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Pallial mucus of the oyster *Crassostrea virginica* regulates the expression of putative virulence genes of its pathogen *Perkinsus marinus* ☆

Emmanuelle Pales Espinosa [a], Erwan Corre [b], Bassem Allam [a,*]

[a] School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY 11794-5000, USA
[b] Analyses and Bioinformatics for Marine Science, Station Biologique de Roscoff, 29688 Roscoff Cedex, France

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ABSTRACT

Perkinsus marinus is a pathogen responsible for severe mortalities of the eastern oyster *Crassostrea virginica* along the East and Gulf coasts of the United States. When cultivated, the pathogenicity of this microorganism decreases significantly, hampering the study of its virulence factors. Recent investigations have shown a significant increase of the in vivo virulence of *P. marinus* exposed to oyster pallial mucus. In the current study, we investigated the effect of pallial mucus on *P. marinus* gene expression compared with cultures supplemented with oyster digestive extracts or with un-supplemented cultures. In parallel, parasite cells cultured under these three conditions were used to challenge oysters and to assess virulence in vivo. *Perkinsus marinus* mRNA sequencing was performed on an Illumina GAIIX sequencer and data were analysed using the Tuxedo RNAseq suite for mapping against the draft *P. marinus* genome and for differential expression analysis. Results showed that exposure of *P. marinus* to mucus induces significant regulation of nearly 3,600 transcripts, many of which are considered as putative virulence factors. Pallial mucus is suspected to mimic internal host conditions, thereby preparing the pathogen to overcome defense factors before invasion. This hypothesis is supported by significant regulation in several antioxidant proteins, heat shock proteins, protease inhibitors and proteasome subunits. In addition, mucus exposure induced the modulation of several genes known to affect immunity and apoptosis in vertebrates and invertebrates. Several proteases (proteolysis) and merozoite surface proteins (cell recognition) were also modulated. Overall, these results provide a baseline for targeted, in depth analysis of candidate virulence factors in *P. marinus*.

1. Introduction

Perkinsus marinus (Alveolata, Perkinsozoa) is an obligate pathogen of the eastern oyster *Crassostrea virginica*. Formerly named *Dermo cystidiummarinum* (Mackin et al., 1950) and categorised as a fungus, the systematic position of *P. marinus* has evolved throughout recent decades (Perkins, 1976; Levine, 1978; Reece et al., 1997; Siddall et al., 1997; Bushek et al., 2002; Saldarriaga et al., 2003; Grauvogel et al., 2007; Bachvaroff et al., 2011) to finally being classified in the new Phylum Perkinsozoa, representing the "connecting link between Dinoflagellates and Apicomplexans" (Noren et al., 1999). All members of this phylum (i.e. Perkinsozoa) are parasitic (see the review by Mangot et al., 2011).

"Dermo" disease, or "Perkinsiosis" produced by *P. marinus*, is a major cause of mortality of oysters along the Gulf and East coasts of the USA (Burreson and Calvo, 1996). This disease is relatively chronic and is characterised by the death of oysters several weeks after infection. In their laboratory experiments, Ford et al. (2002) described a two-phase mortality, with an early phase occurring within 2–3 weeks following infection (leading to ~20% of deaths) and a second phase 2–6 weeks later (80–100% deaths). Oysters dying during the first phase usually present light infections whereas it is the opposite for oysters dying during the second phase which usually display very high parasite loads (>10^6 cells g^{-1} oyster wet weight) (Ford et al., 2002; Allam et al., 2013; Pales Espinosa et al., 2013). In addition, dead/moribund oysters from the second mortality phase are characterised by a proteolytic degradation of soft tissues (Ford and Tripp, 1996), particularly obvious in the gills that can be extensively lysed (Mackin, 1951; Ray, 1954; Ewart and Ford, 1993). Several studies have shown that *P. marinus* releases extracellular products that contribute to tissue digestion among which serine proteases (La Peyre et al., 1995, 1996) that degrade proteins

☆ *Note:* Un-filtered sequence data have been deposited in the NCBI Short Read Archive database (SRA/SRP036141).

* Corresponding author. Tel.: +1 631 632 8745; fax: +1 631 632 8915.
E-mail address: Bassem.Allam@stonybrook.edu (B. Allam).

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present in oyster hemolymph, reduce oyster defenses (Garreis et al., 1996) and cause significant cellular and tissular damage (La Peyre et al., 1995). The factors involved in the rapid death of oysters during the first phase of mortality are, however, not known and it is possible, due to the relatively low parasite loads during that phase, that factors other than proteases are involved in oyster death. In fact, no obvious tissue degeneration has been observed in this group of oysters (Allam et al., 2013; Pales Espinosa et al., 2013).

One of the difficulties in investigating *P. marinus* virulence lies in the fact that the parasite quickly loses its virulence and pathogenicity when cultured under standard laboratory conditions (Volety and Chu, 1994; Bushek and Allen, 1996; Bushek et al., 1997; Ford et al., 2002). Wild-type *P. marinus* (freshly isolated from infected oysters) is significantly more virulent compared with cultured parasite cells (Ford et al., 2002). The addition of oyster supplements (plasma or whole body homogenates) to culture media slightly enhanced *P. marinus* infectivity (Earnhart et al., 2004) and protease expression (MacIntyre et al., 2003; Brown et al., 2005), suggesting the presence of factors in oyster tissues modulating the parasite's virulence. In a recent study, we were able to show that *P. marinus* cultures supplemented with pallial mucus caused early and severe mortality in oysters similar to that usually obtained with wild-type parasite cells (Pales Espinosa et al., 2013). Interestingly, parasite cultures with oyster extracts other than pallial mucus added, including those supplemented with digestive extracts or plasma, were less pathogenic and caused minimal mortalities, similar to those obtained with *P. marinus* grown in standard culture media (Allam et al., 2013; Pales Espinosa et al., 2013). These recent findings demonstrate that the mucus of *C. virginica* plays a significant role in the pathogenesis of *P. marinus* by dramatically enhancing the infectivity of this devastating parasite. This is not surprising since pallial mucus is the first host factor encountered by waterborne *P. marinus* cells reaching the oyster and can therefore serve as a trigger for initiating the machinery needed for the parasite to invade host tissues and cause infection (Allam et al., 2013; Pales Espinosa et al., 2013).

In this study, we confirmed the enhanced virulence of mucus-supplemented parasite cells using standard in vivo challenge experiments and used RNASeq methods to characterise the gene expression profiles of parasite cells cultured in the presence or absence of pallial mucus. The resulting transcriptomes were probed with the primary goal of identifying genes potentially involved in the virulence of the pathogen.

2. Material and methods

2.1. Collection of pallial mucus and digestive extracts

Adult *C. virginica* naïve for *P. marinus* were obtained from a commercial source in Maine, USA (Pemaquid Oyster Company, 78.4 ± 5.6 mm in length, mean ± S.D.) to be used for mucus extraction and challenge experiments. Oysters ($n = 15$) were cleaned of sediment and epibionts. The right valve of each oyster was then carefully removed and underlying pallial tissues were rinsed with seawater (ASW15, salinity of 15). Mucus from mantle, gills and labial palps was collected and pooled following the general procedures described by Pales Espinosa et al. (2009). Briefly, mucus was carefully collected using sterile cotton-tipped swabs. Swabs were then immersed in 50 ml of ice-cold ASW15. Following the collection of pallial mucus, the digestive gland of each oyster was dissected by excluding gonad and connective tissues, finely minced using a razor blade and immersed in 50 ml of ice-cold ASW15. Tubes containing swabs or minced digestive gland were placed at 4 °C for 1 h on a rotating shaker. The resulting fluids (pallial mucus and digestive extract) was centrifuged twice (1,000*g*, 30 min, 4 °C), filter sterilised (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, IL) as per the manufacturer's recommendations. Both fluids were diluted with ASW15 to a protein concentration of 2 mg ml^{-1}.

2.2. Pathogen cultures

Perkinsus marinus cells (ATCC-50439, http://www.atcc.org/products/all/50439.aspx) were grown at 23 °C in sterile DME/F12-3 culture medium (Burreson et al., 2005) and used as a starter when cells were in an exponential phase of growth. Nine 250 ml cultures of *P. marinus* were separately grown in 800 ml vented tissue culture flasks as follows. Six cultures were prepared with 25 ml of *P. marinus* starter, 162.5 ml of DME/F12-3 culture medium supplemented with 62.5 ml of either pallial mucus (three replicate flasks) or digestive extract fluids (three flasks) at a final concentration of 0.5 mg protein ml^{-1}. The third series of cultures was prepared by adding 62.5 ml of ASW15 (controls) instead of mucus or digestive extracts. Flasks were incubated at 23 °C for 14 days. After incubation, parasite cells were recovered by centrifugation (400*g*, 15 min, 4 °C), washed with ASW15 twice, resuspended in ASW15 and kept overnight to be used the following day for (i) challenge experiments and (ii) RNA extraction (see Sections 2.3 and 2.4).

2.3. Challenge experiments

Naïve *C. virginica* were carefully notched with bone shears, avoiding damage to mantle tissues. Four days following notching, oysters (20 per treatment) were separately injected using a 23-gauge blunt needle through the notch into the pallial cavity with *P. marinus* grown in supplemented or un-supplemented media (the three replicates for each culture type were pooled before injection; see Section 2.4.) at a concentration of 2.5×10^6 per oyster in a volume of 1 ml. Subsets of oysters were inoculated with artificial seawater as negative controls. Following inoculation, oysters were covered with damp paper towels for 2 h at room temperature and subsequently returned to separate tanks (three replicate tanks/treatment) maintained at 25 °C. For 4 weeks, oysters were fed DT's Live Marine Phytoplankton (Sycamore, Illinois, USA; Pales Espinosa and Allam, 2006) and monitored daily for mortality. Moribund oysters were immediately removed from tanks and the *P. marinus* loads in whole oyster tissues were determined using the alternative Ray's fluid thioglycollate medium (ARFTM; Ellin and Bushek, 2006) and infections were assessed following the general procedures described by Nickens et al. (2002) and Pales Espinosa et al. (2013). After 4 weeks, the surviving oysters were also processed for *P. marinus* infections using ARFTM. To determine the combined effect of both time to death and *P. marinus* infection intensity (parasite load in wet tissue weight), a virulence index ranging from 0 (least virulent) to 10 (most virulent: short time to death combined with high parasite loads) was calculated as described by Chintala et al. (2002) and Pales Espinosa et al. (2013). Statistical analysis of infection intensities was performed using a one-way ANOVA followed by Holm–Sidak post hoc test as needed. For categorical data (virulence indices) a one-way ANOVA on ranks and Dunn's post hoc tests were used. Mortality data, consisting of time of death (i.e. day of experiment) for individual oysters, were compared by Kaplan–Meier log-rank survival analysis with Holm–Sidak post hoc testing for multiple comparisons (Kleinbaum and Klein, 2005). Differences were considered significant when $P < 0.05$.

2.4. RNA sequencing and analysis

Perkinsus marinus suspensions (see Section 2.2.) were centrifuged (400*g*, 15 min, 4 °C) and cell pellets were immediately used for total RNA extraction using TRI Reagent (MRC, Cincinnati, OH, USA). Total RNA samples (2500–3000 ng μl^{-1}) from the same type of culture were pooled, flash frozen at −80 °C before being used for mRNA sequencing. Library construction and sequencing were performed by the Cold Spring Harbor Laboratory Genome Research Center (Cold Spring Harbor, New York, USA) on an Illumina GAIIX sequencer according to the manufacturer's protocols. Briefly, mRNA was selected using oligo(dT) probes, fragmented, then used for cDNA synthesis using random primers modified for attachment to the Illumina flowcell. Barcoded samples were sequenced on two replicate Illumina PE100 lanes generating a total of ~221.1 million reads for a total of 22,290 MB of sequence. Un-filtered sequence data have been deposited at the NCBI Short Read Archive database (SRA/SRP036141). Data were filtered and trimmed using FASTX-Toolkit (reads with ambiguous bases 'N', and reads with more than 10% Q < 20 bases were removed), cleaned from the adapter using cutadapt (Version 1.1; Martin, 2011) and checked using fastQC (Version 0.10.0), then submitted for digital gene expression analysis using Tuxedo RNAseq suite on the *P. marinus* draft genome. Cleaned reads were independently (one mapping per replicate and per condition) mapped on a reduced version of the draft genome of *P. marinus* (containing only contigs > 2000 nucleotides to avoid highly fragmented coding DNA sequences) using Tophat2 (Trapnell et al., 2009, 2012). Trancript discovery was conducted using Cufflinks and differential expression analysis was done using the CuffDiff2 method (Trapnell et al., 2010, 2012). Comparisons focused on transcripts regulated in parasite cells from cultures supplemented with pallial mucus as opposed to those supplemented with ASW or digestive extracts. The Blast2GO software system (Götz et al., 2008, http://www.blast2go.com/b2ghome) was used to annotate differentially expressed transcripts (BLASTx against the NCBI non redundant database, *E*-value cut off of 10*E*-3). Blast2GO was also used for sequence mapping, assignment of functional Gene Ontology (GO) terms, Enzyme Codes (EC) and InterProScan (IPR) annotations, and for performing enrichment analyses by means of Fisher's exact test with multiple testing correction (Benjamini and Hochberg) of false discovery rate ($P < 0.05$).

3. Results

3.1. Challenge experiments

Results from the challenge experiment showed higher mortality in oysters injected with *P. marinus* from cultures supplemented with pallial mucus compared with the other treatments (Fig. 1). Mortality in this batch reached 20% by day 10 and peaked at 40% at the end of the 4 week experiment. Mortality was much lower in oysters injected with unsupplemented *P. marinus* cells or cells supplemented with digestive extracts, reaching 5% and 0%, respectively, at day 10, and peaking at 20% and 10% at the end of the experiment (Fig. 1A). All dead oysters removed before the end of the 4 week experiment were processed immediately for prevalence and intensity of *P. marinus* by ARFTM. *Perkinsus marinus* was detected in all dead oysters from the pallial mucus and digestive tract treatments. The parasite was also found in some of the oysters (two out of four) removed from the batch injected with un-supplemented *P. marinus* cells, while the single oyster removed from the unchallenged control treatment was negative for *P. marinus*. After the 4 week period, all surviving oysters were processed for *P. marinus* prevalence and intensity. The overall prevalence was 100% for the pallial mucus and digestive extract treatments and 89.5% among oysters injected with un-supplemented *P. marinus*, while no infections were detected in the negative control treatment. The parasite loads in oysters injected with *P. marinus* cultures supplemented with pallial mucus and digestive extracts were 8.3×10^2 and 1.93×10^3 hypnospores $gram^{-1}$, respectively (Fig. 1B). These levels were within the same range as parasite loads measured in oysters injected with un-supplemented cultures (1.5×10^3 hypnospores $gram^{-1}$). Overall, the virulence index was significantly higher for *P. marinus* cells from cultures supplemented with pallial mucus (3.3 ± 0.40; mean ± S.E.M.) compared with cultures supplemented with digestive extract (2.3 ± 0.13) or un-supplemented cultures (2.3 ± 0.40) (Fig. 1C).

3.2. RNA sequencing

The reads were aligned against the *P. marinus* draft genome then assembled into a total of 24,976 transcripts with a mean length of 1382 nucleotides (nt) and a median length of 922 nt. There was a specific focus on transcripts uniquely regulated in parasite cells from cultures supplemented with pallial mucus (those common to parasite cells from cultures supplemented with pallial mucus and any other treatment were eliminated from downstream analyses). These comparisons allowed the identification of 2054 and 1660 transcripts that were significantly up- or down-regulated, respectively, in *P. marinus* cultures supplemented with pallial mucus compared with un-supplemented cultures or those supplemented with digestive extract (Supplementary Tables S1 and S2). Among the regulated genes, 195 up-regulated (representing 9.5% of the 2054 genes) and 91 down-regulated (5.5%) genes were not annotated (no BLASThits, GO, EC or IPR). According to the GO molecular function and biological process terms, the others genes were tentatively classified into different major categories such as "metabolic process", "transport" or "cell homeostasis" (Supplementary Tables S1 and S2). In addition, a GO enrichment analysis identified several significantly regulated transcripts regrouped into 36 GO terms. More than 30% of GO terms refer to "ribosome biogenesis" (e.g. structural constituent of ribosome) or "RNA biosynthetic process" (e.g. DNA-directed RNA polymerase activity) and were significantly over-represented in *P. marinus* grown in mucus compared with the whole transcriptome, suggesting an increase in the general protein biosynthetic process. Most specific enriched "Molecular Function" GO terms are presented in Table 1. The transcripts of particular interest (i.e. putative virulence-related factors) were extracted from the pool and grouped into four major categories: (i) response to stress, (ii) cell signaling, signal transduction and integral component of membrane, (iii) apoptosis regulation and (iv) proteases and protease inhibitors. A more detailed description of these categories is given below.

3.2.1. Response to stress

Among the genes involved in adaptation to, and survival under, stressful environments, those coding for antioxidants and heat shock proteins (HSPs) were given special attention. Different families of genes coding for antioxidant enzymes showed different regulation patterns in *P. marinus* grown in media supplemented with pallial mucus compared with un-supplemented parasite cells or with those supplemented with digestive extracts. For instance, four genes coding for iron/manganese-dependent superoxide dismutases (Fe/Mn SOD) were up-regulated whereas three genes coding for copper/zinc-dependent (Cu/Zn SOD) and one for Fe/Mn SOD were simultaneously down-regulated (Fig. 2; Supplementary Tables S1 and S2). In addition, 19 genes coding for antioxidant proteins (genes involved in antioxidant mechanisms) were up-regulated (ascorbate peroxidase; four peroxiredoxins; nine thioredoxins; thoredoxin reductase; two glutharedoxins; two alkyl hydroperoxide reductases) whereas only one peroxiredoxin was

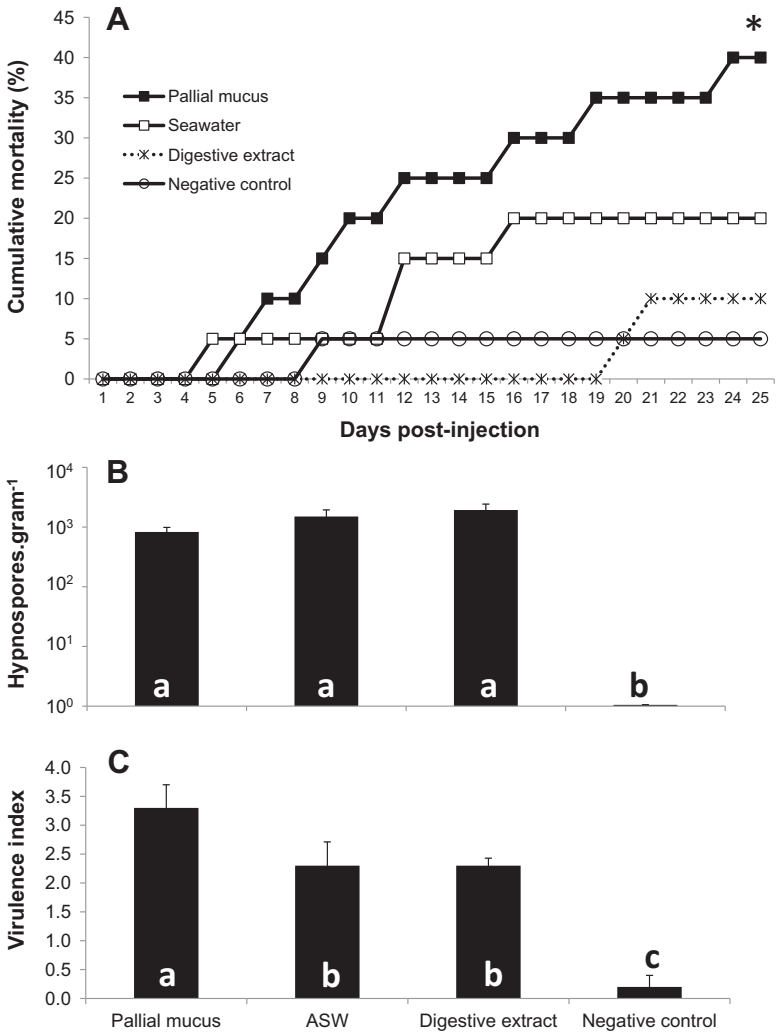

Fig. 1. Cumulative mortality (A), *Perkinsus marinus* loads (B, mean ± S.E.M.) and virulence indices (C, mean ± S.E.M.) in oysters challenged with *P. marinus* grown in media supplemented with pallial mucus, digestive extracts or with sterile seawater. Negative control oysters were injected with sterile seawater. *Higher mortality levels than all other treatments (log-rank test, $P < 0.05$). For each parameter, different letters (a, b and c) indicate statistically significant differences (Holm–Sidak post hoc test, $P < 0.05$; $n = 20$ oysters/treatment) between treatments.

down-regulated (Fig. 2, Supplementary Tables S1 and S2). Another important group of genes with functional roles in response to stress is the HSP. Twenty-four and 28 genes coding for HSPs were up- or down-regulated, respectively, when *P. marinus* was grown in media supplemented with pallial mucus (Fig. 2; Supplementary Tables S1 and S2).

3.2.2. Cell signaling, signal transduction and integral component of membrane

The sequence data analysis showed regulation of multiple genes predicted to be involved in cell signaling and signal transduction (characterised by one or several of the following GO terms: integral to membrane, signal transduction, kinase or phosphatase activity; Supplementary Tables S1 and S2). Exposure of *P. marinus* to pallial mucus also caused significant regulation of another group of genes known as potential virulence factors in other pathogens, namely the serine/threonine kinases (Fig. 2): 23 were up- and 25 were down-regulated in *P. marinus* grown in media supplemented with pallial mucus.

Among genes potentially able to manipulate host cell metabolism, particular attention was given to those known to represent virulence factors in other pathogens. Thus, several genes related to the glycosylphosphatidylinositol (GPI) anchor biosynthesis were up- (nine) or down- (four) regulated (Fig. 2). Other genes potentially involved in the biosynthesis of the GPI anchor were also significantly regulated including those with the GO term "transferring glycosyl groups" (13 up- and 33 down-regulated; Fig. 2) or those described as "merozoite surface protein" (13 up- and five down-regulated; Fig. 2).

One gene coding for a macrophage migration inhibitory factor as well as three genes coding for immunophilins (i.e. cyclophilins) were found to be up-regulated in *P. marinus* grown in media supplemented with pallial mucus. No genes with immunosuppression function were found to be down-regulated (Table 2).

3.2.3. Apoptosis regulation

Several genes involved in the regulation of apoptosis were found to be modulated in *P. marinus* grown in media supplemented with pallial mucus compared with the other two culture conditions. For instance, 14 genes with pro-apoptotic activity were up-regulated while six were down-regulated (Table 3). Similarly, 11 genes coding for proteins with anti-apoptotic properties were up-regulated whereas only one gene with anti-apoptotic functions was down-regulated (Table 3).

Table 1
Most specific Gene Ontology terms enriched in *Perkinsus marinus* supplemented with pallial mucus compared with the whole transcriptome. Only Gene Ontology terms under "Molecular Function" are presented out of 36 differentially regulated Gene Ontology terms (Fisher's exact test with Benjamini and Hochberg correction).

	Molecular function Gene Ontology terms	Regulated transcripts (%)	Transcriptome (%)	Enrichment (fold)
Up-regulated	Structural constituent of ribosome	13.55	1.50	9.05
	rRNA binding	0.75	0.12	6.10
	DNA-directed RNA polymerase activity	1.84	0.69	2.69
	Protein disulfide oxidoreductase activity	1.17	0.36	3.25
	Protein domain specific binding	0.75	0.16	4.74
	Riboflavin synthase activity	0.25	0.05	4.95
	Proton-transporting ATP synthase activity, rotational mechanism	0.83	0.21	3.95
	Phosphotransferase activity, phosphate group as acceptor	0.75	0.17	4.27
Down-regulated	Serine-type carboxypeptidase activity	3.12	0.57	5.47
	Cysteine-type endoypeptidase activity	2.64	0.79	3.34
	ATP-dependent helicase activity	4.81	2.56	1.88
	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	1.49	0.44	3.39

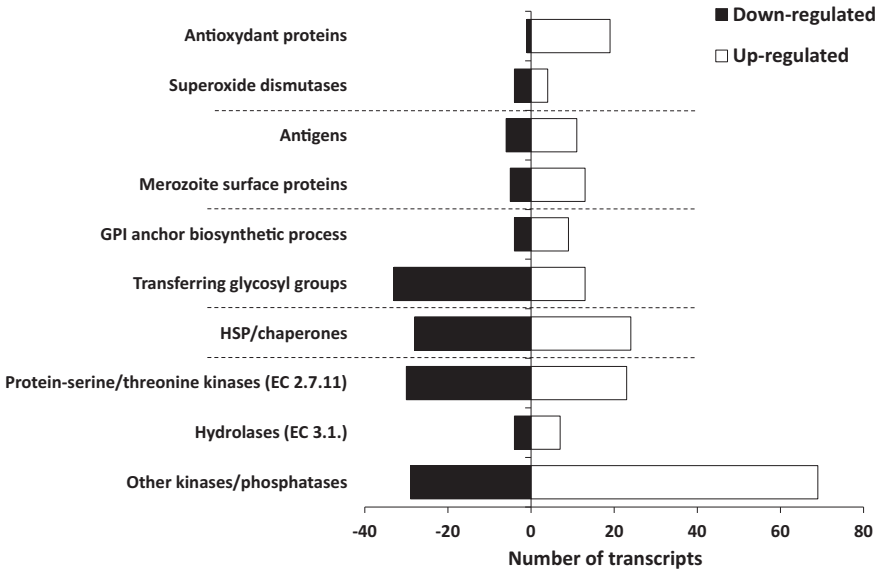

Fig. 2. Transcripts with known links to virulence regulated in *Perkinsus marinus* supplemented with oyster pallial mucus. Dashed lines separate different functional groups.

Table 2
Transcripts with Gene Ontology terms related to "immunosuppression function" which were up-regulated in *Perkinsus marinus* grown with oyster pallial mucus. Additional information on these transcripts is given in Supplementary Table S1.

Code	Protein identification	Gene Ontology terms
CUFF.2454.1	Cyclophilin	F:peptidyl-prolylcis-trans isomerase activity; P:protein folding
CUFF.4228.1	Cyclophilin ovcyp-2	P:protein folding; F:peptidyl-prolyl cis-trans isomerase activity; C:plasma membrane
CUFF.7030.1	Macrophage migration inhibitory factor	P:negative regulation of apoptotic process
CUFF.21597.1	Peptidyl-prolyl cis-transisomerase cyclophilin type	P:protein folding; F:peptidyl-prolyl cis-trans isomerase activity; F:DNA binding

3.2.4. Proteases and protease inhibitors

Forty-two and 106 genes coding for proteases were, respectively, up- or down-regulated in *P. marinus* supplemented with pallial mucus compared with the two other culture conditions (Fig. 3; Supplementary Tables S1 and S2). This trend is also supported by the results of the enrichment analysis showing that the GO terms "Proteolysis" and more specifically "cysteine-type endopeptidase activity" and "serine-type carboxypeptidase activity" were significantly under-represented in *P. marinus* grown in mucus compared with the whole transcriptome (Table 1). The major groups of up-regulated proteases include the threonine-type endopeptidases (12 members), the aspartic endopeptidases (seven) and the serine endopeptidases (three) while the down-regulated proteases include mostly the cysteine-type endopeptidases (32) and serine-type carboxypeptidases (30). In addition, two extracellular peptidase inhibitor-like and a cysteine-type endopeptidase inhibitor were up-regulated when *P. marinus* cultures were supplemented with pallial mucus (Table 4).

Table 3
Transcripts with Gene Ontology terms related to apoptotic and anti-apoptotic functions which were regulated in *Perkinsus marinus* grown with oyster pallial mucus. Additional information on these transcripts is given in Supplementary Tables S1 and S2.

	Code	Protein identification	Gene Ontology terms	Signal peptide
Up-regulated	*Apoptotic properties*			
	CUFF.17292.1	60s Ribosomal protein	P:induction of apoptosis; F:protein serine/threonine kinase activity	No
	CUFF.19295.1	Apoptosis inducing factor	F:FAD binding; F:oxidoreductase activity; P:oxidation reduction	Yes
	CUFF.8331.1	Apoptosis inducing factor	F:FAD binding; F:oxidoreductase activity; P:oxidation reduction	No
	CUFF.13173.1	Exosome complex exonuclease	C:cytoplasm; P:apoptosis	Yes
	CUFF.12290.1	Hypothetical protein	C:cytoplasm; P:apoptosis	No
	CUFF.10911.1	LPS-induced transcription factor regulating TNF	–	Yes
	CUFF.12417.1	LPS-induced TNFa	C:cytoplasm; P:regulation of transcription	No
	CUFF.7394.1	LPS-induced TNFa	F:motor activity; P:cellular process; F:nucleotide binding	No
	CUFF.22051.2	Liver stage antigen-	P:apoptosis; F:ATP binding; F:protein kinase activity	Yes
	CUFF.7030.1	Macrophage migration inhibitory (MIF)		Yes
	CUFF.18363.1	Phosducin-like protein	P:apoptosis; C:cytoplasm	No
	CUFF.9903.1	Programmed cell death	C:spliceosomal complex; P:nuclear mRNA splicing, via spliceosome	No
	CUFF.1095.1	Pterin-4-alpha-carbinolamine dehydratase	P:activation of pro-apoptotic gene products	No
	CUFF.12513.1	Ubiquitin family protein	P:induction of apoptosis by extracellular signals; P:anti-apoptosis	No
	Anti-apoptotic properties			
	CUFF.13098.1	14-3-3 protein	F:protein domain specific binding	No
	CUFF.1785.1	14-3-3 protein	F:protein domain specific binding	Yes
	CUFF.19751.1	14-3-3 protein	F:protein domain specific binding	No
	CUFF.3899.1	14-3-3 protein	F:protein domain specific binding	No
	CUFF.9463.2	14-3-3 protein	F:protein domain specific binding	No
	CUFF.7441.1	Adenylate kinase	P:negative regulation of apoptosis; F:p53 binding	Yes
	CUFF.18724.1	Apoptosis antagonizing transcription factor	C:nucleus	No
	CUFF.4342.1	Basic transcription factor	P:negative regulation of apoptosis	No
	CUFF.17794.1	Fas apoptotic inhibitory	C:membrane	No
	CUFF.17795.1	Fas apoptotic inhibitory	F:ATP binding; F:cysteine-tRNA ligase activity	No
	CUFF.12513.1	Ubiquitin family protein	P:induction of apoptosis by extracellular signals; P:anti-apoptosis	No
Down-regulated	*Apoptotic properties*			
	CUFF.19150.1	Apoptosis-inducing factor, putative	–	Yes
	CUFF.22051.1	Liver stage antigen-	P:apoptosis; F:ATP binding; F:protein kinase activity	Yes
	CUFF.21693.1	Polyubiquitin-partial	P:induction of apoptosis by extracellular signals; P:anti-apoptosis	Yes
	CUFF.11240.1	Programmed cell death	F:RNA binding; P:mRNA processing; C:nucleus	No
	CUFF.3981.1	Programmed cell death	F:RNA binding; P:mRNA processing; C:nucleus	No
	CUFF.7920.1	Programmed cell death	F:RNA binding; P:mRNA processing; C:nucleus	No
	Anti-apoptotic properties			
	CUFF.21693.1	Polyubiquitin-partial	P:induction of apoptosis by extracellular signals; P:anti-apoptosis	Yes

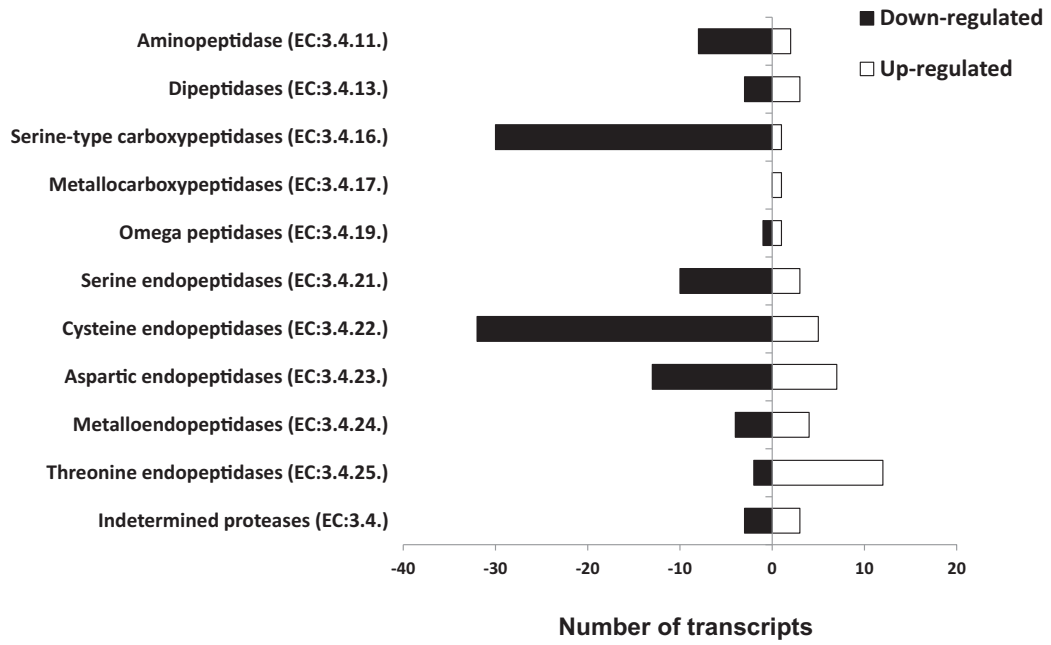

Fig. 3. Number of proteases, grouped by enzyme code (EC) regulated in *Perkinsus marinus* supplemented with oyster pallial mucus.

Table 4
Transcripts with Gene Ontology terms related to protease and protease inhibitor activities (other than proteasome subunits) which were up-regulated in *Perkinsus marinus* grown with oyster pallial mucus. Additional information on these transcripts is given in Supplementary Table S1.

Code	Protein identification	Gene Ontology terms	Signal peptide
Protease inhibitors			
CUFF.2659.1	Extracellular peptidase inhibitor-like	F:peptidase inhibitor activity; C:extracellular region	Yes
CUFF.5850.1	Extracellular peptidase inhibitor-like	F:peptidase inhibitor activity; C:extracellular region	Yes
CUFF.18281.1	Ribosomal l1 domain-containing	F:cysteine-type endopeptidase inhibitor activity	Yes
Proteases			
CUFF.15198.2	Aspartylaminopeptidase	F:cofactor binding; F:ligase activity	Yes
CUFF.22340.1	Methionineaminopeptidase	F:damaged DNA binding	Yes
CUFF.13862.1	Peptidase u34 dipeptidase	F:dipeptidase activity; P:proteolysis	Yes
CUFF.18419.1	Peptidase u34 dipeptidase	F:dipeptidase activity; P:proteolysis	No
CUFF.22288.1	Small conserved	F:dipeptidase activity; P:proteolysis	No
CUFF.810.1	Serinecarboxypeptidase iii precursor	F:serine-type carboxypeptidase activity; P:proteolysis	Yes
CUFF.5544.1	Hypothetical protein	F:metallocarboxypeptidase activity; F:zinc ion binding	Yes
CUFF.18713.1	Peptidasepyroglutamyl peptidase i-like	P:proteolysis	Yes
CUFF.1414.1	cna protein b-type domain	F:serine-type endopeptidase activity; P:proteolysis	Yes
CUFF.6674.1	Hypothetical protein	F:serine-type endopeptidase activity; P:proteolysis	Yes
CUFF.9702.1	Signal peptidase	F:serine-type endopeptidase activity	No
CUFF.2697.1	Alpha beta	C:mitochondrialintermembrane space; P:gluconeogenesis	No
CUFF.19998.1	Preprocathepsin c	P:nuclear mRNA splicing, via spliceosome	Yes
CUFF.8225.1	Preprocathepsin c	P:nuclear mRNA splicing, via spliceosome	Yes
CUFF.6355.1	Protein	F:nucleic acid binding; C:nucleus	No
CUFF.16942.1	Thiolproteinase	F:metalloendopeptidase activity; P:proteolysis	Yes
CUFF.16100.2	60s Ribosomal protein	F:aspartic-type endopeptidase activity; P:proteolysis	Yes
CUFF.10095.1	Aspartyl	F:ligase activity; P:metabolic process; F:catalytic activity	No
CUFF.13610.1	gag pol env	F:aspartic-typeendopeptidase activity; P:proteolysis	No
CUFF.6254.1	gag pol env	F:aspartic-typeendopeptidase activity; F:zinc ion binding	No
CUFF.16100.1	Ribosomal protein l30	F:aspartic-type endopeptidase activity; P:proteolysis	Yes
CUFF.20188.1	Ribosomal protein l30	F:aspartic-type endopeptidase activity; P:proteolysis	Yes
CUFF.8136.1	Gastricsin	F:aspartic-typeendopeptidase activity	Yes
CUFF.9375.1	Mitochondrial processing peptidase alpha	F:metalloendopeptidase activity; P:proteolysis	Yes
CUFF.9876.1	Mitochondrial processing peptidase alpha	F:metalloendopeptidase activity; P:proteolysis	Yes
CUFF.1997.1	Peptidase m23	C:intracellular; F:GTP binding	No
CUFF.9177.1	Thimetoligopeptidase	F:binding	No
CUFF.9425.1	Metallopeptidase m24	F:peptidase activity, acting on L-amino acid peptides	Yes
CUFF.21836.1	Microsomal signal peptidase 23 kd	F:peptidase activity; P:signal peptide processing	No
CUFF.12214.1	Carboxypeptidase y	F:serine-typecarboxypeptidase activity	No

4. Discussion

Mucus covering invertebrates has multiple roles including protection of epithelial cells from the environment and infectious agents (Reel and Fuhrman, 1981; Iguchi et al., 1982; Sze and Lee, 1995; Ritchie, 2006), locomotion (Denny, 1980; Davies and Beckwith, 1999), adhesion (Smith et al., 1999), cleansing of body surface (Bavington et al., 2004), and nutrition (Duerden, 1906; Ward and Shumway, 2004; Pales Espinosa et al., 2009). It is therefore not surprising that its composition is also diverse. For instance, invertebrates mucus contains large mucin-type glycoproteins (review by Davies and Hawkins, 1998), polysaccharides (Coddeville et al., 2011), water, electrolytes, epithelial and blood cells, and a wide range of bioactive molecules (Schachter and Williams, 1982; Rollins-Smith et al., 2005; Moraes et al., 2006; Pales Espinosa et al., 2009). For example, lysozymes (McDade and Tripp, 1967; Canicatti and Dancona, 1990), terpenoids (Miyamoto et al., 1994), antimicrobial peptides (Iguchi et al., 1982), antioxidants (Moraes et al., 2006), proteases (Canicatti and Dancona, 1990; Brun et al., 2000), agglutinins (Fisher, 1992) and lectins (Bulgheresi et al., 2006; Pales Espinosa et al., 2009, 2011; Itoh et al., 2011) have been identified or suspected to be present in invertebrate mucus. Mucus from marine organisms is also known to regulate the virulence of infectious microbes (Denkin and Nelson, 1999; Uttakleiv Ræder et al., 2007). In previous challenge experiments, we showed enhanced virulence in *P. marinus* exposed to oyster pallial mucus causing early and severe mortality of oysters whereas oysters injected with cultures supplemented with seawater, digestive extracts or plasma showed minimal mortalities (Winnicki, 2010. The influence of pallial mucus from the oyster, *C. virginica*, on the virulence of its pathogenic alveolate, *P. marinus*. MSc Thesis, Stony Brook University, Stony Brook, New York, USA; Pales Espinosa et al., 2013). Results from the challenge experiment in the present study corroborate our previous data and show significantly higher mortality and higher virulence indices in oysters injected with *P. marinus* grown in media supplemented with pallial mucus compared with parasite cells from cultures added with seawater or digestive extracts. Interestingly, high mortality was measured in oysters injected with *P. marinus* from cultures supplemented with pallial mucus even though parasite loads were similar across all treatments, suggesting that mucus plays a significant role in the virulence of *P. marinus*.

The main objective of the current study was to identify genes regulated following exposure of *P. marinus* to oyster pallial mucus and generate an inventory of potential virulence candidates. To our knowledge, this report is the first transcriptomic profiling study comparing *P. marinus* cells with varying levels of virulence. Results showed significant regulation of gene transcription in response to mucus exposure with 2054 up- and 1660 down-regulated genes. A small percentage of regulated transcripts (9% of the up- and 5.5% of the down-regulated) was not annotated and requires additional investigation for the probing of potential virulence factors. Remaining genes offer a unique snapshot of the transcriptomic profile of virulent *P. marinus*.

Two non-exclusive scenarios are proposed and discussed regarding the effect of mucus on *P. marinus*: (i) mucus may mimic the internal oyster environment, preparing the pathogen to cope with stressful conditions and making it more resistant to oyster defenses; and/or (ii) mucus exposure may trigger the production of virulence factors by *P. marinus*, leading to oyster mortality.

Several genes involved in the neutralisation of reactive oxygen species (ROS) were regulated in response to mucus exposure. Oxidation is a natural process within cells leading to the production of toxic ROS. ROS (including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HClO), and hydroxyl radical (OH)) play important roles in cell signaling and homeostasis but if produced in too high a quantity, they can become harmful to the cell. In order to prevent oxidative stress, antioxidant molecules inhibit the action of ROS, remove the excess of free radicals and maintain the oxidative system in balance. In molluscs, hemocytes are known to play a major role in defense against non-self. Among their various weapons, hemocytes produce ROS that play an important part in hemocyte-mediated cytotoxicity (Adema et al., 1991). As an intracellular parasite, *P. marinus*, however, has developed means to avoid this defense mechanism by producing and secreting molecules that restrain the deleterious effects of ROS (Volety and Chu, 1995; Anderson, 1999). Thus, superoxide radicals are dismutated by the superoxide dismutases (SOD) to hydrogen peroxide that could then be scavenged by catalases (which seem to be lacking in *P. marinus* Chu et al., 1998; Schott et al., 2003) or peroxidases. In addition, several proteins act as antioxidants by providing electrons to catalases or peroxidases. These different mechanisms provide *P. marimus* with a remarkable ability to resist high levels of superoxide and hydrogen peroxide (Schott et al., 2003). In our study, four genes coding for Fe/Mn SOD were up-regulated while three Cu/Zn SOD and one Fe/Mn SOD genes were simultaneously down-regulated in *P. marinus* exposed to mucus. These changes in SOD regulation in *P. marinus* support the hypothesis that oyster pallial mucus may be stressful for the parasite and suggest that Fe/Mn SOD and Cu/Zn SOD may have different and specialised roles in response to oxidative stress, as suggested for plants (Kliebenstein et al., 1998). Interestingly, only Fe-SOD type activity has been detected in cell extracts (Ahmed et al., 2003) and culture supernatant of *P. marinus* (Wright et al., 2002), suggesting that some Fe-SOD may be excreted and involved in the suppression of the oxidative burst associated with phagocytosis. Among the up-regulated SOD found in our study, one (CUFF.8411.1; *E*-value = $4E^{-165}$) presents strong similarities with the already described iron dependent *Pm*SOD1 suggested to enhance the survival of *P. marinus* in host cells and tissues (Wright et al., 2002; Joseph et al., 2010). Subsequent to the SOD dismutation, some of the 19 antioxidant proteins (i.e. peroxidases and electron donors: ascorbate peroxidase; four peroxiredoxins; nine thioredoxins; thioredoxin reductase; two glutharedoxins; two alkyl hydroperoxidereductases) up-regulated in *P. marinus* exposed to mucus may contribute to the elimination of the hydrogen peroxide (H_2O_2). Interestingly, only one peroxiredoxin was down-regulated in *P. marinus* exposed to mucus.

Another group of genes that can be involved in *P. marinus* resistance to host defenses is the threonine-type endopeptidases group (12 up-regulated). These endopeptidases are part of the proteasome and are involved in the degradation of unneeded or damaged proteins (Fornari et al., 2012). Their regulation may also reflect a response to the stress caused by the exposure of *P. marinus* to host defense molecules present in oyster mucus. This hypothesis is also supported by the fact that important regulation occurred for genes coding for HSP/chaperones (52 up- or down-regulated genes). Production of high levels of HSPs (or stress proteins) is known to be triggered by exposure to different environmental or biological stressors such as temperature changes, hypoxia, infection or inflammation (Borges and Ramos, 2005).

Interestingly, two extracellular peptidase inhibitors were up-regulated when *P. marinus* was grown with pallial mucus. Proteases inhibitors in human pathogens are known to regulate or inactivate proteolysis induced by host factors (Santos et al., 2005; Pandey et al., 2006) and act as virulence factors. Proteases are present in oyster hemolymph (e.g. aminopeptidases; Yoshino and Cheng, 1976) and mucus (McDade and Tripp, 1967; Brun et al., 2000; Pales Espinosa et al., unpublished data) and probably act as a first barrier of defense against pathogens. *Perkinsus marinus* peptidase inhibitors may therefore be used to inactivate proteolysis, cancelling this defense.

Taken together, the regulation of several SOD, antioxidant proteins, proteasome subunits, stress proteins and peptidase inhibitors support the idea that *P. marinus* is stressed by exposure to oyster pallial mucus which may mimic intra-tissular conditions. The fact that *P. marinus* responds to mucus stimuli suggests its excellent adaptation to overcome host defenses as mentioned previously by Schott et al. (2003). The incubation in pallial mucus may have prepared the parasite for host invasion. Before tissue infiltration, *P. marinus* would already be equipped to resist harsher intracellular conditions, facilitating its survival and spread in oyster tissues. The parasite's ability to counter host defenses, however, is unlikely to be responsible for the early death of oysters observed in the current study and in previous work (Ford et al., 2002; Pales Espinosa et al., 2013). For instance, these early mortalities are typically associated with relatively low parasite loads in oyster tissues (Ford et al., 2002; Pales Espinosa et al., 2013), suggesting the involvement of potent virulence factors other than those that simply facilitate the parasite's survival.

In addition, several genes potentially involved in the manipulation of host immunity were also regulated. Signal transduction plays a central role in host–pathogen interactions, allowing molecular crosstalk between signaling molecules and cell surface receptors, leading to specific physiological responses in both partners. The nature of extracellular signaling molecules (e.g. those produced by *P. marinus*) and receptors (e.g. oyster receptors) are various as well as the host cellular response. The initial stimulus can trigger the dysregulation of a large number of genes in host cells, eventually leading to alterations and apoptosis. In this study, multiple genes linked to cell signaling and signal transduction were modulated in *P. marinus* exposed to mucus (Fig. 2), some of which were identified as virulence factors in other pathogens.

One of the largest groups of genes modulated in *P. marinus* supplemented with mucus is the protein kinases/phosphotransferases. These enzymes transfer phosphate groups from donors to substrate receptors such as proteins, lipids, carbohydrates or other kinases. They are extensively used to transmit signals and control complex cellular processes. Some kinases (e.g. serine/threonine (S/T) kinases, or rhoptry kinases (ROPs)) are known to be secreted by pathogens (e.g. *Toxoplasma gondii*; Bradley and Sibley, 2007; El Hajj et al., 2007) and are used as virulence factors to proliferate in host tissues (ROP18, El Hajj et al., 2007; Taylor et al., 2006) or to manipulate host gene expression using STAT signaling pathways (ROP16, Saeij et al., 2007). In our study, 99 kinases/phosphotransferases (among them 23 S/T kinases) were up-regulated while 63 (among them 25 S/T kinases) were down-regulated in *P. marinus* supplemented with mucus. It should be noted that ROPs 16 and 18 or similar molecules have not yet been described in *P. marinus*. Nevertheless, the modulation of such an important number of kinases suggests a significant shift in signal transduction and consequently an activation of multiple metabolic pathways likely associated with the increased virulence of the parasite following exposure to mucus.

Severe infection with protozoan parasites (e.g. *Leishmania* spp., *T. gondii, Plasmodium* spp.) commonly results in the production of high levels of inflammatory cytokines such as interleukins (ILs) and tumor necrosis factor alpha (TNF-α) in vertebrates (Schofield and Hackett, 1993) and probably in invertebrates as well (Hughes et al., 1990). The TNF superfamily contributes to host protection because it regulates its immune system and acts against microbial infections (Debierre-Grockiego and Schwarz, 2010). These

cytokines, however, also have a "dark side" by contributing to cancer, apoptosis, autoimmune and others diseases (see the review by Aggarwal, 2003). If produced in *C. virginica*, they may cause damaging effects. In this study, several genes known to modulate the production of TNF-α were regulated. For example, this is the case for the lipopolysaccharide-induced tumor necrosis factor (i.e. LITAF; Jouault et al., 1994; Aggarwal, 2003), or the glycosylphosphatidylinositol anchors (GPIs; 13 genes regulated, see Fig. 2; Schofield and Hackett, 1993; Debierre-Grockiego et al., 2003). The GPIs, free or associated with surface antigens of several parasites, have been described to induce the production of cytokines and/or nitric oxide (NO, a powerful reactive nitrogen species) leading to inflammatory responses. This is the case for *Plasmodium*, in which GPIs were first named as "parasite-derived toxin" (Schofield and Hackett, 1993; Schofield et al., 1996) but also *Trypanosoma brucei* and *Trypanosoma cruzi*, (Tachado and Schofield, 1994; Tachado et al., 1997; Almeida et al., 2000), *Leishmania mexicana* (Tachado et al., 1997) and *T. gondii* (Debierre-Grockiego et al., 2003). Interestingly, the TNF-1 receptor has been found to be up-regulated in *C. virginica* infected with *P. marinus* (Tanguy et al., 2004), suggesting that if TNF-α is over-produced, it could easily bind to its receptors (also over-expressed) and induce harmful effects in oysters.

In addition to the TNF superfamily, other molecules are also able to induce apoptosis. For example, it has been demonstrated in vertebrates that arginine-glycine-aspartic acid-(RGD-) containing peptides are able to induce apoptosis in diverse types of cells (Buckley et al., 1999; Yamada, 1991). In *Crassostrea gigas*, RGD- or arginine-glycine-glutamicacid (RGE)-containing peptides (six amino-acid peptides simulating recognition sequences of fibronectin) were able to trigger apoptosis in hemocytes, probably via integrin-like receptors on the hemocyte cell surface (Terahara et al., 2003, 2005). Our data showed the up-regulation of one poly-ubiquitin (CUFF.12513.1, Supplementary Table S1) containing two retro RGD triplets. This sequence presents high similarity with the exogenous ubiquitin described by (Majetschak (2011). Interestingly, extracellular ubiquitins are known to induce apoptosis in human hematopoietic cells (Daino et al., 2000).

Several genes coding for proteins known to have immunosuppressive functions were also found to be up-regulated in *P. marinus* grown in media supplemented with pallial mucus (e.g. macrophage migration inhibitory; cyclophilin; cyclophilin ovcyp-2; peptidyl-prolylcis-trans isomerase cyclophilin type). Macrophage migration inhibitory factors (MIF) are often thought of as pro-inflammatory cytokines with the ability to induce various immunomodulatory and pro-inflammatory responses (Calandra et al., 1994; Kanai et al., 2003; Lue et al., 2002; Kamir et al., 2008; Miller et al., 2012). MIFs are relatively conserved throughout eukaryotes and have been found in vertebrates (Bozza et al., 1995), invertebrates (Cui et al., 2011) and in some pathogens that express MIF-like proteins (Augustijn et al., 2007; Silverman et al., 2008; Dobson et al., 2009; Thorat et al., 2010; Miller et al., 2012). Because the MIF-like proteins found in pathogens share similarities with MIFs from higher eukaryotes (Dobson et al., 2009), it is reasonable to think that they could also have similar roles. Previous studies have shown that MIFs from pathogens can modulate host cell apoptosis (Augustijn et al., 2007). Others studies are more cautious regarding the agonist function of parasite MIFs and suggest that these molecules may rather play a role in parasite survival (Thorat et al., 2010; Miller et al., 2012) by having, for example, an anti-inflammatory effect at higher MIF levels (Jang et al., 2011). In addition to MIF, three cyclophilin-type proteins were up-regulated in *P. marinus* exposed to mucus. These molecules are known to have an immunosuppressive action as well as a diverse array of additional cellular functions, including roles as chaperones and in cell signaling (Wang and Heitman, 2005). Nevertheless, most previous studies on these immunosuppressive proteins have been performed using vertebrate models and inferring a similar role in oyster-*Perkinsus* interactions remains speculative.

Additionally, our results displayed 11 and 13 up-regulated genes coding for antigen and merozoite surface proteins (MSPs), respectively, attached via a GPI anchor to the cell membrane (Harvey et al., 2012). In *Plasmodium falciparum*, some MSPs are thought to mediate initial host cell recognition, erythrocyte binding and cell invasion (Pachebat et al., 2001; Goel et al., 2003; Kadekoppala and Holder, 2010; Harvey et al., 2012) and the same function could be proposed for *P.* marinus MSPs. The up-regulation of several MSPs on the *P. marinus* cell surface might therefore facilitate the parasite binding to host cells and tissue invasion.

The public protein database of *P. marinus* includes 536 known proteases (Merops http://merops.sanger.ac.uk) involved in many physiological processes. Some of these are released in the extracellular products (ECPs), degrade host proteins or act as immunosuppressants reducing oyster hemocyte mobility (Garreis et al., 1996) and are therefore considered as virulence factors causing cellular and tissular damage (La Peyre and Faisal, 1995; La Peyre et al., 1995; Faisal et al., 1999). In particular, the low molecular weight serine proteases (chymotrypsin or chymotrypsin-like) are considered some of the major proteases produced and secreted by *P. marinus* cells in culture (La Peyre et al., 1996; Faisal et al., 1999; MacIntyre et al., 2003). In addition, aspartic proteases were suggested to be present in *P. marinus* ECP, but not cysteine proteases or metalloproteases (Faisal et al., 1999). Our results showed an up-regulation in the expression of 42 proteases (including 12 threonine-type endopeptidases that were discussed above) while 106 (e.g. cysteine-type endopeptidases (32), serine-type carboxypeptidases (30), aspartic endopeptidases (13), and serine endopeptidases (10)) were down-regulated in *P. marinus* grown in media supplemented with pallial mucus. This trend (a high number of down-regulated proteases) was unexpected because previous studies have reported an increase in the production of serine proteases in *P. marinus* ECP when cultures were supplemented with *C. virginica* tissue extracts (MacIntyre et al., 2003; Brown et al., 2005) and because proteases are widely considered the main virulence factors of *P. marinus* (LaPeyre, 1996; Tall et al., 1999). One would expect proteases to increase in virulent *P. marinus* cultures such as those exposed to pallial mucus. Our previous work showed different effects of various oyster supplements on protease activity in *P. marinus* ECP. For instance, protease activity in ECP increased when *P. marinus* cultures were supplemented with plasma or gill mucus but remained unchanged in cultures with added digestive extracts or mantle mucus, despite the fact that this latter treatment increased parasite virulence (Winnicki, 2010, MSc thesis, cited earlier; Pales Espinosa et al., 2013). Overall, the down-regulation of a large number of diverse types of proteases suggests a significant modification in the physiology of *P. marinus* when exposed to host mucus. The mechanisms leading to this down-regulation are unclear even though it could be hypothesised that the production of these enzymes is directly inhibited by specific molecules present in mucus or because such proteases are not necessary (mucus could be a good source of small peptides) or inadequate for the digestion of proteins present in mucus. More importantly, despite the fact that proteases probably play an important role in the pathogenesis of the disease, particularly in the later stages of the infection (La Peyre et al., 1995), most of these enzymes may not be involved in the early mortality seen in oysters exposed to wild-type *P. marinus* (Ford et al., 2002) or to parasite cultures supplemented with mucus. Several studies (Earnhart et al., 2004; Winnicki, 2010, MSc thesis, cited earlier; Pales Espinosa et al., 2013) have been unable to correlate *P. marinus* virulence with protease production. At the same time, we cannot rule out the possibility that some up-regulated proteases are involved in *P. marinus* virulence since similar enzymes in other parasites are known to significantly affect host health. For instance, the pyroglutamyl peptidase

(PGP) in *P. marinus* (Cuff.18713.1) is related to the PGP-I in *T. brucei* (*E*-value: 4*E*-08), known for degrading human neuropeptides (Morty et al., 2006). In addition, two of the seven aspartic endopeptidases up-regulated in *P. marinus* (Cuff.16100.2, *E*-value: 1.00*E*-09 and Cuff.10095.1, *E*-value: 3.00*E*-24) contain similarities with plasmepsins involved in the catabolic pathway of hemoglobin in several *Plasmodium* spp. (Goldberg et al., 1991; Sharma et al., 2005; Drew et al., 2008). Finally, three serine-type endopeptidases were found to be up-regulated and may be among those previously described by La Peyre et al. (1995). Overall, these enzymes are considered important potential virulence factors and warrant additional studies.

As stated above, several triggers can initiate molecular pathways leading to irreversible cellular alterations and apoptosis. The regulation of apoptosis is a prominent feature of host–pathogen interactions. Some intracellular parasites that depend on survival of the host cells for their persistence, proliferation and dissemination within host tissue are able to regulate and/or prevent host cell death (see the review by Sokolova, 2009). This characteristic was well described in human pathogens such as *Leishmania* spp. (Lüder et al., 2001; Aga et al., 2002; Ruhland et al., 2007) and it is also the case for *P. marinus* which is able to quickly modulate hemocyte apoptosis, allowing it to establish successful infection (Hughes et al., 2010). According to our results, several genes with anti-apoptotic properties (see Table 3), including the well-described 14-3-3 proteins (Zhang et al., 1999; Silverman et al., 2008; Clapp et al., 2012) or the Fas apoptotic inhibitory molecule (FAIM) (Huo et al., 2009), were up-regulated in *P. marinus*, providing targets for the investigations of molecular pathways of apoptosis regulation in oysters cells by *P. marinus*. The 14-3-3 proteins are of particular interest since they were previously detected in the secretomes of *Leishmania donovani* (intracellular pathogen) and are thought to prolong the lifespan of infected cells (Silverman et al., 2008). At the same time, several pro-apoptotic genes were also up-regulated in *P. marinus* exposed to mucus. The significance of these genes is not clear since mucus exposure does not alter parasite proliferation (Allam et al., 2013; Pales Espinosa et al., 2013), and their regulation may also be related to the control of host cell apoptosis rather than (or in addition to) parasite cells themselves. This is supported by the fact that several of these transcripts display sequences matching putative signal peptides (Table 3), suggesting that they may be secreted. A refined two-way control of host cell apoptosis by both pro- and anti-apoptotic factors has been observed in other intracellular parasites (Schaumburg et al., 2006) and may provide a mechanistic explanation of dynamic changes in the apoptosis of oyster hemocytes exposed to *P. marinus* for different time intervals (Hughes et al., 2010). Overall, this is an area of research that should be given high priority in the case of oyster–*Perkinsus* interactions due to both fundamental and applied implications.

Pallial mucus significantly modulates gene expression of multiple putative virulence factors of *P. marinus*. Some of the regulated genes are likely involved in the management of stress induced by mucus exposure itself and their regulation may prepare the pathogen for tissue invasion and contact with oyster defenses. While this preparation would increase the chances of *P. marinus* successfully colonising the host, it is unlikely sufficient to induce oyster death and other virulence factors have to be considered. Regulated genes involved in the attachment of the pathogen, in the modulation of host immunity, and cell signaling and survival represent good candidates for enhanced parasite virulence. Results also underline the importance of mucus covering pallial organs in disease pathogenesis. An in-depth biochemical characterisation of oyster pallial mucus (protein and carbohydrate composition) is underway to identify specific mucus factors that trigger observed physiological changes in *P. marinus*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2014.01.006.

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