. Wang, E.R. Unger, Characterization of the human cervical mucous proteome, Clin. Proteomics 6 (2010) 18–28.
[123] Q. Xue, N. Itoh, K.L. Schey, R.K. Cooper, J.F. La Peyre, Evidence indicating the existence of a novel family of serine protease inhibitors that may be involved in marine invertebrate immunity, Fish Shellfish Immunol. 27 (2009) 250–259.
[124] D.M. Chipman, N. Sharon, Mechanism of lysozyme action, Science 165 (1969) 454–465.
[125] P. Jollès, Lysozymes: Model Enzymes Biochemistry and Biology, Springer, 1996.
[126] B. Allam, C. Paillard, Defense factors in clam extrapallial fluids, Dis. Aquat. Org. 33 (1998) 123–128.
[127] B. Allam, C. Paillard, M. Auffret, Alterations in hemolymph and extrapallial fluid parameters in the Manila clam, *Ruditapes philippinarum*, challenged with the pathogen *Vibrio tapetis*, J. Invertebr. Pathol. 76 (2000) 63–69.
[128] J.G. McHenery, T.H. Birkbeck, J.A. Allen, The occurrence of lysozyme in marine bivalves, Comp. Biochem. Physiol. B Comp. Biochem. 63 (1979) 25–28.
[129] Ø.M. Olsen, I.W. Nilsen, K. Sletten, B. Myrnes, Multiple invertebrate lysozymes in blue mussel *Mytilus edulis*, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 136 (2003) 107–115.
[130] F.-L.E. Chu, J.F. La Peyre, Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (*Crassostrea virginica*), J. Invertebr. Pathol. 54 (1989) 224–232.
[131] Q.-G. Xue, K.L. Schey, A.K. Volety, F.-L.E. Chu, J.F. La Peyre, Purification and characterization of lysozyme from plasma of the eastern oyster *Crassostrea virginica*, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 139 (2004) 11–25.
[132] S. Daffre, P. Kylsten, C. Samakovlis, D. Hultmark, The lysozyme locus in *Drosophila melanogaster*: an expanded gene family adapted for expression in the digestive tract, Mol. Gen. Genet. MGG. 242 (1994) 152–162.
[133] R. Chandan, K. Shahani, R. Holly, Lysozyme Content of Human Milk, 1964.
[134] X. Ye, H. Wang, T. Ng, First chromatographic isolation of an antifungal thaumatin-like protein from French bean legumes and demonstration of its antifungal activity, Biochem. Biophys. Res. Commun. 263 (1999) 130–134.
[135] L.C. Loon, Pathogenesis-related proteins, Plant Mol. Biol. 4 (1985) 111–116.
[136] B. Altincicek, E. Knorr, A. Vilcinskas, Beetle immunity: identification of immune-inducible genes from the model insect *Tribolium castaneum*, Dev. Comp. Immunol. 32 (2008) 585–595.
[137] Z. Bai, Y. Yin, S. Hu, G. Wang, X. Zhang, J. Li, Identification of genes involved in immune response, microsatellite, and SNP markers from expressed sequence tags generated from hemocytes of freshwater pearl mussel (*Hyriopsis cumingii*), Mar. Biotechnol. 11 (2009) 520–530.
[138] B. Allam, E. Pales Espinosa, A. Tanguy, F. Jeffroy, C. Le Bris, C. Paillard, Transcriptional changes in Manila clam *Ruditapes philippinarum* in response to Brown Ring Disease, Fish Shellfish Immunol. (2014).
[139] M. Gerdol, C. Manfrin, G. De Moro, A. Figueras, B. Novoa, P. Venier, et al., The C1q domain containing proteins of the Mediterranean mussel *Mytilus galloprovincialis*: a widespread and diverse family of immune-related molecules, Dev. Comp. Immunol. 35 (2011) 635–643.
[140] U. Kishore, C. Gaboriaud, P. Waters, A.K. Shrive, T.J. Greenhough, K.B.M. Reid, et al., C1q and tumor necrosis factor superfamily: modularity and versatility, Trends Immunol. 25 (2004) 551–561.
[141] M. Gerdol, P. Venier, A. Pallavicini, The genome of the Pacific oyster *Crassostrea gigas* brings new insights on the massive expansion of the C1q gene family in bivalvia, Dev. Comp. Immunol. 49 (2015) 59–71.
[142] M.E. Johansson, K.A. Thomsson, G.C. Hansson, Proteomic analyses of the two mucus layers of the colon barrier reveal that their main component, the Muc2 mucin, is strongly bound to the Fcgbp protein, J. Proteome Res. 8 (2009) 3549–3557.
[143] E. Ottaviani, A. Franchini, S. Cassanelli, S. Genedani, Cytokines and invertebrate immune responses, Biol. Cell. 85 (1995) 87–91.
[144] T. Calandra, J. Bernhagen, R.A. Mitchell, R. Bucala, Macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor, J. Exp. Med. 179 (1994) 1895–1902.
[145] A.B. Garcia, R.J. Pierce, B. Gourbal, E. Werkmeister, D. Colinet, J.-M. Reichhart, et al., Involvement of the cytokine MIF in the snail host immune response to the parasite *Schistosoma mansoni*, PLoS Pathog. 6 (2010), e1001115.
[146] J.L. Miller, A. Harupa, S.H.I. Kappe, S.A. Mikolajczak, *Plasmodium yoelii* macrophage migration inhibitory factor is necessary for efficient liver-stage development, Infect. Immun. 80 (2012) 1399–1407.
[147] M.-G. Parisi, M. Toubiana, V. Mangano, N. Parrinello, M. Cammarata, P. Roch, MIF from mussel: coding sequence, phylogeny, polymorphism, 3D model and regulation of expression, Dev. Comp. Immunol. 36 (2012) 688–696.
[148] S. Cui, D. Zhang, S. Jiang, H. Pu, Y. Hu, H. Guo, et al., A macrophage migration inhibitory factor like oxidoreductase from pearl oyster *Pinctada fucata* involved in innate immune responses, Fish Shellfish Immunol. 31 (2011) 173–181.
[149] G. Beck, T.W. Ellis, G.S. Habicht, S.F. Schluter, J.J. Marchalonis, Evolution of the acute phase response: iron release by echinoderm *Asterias forbesi* coelomocytes, and cloning of an echinoderm ferritin molecule, Dev. Comp. Immunol. 26 (2002) 11–26.
[150] M. Von Darl, P.M. Harrison, W. Bottke, cDNA cloning and deduced amino acid sequence of two ferritins: soma ferritin and yolk ferritin, from the snail *Lymnaea stagnalis* L, Eur. J. Biochem. 222 (1994) 353–366.
[151] P. Wang, J. Heitman, The cyclophilins, Genome Biol. 6 (2005) 226.
[152] J. Liu, J.D. Farmer Jr., W.S. Lane, J. Friedman, I. Weissman, S.L. Schreiber, Calcineurin is a common target of cyclophilin–cyclosporin A and FKBP-FK506 complexes, Cell 66 (1991) 807–815.
[153] D.S.T. Ong, L. Wang, Y. Zhu, B. Ho, J.L. Ding, The response of ferritin to LPS and acute phase of *Pseudomonas* infection, J. Endotoxin Res. 11 (2005) 267–280.
[154] B. Halliwell, Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life, Plant Physiol. 141 (2006) 312–322.
[155] A.L. Fink, Chaperone-mediated protein folding, Physiol. Rev. 79 (1999) 425–449.
[156] K. Rokutan, Role of heat shock proteins in gastric mucosal protection, J. Gastroenterol. Hepatol. 15 (2000) 12–19.
[157] D. Moraga, A.L. Meistertzheim, S. Tanguy-Royer, I. Boutet, A. Tanguy, A. Donval, Stress response in Cu^{2+} and Cd^{2+} exposed oysters (*Crassostrea gigas*): an immunohistochemical approach, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141 (2005) 151–156.
[158] N. Itoh, Q.-G. Xue, K.L. Schey, Y. Li, R.K. Cooper, J. La Peyre, Characterization of the major plasma protein of the eastern oyster, *Crassostrea virginica*, and a proposed role in host defense, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 158 (2011) 9–22.
[159] Q. Xue, J. Gauthier, K. Schey, Y. Li, R. Cooper, R. Anderson, et al., Identification of a novel metal binding protein, segon, in plasma of the eastern oyster, *Crassostrea virginica*, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 163 (2012) 74–85.
[160] J.F. Beguel, Q. Xue, J.F. La Peyre, Potential role of the two most dominant plasma proteins, dominin and segon, in eastern oyster shell mineralization, Abstracts of Technical Papers Presented at the 106th Annual Meeting National Shellfisheries Association, Jacksonville, Florida Journal of Shellfish Research 2014, p. 590.