Full length article

Regulation of oyster (*Crassostrea virginica*) hemocyte motility by the intracellular parasite *Perkinsus marinus*: A possible mechanism for host infection

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ABSTRACT

Hemocytes associated with the mucus lining of pallial (mantle, gill) surfaces of the oyster *Crassostrea virginica* have been recently suggested to facilitate infection by the Alveolate parasite *Perkinsus marinus* by mediating the uptake and dispersion of parasite cells. These "pallial hemocytes", which are directly exposed to microbes present in surrounding seawater, are able to migrate bi-directionally between mucosal surfaces and the circulatory system, potentially playing a sentinel role. Interestingly, *P. marinus* was shown to increase trans-epithelial migration of hemocytes suggesting it may regulate cell motility to favor infection establishment. The purpose of this study was to investigate the effect of *P. marinus* on hemocyte motility and identify specific molecular mechanisms potentially used by the parasite to regulate hemocyte migration. In a first series of experiments, various components of *P. marinus* (live *P. marinus* cells, extracellular products, fragments of *P. marinus* cell membrane, membrane-modified live *P. marinus* cells, heat-killed *P. marinus*) along with components of the opportunistic bacterial pathogen *Vibrio alginolyticus* (bacterial cells and extracellular products) were investigated for their effects on hemocyte motility. In a second series of experiments, inhibitors of specific molecular pathways involved in motility regulation (Y-27632: inhibitor of Rho-associated protein kinase, RGDS: integrin inhibitor, CK-666: Arp2/3 inhibitor) were used in conjunction with qPCR gene expression experiments to identify pathways regulated by *P. marinus* exposure. Results showed a specific increase in hemocyte motility following exposure to live *P. marinus* cells. The increase in motility induced by *P. marinus* was suppressed by RGDS and CK-666 implicating the involvement of integrins and Arp2/3 in cell activation. Gene expression data suggest that Arp2/3 is possibly regulated directly by an effector produced by *P. marinus*. The implications of increased hemocyte motility prompted by *P. marinus* during the early stage of the infection process are discussed.

1. Introduction

The eastern oyster (*Crassostrea virginica*) has an open circulatory system with hemocytes circulating throughout its blood vessels, sinuses and soft tissues. In addition to circulatory hemolymph, hemocytes have also been found associated with the mucus lining of pallial surfaces (epithelial tissues associated with mantle, gills, etc.) of *C. virginica* [24]. Given their physical location, these "pallial hemocytes" are exposed to a microbe-rich milieu that includes potential pathogens present in seawater. Furthermore, pallial hemocytes were shown to transit bi-directionally between the pallial surfaces and the circulatory system suggesting they may play a sentinel role by monitoring the microbial make-up of oyster surroundings and transmitting information to underlying tissues and hemolymph [23].

Perkinsiosis, commonly known as dermo disease, is caused by the protozoan parasite *Perkinsus marinus*. The primary portal of entry for the parasite into host tissues was presumed to be gut epithelium [29,30], however, more recent studies support that pallial organs such as labial palps, gills, and the mantle are likely to play important roles in the infection process [1,5]. Recent investigations further suggested that pallial hemocytes facilitate the establishment of *P. marinus* infection by mediating the uptake and dispersion of parasite cells [23,24]. Previous studies have shown that *P. marinus* is readily phagocytosed by *C. virginica* hemocytes; however, it is able to evade degradation [20,43]. Interestingly, the introduction of *P. marinus* into the pallial cavity was shown to increase trans-epithelial migration of hemocytes at the pallial interface suggesting it may regulate cell motility to favor infection establishment [23].

A number of pathogens are able to modify host cell cytoskeleton and blood cell migration to aid infection and disease development

[14,15,22,32,39]. For instance, neutrophil motility is inhibited by Gp63 protease produced by *Leishmania major* [39]. *Salmonella* induces membrane ruffling in non-phagocytic cells of the intestinal epithelium to facilitate entry into these cells [15,32]. *Toxoplasma gondii* elicits increased motility in dendritic cells to aid in transmigration across endothelial cells [22]. Similarly, *P. marinus* cells have been shown to enhance *C. virginica* hemocyte motility while parasite extracellular products reduce host hemocyte motility [11]. Although the establishment of some pathogens may depend on modification to host cell motility, studies on the molecular mechanisms of these interactions in marine invertebrates, such as oysters, are limited.

There is a wide range of receptors and molecules that regulate cell motility particularly at the steps of adhesion and actin polymerization. Previous investigations have shown changes in some of the factors regulating host cell motility in response to pathogen exposure. In *C. virginica*, *P. marinus* has been found to upregulate several motility related genes including tetraspanin, RhoGTP, RaIB binding protein, and calmodulin in hemocytes and/or gill tissues [41]. Analysis of oyster genomic resources also revealed a number of genes involved in cytoskeletal remodeling and motility including focal adhesion kinase (FAK), actin related protein 2 (Arp2), cofilin, cyclase associated protein 1 (CAP1) and profilin [33,33,44,48] some of which are known to be regulated during host-parasite interactions. For example, the bacterium *Salmonella enterica* was shown to activate cell division control protein 42 (Cdc42) homolog in mammalian cells to manipulate host cell cytoskeleton and mediate entry [32]. Similarly, *Shigella flexneri* (also a bacterium) can modify cell motility by producing effectors that interact with Neural Wiskott-Aldrich Syndrome Protein (N-WASP) which subsequently recruits the Arp2/3 complex [3]. In a recent study on *Crassostrea ariakensis*, Ca-TSP, a novel tetraspanin family member gene involved in motility regulation, was shown to be upregulated following exposure to lipopolysaccharide (LPS) [28].

The aim of this study was to investigate the effect of *P. marinus* on hemocyte motility in *C. virginica* and identify specific molecular mechanisms potentially used by the parasite to regulate hemocyte migration. In a first series of experiments, various components of *P. marinus* (live *P. marinus* cells, extracellular products, fragments of *P. marinus* cell membrane, membrane-modified live *P. marinus* cells, heat-killed *P. marinus*) along with components of the opportunistic bacterial pathogen *Vibrio alginolyticus* (bacterial cells and extracellular products) were investigated for their effects on hemocyte motility. *V. alginolyticus* was used to test the hypothesis that the obligate *P. marinus* may have different effect on hemocyte motility as compared to this opportunistic bacterium. In a second series of experiments, inhibitors of specific molecular pathways involved in motility regulation (Y-27632: inhibitor of Rho-associated protein kinase, RGDS: integrin inhibitor, CK-666: Arp2/3 inhibitor) were used in conjunction with qPCR gene expression experiments to identify pathways regulated by *P. marinus* exposure. Results are discussed with a focus on the effect of *P. marinus*-hemocyte interactions on the success or failure of infection establishment.

2. Methods

2.1. Oysters

Adult Eastern oysters, *C. virginica*, were obtained from Frank M. Flower and Sons (Oyster Bay, NY). Oysters were stripped of debris and fouling organisms and acclimated in aerated UV-filtered seawater (28–30 ppt, 23 °C) for 7–10 days prior to the experiments. They were fed daily with a commercial diet (DT's Live Marine Phytoplankton, Sycamore, Illinois, USA).

2.2. Pathogens

P. marinus (ATCC 50439) was grown in DME/F12-3 media at 23 °C in an Ambi-Hi-Lo chamber (Lab-Line Instruments Inc). For gene expression experiments, 325 ml cultures in 750 ml polystyrene flasks (Corning) were grown in duplicate. Log phase *P. marinus* (primarily trophozoites) cultures were mixed from both flasks and collected via centrifugation (1200 g, 10 min, 23 °C) washed and resuspended (final concentration of 1×10^6 in FSW) with 28 ppt 0.22 μm sterile filtered seawater (FSW) one day prior to the gene expression experiment. For the in-vitro hemocyte trafficking experiments, *P. marinus* cultures were grown in duplicate (16 ml in 25 ml flasks). Log phase cultures were incubated in FSW overnight and collected as described above with supernatant reserved for extracellular products (ECP) experimental treatment. Different *P. marinus* preparations were made from the collected cultures for the experimental treatments, including heat-killed *P. marinus* cells, membrane-modified live *P. marinus* cells, fragments of *P. marinus* cell membrane, *P. marinus* extracellular products (ECP), and washed live *P. marinus* cells. Heat-killed *P. marinus* cells were prepared by incubating 2 ml of *P. marinus* culture at 100 °C for 15 min. *P. marinus* cell membranes were modified by incubating 2 ml of *P. marinus* with the lectin Concavalin A (Con A; used to saturate mannosyl and glucosyl residues associated with cell surface and potentially alter *P. marinus* and *C. virginica* cell-cell interactions) for 1 h at room temperature, centrifuged (400 g, 4 min), washed with FSW, and resuspended. To isolate *P. marinus* membrane, 5 ml of *P. marinus* culture was frozen and thawed twice (−20 °C, 10 min), sonicated (Virsonic 50 at 100%) in short bursts (on ice, 10 min), washed in FSW and centrifuged (12000g, 5 min), and stored at −20 °C. The remaining collected *P. marinus* culture was used for the live *P. marinus* cell treatment. Live *Vibrio alginolyticus* cells, and *V. alginolyticus* ECP were also used for hemocyte trafficking experiments. *V. alginolyticus* was grown on shaker at room temperature in marine broth. Three ml of the culture (0.2 OD at 600 nm wavelength, approximately 5×10^7 cells) was centrifuged (10000 g, 2 min), washed and resuspended with FSW (14 ml), incubated overnight (room temperature), collected via centrifugation (10000 g, 5 min) and resuspended in FSW (OD 0.2) with supernatant retained for *V. alginolyticus* ECP treatment.

2.3. Effects of pathogen exposure on hemocyte trafficking

To mimic hemocyte trans-epithelial trafficking, an in-vitro model was developed using 24-well cell culture inserts (8 μm pores) (Millipore) as a proxy for epithelia. Circulatory hemocytes were collected as a proxy for pallial hemocytes due the relatively limited quantity of the latter for extensive functional testing. Ten pools (2 oysters/pool) of hemolymph were collected from the adductor muscle via a 16-gauge syringe and diluted 1:1 (final concentration approximately 1×10^6) with ice-cold FSW. To evaluate the effect of direct pathogen contact on hemocyte motility, hemocytes were co-incubated with FSW (negative control) or one of the following: live *P. marinus*, heat-killed *P. marinus*, membrane modified *P. marinus*, *P. marinus* membrane only, *P. marinus* extracellular products (ECP), live *V. alginolyticus*, *V. alginolyticus* ECP, or 6 μm fluorescent beads (to mimic *P. marinus* phagocytosis). Cell culture inserts were immersed in 24-well culture plates (BD Falcon) with the chemoattractant LPS (1300 μl, 40 μg/ml resuspended in FSW, Sigma) [40] for 5 min. Pathogen preparations were then added (100 μl containing 10^5 beads or *P. marinus* cell equivalent, or 5×10^6 bacteria) into the cell culture insert and allowed to settle for 1 min before 100 μl diluted hemolymph from each pool (yielding 1:1 hemocyte:*P. marinus* and 1:50 hemocyte:*V. alginolyticus* ratios) were added. Following incubation (1 h at room temperature in the dark), the top membrane of the inserts was swabbed twice and the inserts were incubated with the viability stain calcein AM (4 μM final concentration in 500 μl FSW, 40 min, on ice). Membranes were then fixed (500 μl 1% formalin in FSW, 10 min, on ice) and stored (900 μl of FSW, in the dark, at 4 °C) until microscopic observations (typically within the next 8 h). Each cell culture insert was photographed in 5 different microscopic fields (10× objective) viewed on an inverted microscope (Nikon Eclipse TE-2000S). Fluorescent cells on each image were counted

Table 1
Primers designed for assessing transcript levels of motility-related genes in *C. virginica* hemocytes.

Gene	Forward (5′-3′)	Reverse (3′-5′)
Tetraspanin	GAAATTCGGCTTGTGCCGTT	GCTTGGTGCTGGTTTTGAGG
Rho GTP	GAGGTCGATGGAAAGCAGGT	GTGGGCGGAGTCTGTCATAG
Profilin	CACGCCCAGTCTGTTCATCT	CCTCCCACCCAGTGTGAATC
RalB	CTGGCAATGATGTTGGGCTC	TGCTGAGGGACTGCTACAAC
Arp2	GTAGCGGTCCTATGCGGAAG	ATGTATCTTTCGGGGCAGGG
FAK	GTATCGTCTCACGACTGCGT	TCGACTCAGGAGCCATCCAT
CAP	CAACCTGGGATGGCTCCAAA	TGTGACGGAATGGTGCAAGA
CDC42	ACCCACTTTTCTCGGACGTTT	GATGATTGGTGGAGAGCCGT
Calmodulin	AGTCGGTCAAAACAACCCGT	TCCCAGAACCTGTTGCACTG
β-actin	TTGGACTTCGAGCAGGAGATGGC	ACATGGCCTCTGGGCACCTGA

manually and/or automatically using ImageJ and the average number of hemocytes in the 5 different fields was used to calculate the number of migratory hemocytes using the equation: % Migratory hemocytes = ((Area of the insert membrane/Area of the microscopic field) x average number of fluorescent hemocytes)/(total number of hemocytes added to the cell insert) $\times 100$.

2.4. Pathogens as chemoattractants

To evaluate whether oyster hemocytes are chemoattracted to *P. marinus*, hemocytes were placed in the cell insert and exposed to different pathogen components delivered to the culture well below the cell insert. The experiment was run concurrently with the direct pathogen exposure experiment using some of the same pathogen treatments as well as the same oyster hemolymph pools. A total of 1300 μl of FSW (negative control), LPS (positive control), live *P. marinus*, live *V. alginolyticus*, *P. marinus* ECP, and *V. alginolyticus* ECP were added into the wells below the cell insert. Cell inserts were immersed in the 6 different treatments for 5 min before diluted hemolymph (100 μl, containing 10^5 hemocytes) was added into each cell insert (100 μl FSW in control preparation). Following incubation (1 h, room temperature in the dark), cell inserts were collected, stained and processed as described above.

2.5. Migration inhibition assays

Chemical inhibitors were used in conjunction with cell inserts to further explore the mechanisms used by *P. marinus* to regulate hemocyte motility. Arg-Gly-Asp-Ser tetrapeptide (RGDS) (Tocris), Y-27632 (Tocris) and CK-666 (R&D Systems Incorporated) were selected based on pathways of interest and commercial availability. Each inhibitor was evaluated via calcein AM viability staining (4 μM final concentration) and flow cytometry to assess the maximum tolerable dose on *C. virginica* hemocytes (data not shown). Ten pools (3 oysters/pool) of hemolymph were used in these experiments. BD FluoroBlok cell culture inserts (8 μm pores) were placed in 24-well plates (BD Falcon) and equilibrated with 700 μl LPS (40 μg/ml resuspended in FSW, Sigma) [40]. Hemocytes (100 μl, 1×10^6 cells/ml diluted in FSW) were aliquoted into cell culture inserts and 30 μl of each of the following treatments was added: RGDS (10 μM), Y-27632 (100 μM), CK-666 (10 μM) and 2 inserts received only FSW. Inserts were incubated for 5 min before 100 μl of *P. marinus* (experimental treatments and positive control; 4 inserts/hemocyte pools) or beads (1 control insert for phagocytosis) were added into the cell culture inserts. Samples were incubated (1 h in the dark at 23 °C), before inserts were collected and processed as described above.

2.6. Hemocyte gene expression

Five pools (20–25 oysters/pool yielding 60 ml/pool at approximately 2.5×10^6 hemocytes/ml) of hemolymph were centrifuged (800 g, 10 min, 4 °C) and resuspended in 30 ml ice-cold FSW (28 ppt, final concentration approximately 5×10^6 hemocytes/ml). Each hemocyte pool was aliquoted into one of the four treatments (3 ml/treatment), which included 3 ml of: FSW (negative control), *P. marinus*, heat killed *P. marinus*, or 8 μm beads (positive control) to yield approximately 1:1 hemocyte:test particle ratio. Each treatment was duplicated to allow for collection at two separate time points except for FSW, which was in triplicate to account for a time 0 collection. All treatments were collected at 6 h and 24 h post-exposure, while hemocytes with FSW alone was also collected at time 0. Samples were centrifuged (1200 g, 10 min at 4 °C) resuspended and homogenized in Trizol (Molecular Research Center, Inc. Cincinnati, OH) and stored at −80 °C for RNA extraction no greater than 36 h post-collection. The whole experiment was repeated again on the next day to generate a total of 10 discrete pools. Total RNA was extracted following the manufacturer's protocol with the addition of Proteinase K and additional ethanol wash with molecular grade glycogen (Thermo Scientific, Wilmington, Delaware, USA) to enhance yield of high quality RNA. Samples were analyzed with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) before cDNA was reverse-transcribed from RNA samples (2.5 μg) utilizing Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV; Promega, Madison, Wisconsin, USA) following the manufacturer's protocol.

Quantitative real-time (qPCR) primers were designed to amplify 8 motility-related genes in *C. virginica* hemocytes (Table 1) based on the NCBI database and previous studies [16,31,33,41,44]. These included tetraspanin, calmodulin, Rho GTP, profilin, RalB, Arp2, CDC42, FAK, and CAP. Each primer pair was tested for the optimal efficiency and amplification products were confirmed on gel electrophoresis. Preliminary assays were preformed to ensure amplification was specific to the organism of interest since treatment samples contained both *P. marinus* and *C. virginica* cDNA. β-actin [31] was confirmed as a reliable housekeeping gene during preliminary assays. qPCR reactions containing 5 ng cDNA template, 100 nM of forward and reverse primer and 5 μl of 2Brilliant SYBR Green qPCR master mix (Agilent, Santa Clara, California, USA) in 10 μl volumes were performed on a Eppendorf Mastercycler Realplex (Eppendorf, Hauppauge, New York, USA). The qPCR thermal profile was as follows: 95 °C for 10 min, 40 cycles of amplification with denaturation at 95 °C for 15 s, annealing and extension for 1 min (at 56 °C), and melting curve analysis. The relative expression of genes of interest was calculated based on the comparative CT method ($2^{-\Delta\Delta Ct}$) utilizing β-actin as a reference gene [26].

2.7. Data analysis

All statistical analyses were made in SPSS and SigmaStat. Repeated measures ANOVA and Student-Newman-Keuls post-hoc tests were performed on arcsine transformed percent migratory hemocyte data. ΔCt data derived from the gene expression experiment were also submitted to repeated measures analyses with Student-Newman-Keuls post-hoc test. All differences were considered significant at p < 0.05. Discriminant analysis (DA) and principal components analysis (PCA) were used to evaluate the overall effect of each treatment on the
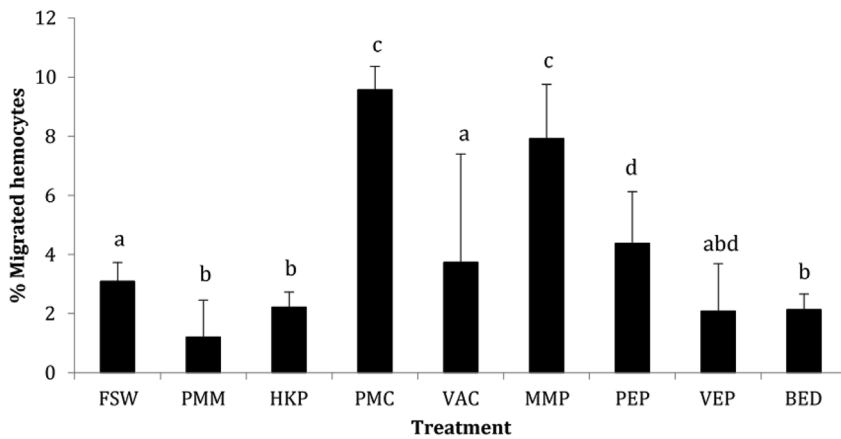
Fig. 1. Effect of different treatments on hemocyte motility (mean ± SE). FSW: seawater, PMM: *P. marinus* cell membranes, HKP: heat-killed *P. marinus* cells, PMC: *P. marinus* cells, VAC: *V. alginolyticus* cells, MMP: membrane modified *P. marinus*, PEP: *P. marinus* extracellular products, VEP: *V. alginolyticus* extracellular products, BED: 6 μm beads. Different letters (a, b, c and d) denote significant difference between treatments (SNK post-hoc test, p < 0.05, n = 10).

expression of motility-related genes at both 6 h and 24 h post-exposure.

3. Results

3.1. Effects of pathogen exposure on hemocyte trafficking in vitro

The effect of various pathogen components on hemocyte migration was evaluated (Fig. 1). *P. marinus* co-incubation with hemocytes resulted in significantly greater migrated hemocytes (9.5%) compared to the FSW control (3.1%) and all other treatments (heat-killed *P. marinus* 2.2%, *P. marinus* membrane 1.2%, *V. alginolyticus* cells 3.7%, *V. alginolyticus* ECP 2.1%, *P. marinus* ECP 4.4%, beads 0.98%) except Con A membrane-modified *P. marinus* (7.9%). Exposure of hemocytes to *P. marinus* extracellular products (4.4%) resulted in significantly greater migration compared to heat-killed *P. marinus*, *P. marinus* membrane only, *V. alginolyticus* cells, beads, and FSW. Conversely, heat-killed *P. marinus* and *P. marinus* membrane resulted in significantly less hemocyte migration compared to the FSW control and other *P. marinus* treatments. No significant difference was observed between control and *V. alginolyticus* cells or *V. alginolyticus* ECP.

3.2. Pathogens as chemoattractants

Hemocytes were exposed to *P. marinus* and *V. alginolyticus* cells or their extracellular products to assess the chemoattractant effects of these pathogens. *P. marinus* cells elicited a significant increase in the proportion of hemocytes (33%) that migrated across the cell insert filter as compared to all other treatments, which ranged from 2.1 (*V. alginolyticus* cells) to 5.5% (*V. alginolyticus* ECP). The remaining treatments (*V. alginolyticus*, *V. alginolyticus* ECP and *P. marinus* ECP) did not elicit a significant change in hemocyte migration as compared to the LPS positive control (Fig. 2).

### 3.3. Migration inhibition assays

Similar to the results of the pathogen exposure experiment described above, hemocytes exposed to *P. marinus* cells displayed greater transmembrane motility than hemocytes exposed to beads (Fig. 3). Samples treated with motility inhibitors indicated that RGD and CK666 significantly reduced hemocyte motility compared to hemocytes added with *P. marinus* alone (control). The percent of hemocytes migrated after exposure to RGDS and CK666 was comparable to hemocytes exposed to beads alone. Conversely, Y-27632 did not significantly decrease motility of hemocytes exposed to *P. marinus*.

3.4. Regulation of hemocyte gene expression by pathogens

Six hours post-treatment, transcription levels of Rho GTP in
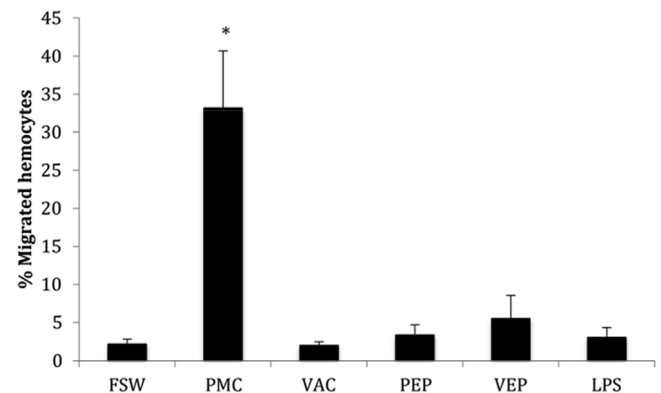
Fig. 2. Effect of different treatments as hemocyte chemoattractants (mean ± SE) FSW: seawater, LPS: lipopolysaccharide, PMC: *P. marinus* cells, VAC: *V. alginolyticus* cells, PEP: *P. marinus* extracellular products, or VEP: *V. alginolyticus*. * indicates significant difference in percent of migrated hemocytes compared to the LPS control (SNK post-hoc test, p < 0.05, n = 10).
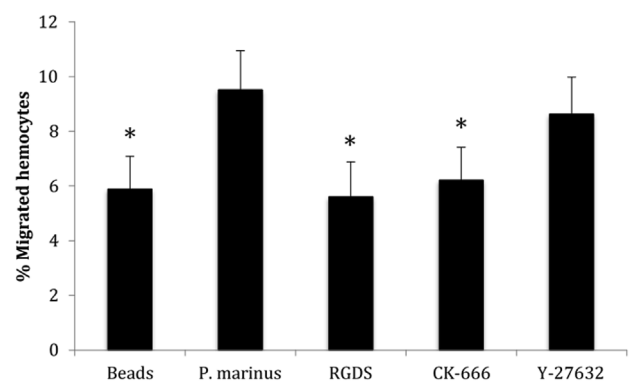
Fig. 3. Effect of various inhibitors on hemocytes migration (mean ± SE). Cell culture inserts with beads (negative control), *P. marinus* (positive control), and *P. marinus* and inhibitors (RGDS, CK666, Y-276320). * indicates significant difference from *P. marinus* (SNK post-hoc test, p < 0.05, n = 7).

hemocytes exposed to *P. marinus* were significantly upregulated compared to all other treatments (Fig. 4). Profilin expression was significantly upregulated in hemocytes exposed to beads compared to hemocytes exposed to live or heat-killed *P. marinus*. CDC42 expression was also significantly upregulated in hemocytes exposed to beads versus all other treatments. RalB expression was significantly upregulated in hemocytes exposed to all treatments compared to the control. Changes for FAK, calmodulin Arp2, CAP, and tetraspanin were not statistically significant.
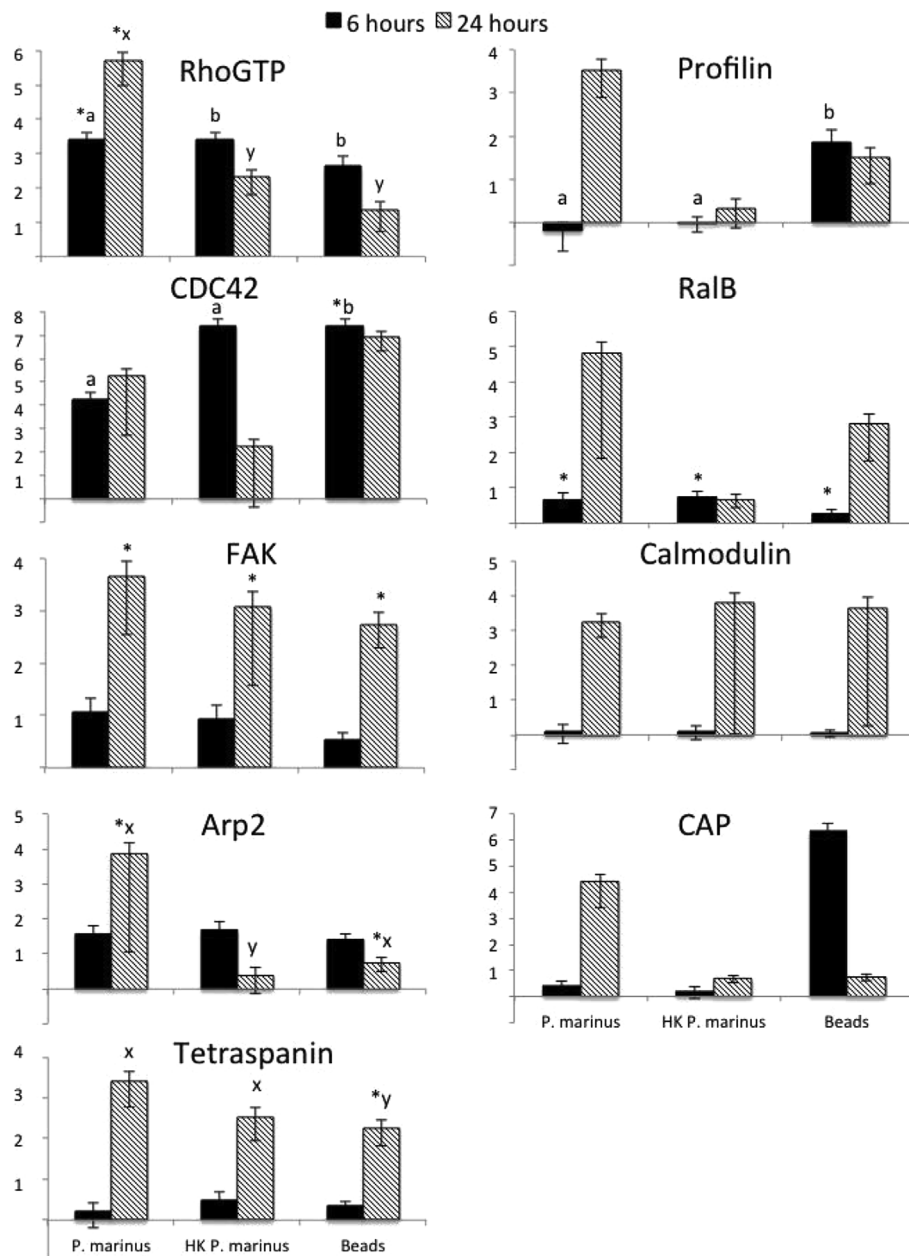
Fig. 4. Fold change (Log10, mean ± SE) of motility-related genes in *C. virginica* hemocytes at 6 and 24 h after exposure to *P. marinus*, heat-killed *P. marinus*, or beads. * Denotes significant difference from untreated control hemocytes (represented by the x-axis). Different letters (6 h: a and b; 24 h: x and y) indicate significant difference between treatments within each sampling time (SNK post-hoc test, p < 0.05, n = 10).

At 24 h post-treatment, hemocytes exposed to live *P. marinus* continued to exhibit significant upregulation of Rho GTP compared to all other treatments (Fig. 4). Arp2 was significantly upregulated in hemocytes exposed to *P. marinus* or beads compared to the control and hemocytes exposed to heat-killed *P. marinus*. Tetraspanin was significantly downregulated in hemocytes exposed to beads compared to the *P. marinus* treatments. FAK expression was significantly upregulated in all treatments compared to the control.

Discriminant analysis (DA) of motility-related gene expression levels at 6 h post-exposure showed significant separation of hemocytes submitted to the different treatments along function 1 with most contrast noted between hemocytes exposed to live *P. marinus* and those exposed to beads (88.1% variance explained, Eigenvalue = 19.206, Wilks Lambda = 0.13, P < 0.0001) (Fig. 5A). At 24 h post-exposure, the separation was less obvious even though hemocytes exposed to *P. marinus* remained well separated along function 1 from those exposed

to beads (97% variance explained, Eigenvalue = 69.35, Wilks Lambda = 0.004, P < 0.23) (Fig. 5B).

Principal Component Analysis (PCA) analysis of gene expression levels indicate that 6 h post-exposure, Rho GTP clustered with RalB, while calmodulin clustered with Arp2 and tetraspanin (Fig. 6A). Similarly, CDC42 clustered with CAP and profilin, and FAK was separated from all other genes. Clustered genes were separated along component 1 and component 2 (63.2% total variance explained). At 24 h post-exposure, clustering of genes was much less delineated, with 82.8% total variance explained by components 1 and 2 (Fig. 6B). These results corresponded well to correlation analysis of gene transcript levels (Supplementary Table 1).

4. Discussion

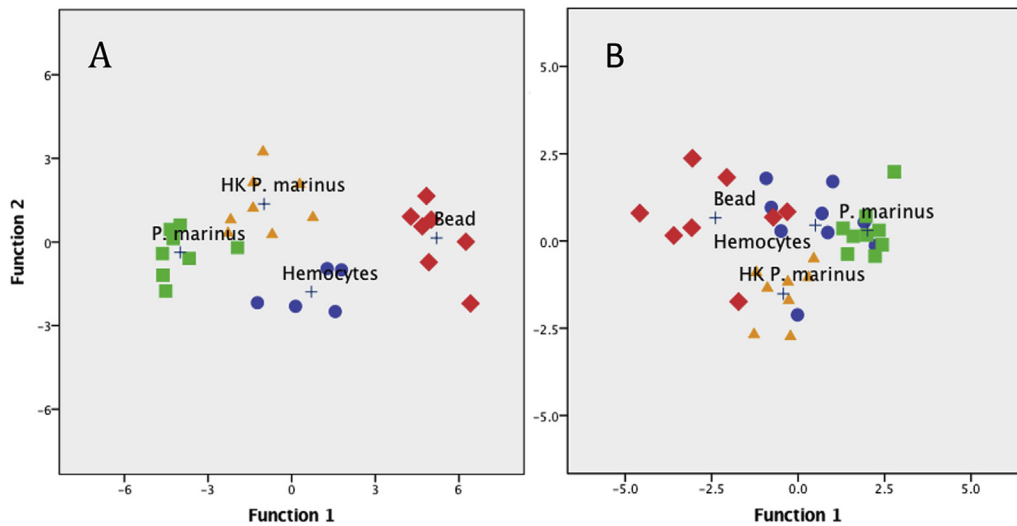Bi-directional movement of hemocytes across the pallial epithelium

Fig. 5. Discriminant analysis (DA) of motility-related gene expression (Rho GTP, Tetraspanin, FAK, Arp2, CDC42, Profilin, RaIB, Calmodulin, and CAP) at (A) 6 h, and (B) 24 h post-exposure. Different treatment groups are indicated by different symbols and the positions of the group centroids are indicated by the cross symbols.
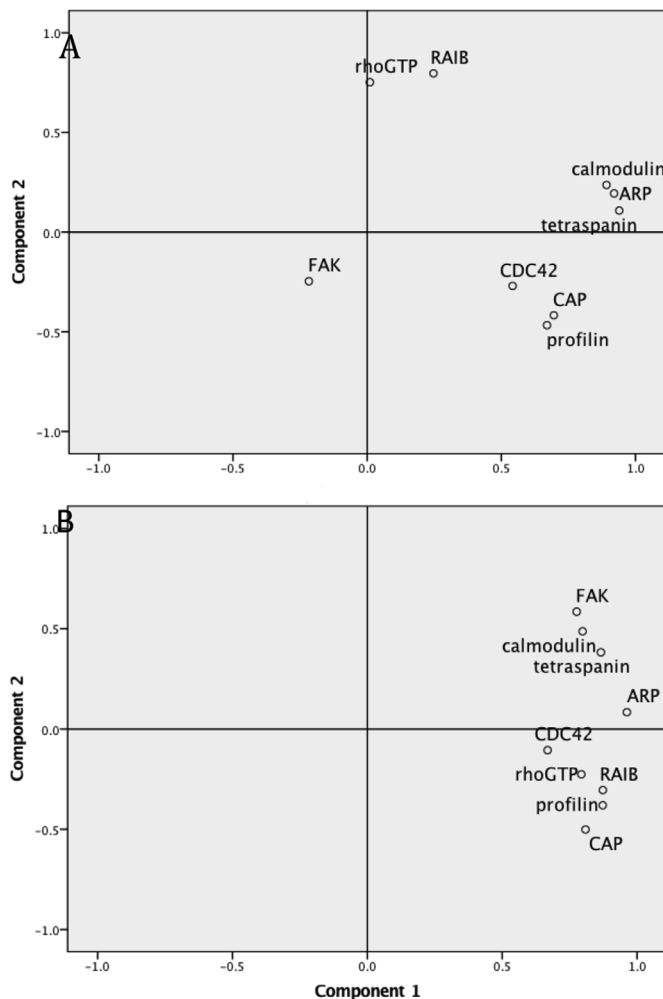

Fig. 6. Principal component analysis of motility-related gene expression at A) 6 h and B) 24 h post-exposure. All treatments were combined to generate the analysis.

and increased migration to the pallial epithelial surfaces following *P. marinus* exposure has been recently reported [23]. This study indicates that while *P. marinus* ECP increases hemocyte motility, *P. marinus* cells induce the greatest response. Consistent with the results of this study, Garreis *et al.* [11] have shown that low concentrations of *P. marinus* extracellular proteins increase hemocyte motility even though the trend was inverted when high concentrations of parasite ECP were used. Interestingly, *P. marinus* cell membrane alone elicited a decrease in hemocyte motility and similar results were observed with heat-killed *P. marinus*. These results suggest that membrane components are not sufficient to induce motility. Live *P. marinus* cells may induce additional biological processes post-phagocytosis that can enhance hemocyte motility. The fact that *V. alginolyticus* did not cause significant changes in hemocyte motility indicates that motility regulation is likely pathogen-specific. Given the ability for *P. marinus* to evade normal degradation processes within hemocytes [19,43] and the ability of hemocytes to migrate across pallial epithelia [23], enhancing hemocyte motility by *P. marinus* could prove to be advantageous to the infection process.

Results also indicate that *P. marinus* cells are significantly stronger chemoattractants than the control (LPS) and other treatments (*P. marinus* ECP, *V. alginolyticus*, *V. alginolyticus* ECP). Since *P. marinus* cells settle to the bottom of the well below the cell insert, they are not in direct contact with the hemocytes. Therefore, increased motility across the membrane is likely due to a secreted compound produced by *P. marinus* cells. Pathogens and their secreted products have been shown to affect hemocyte motility in various host organisms [4,8,11,38]. Compounds secreted by pathogens such as bacteria have been shown to increase chemotaxis in *C. virginica* hemocytes [4]. Consistent with the data from Garreis et al. [11]; greater hemocyte motility induced by *P. marinus* cells compared to *P. marinus* ECP was also observed in this study. ECP has been demonstrated to play an important role in mediating oyster-*P. marinus* interactions [11,40]. Previous studies have reported that hemocytes exposed to *P. marinus* ECP may result in a decreased ability to clear *Vibrio vulnificus* [40]. ECP have also been shown to attenuate *C. virginica* immunity via decreased lysozyme activity, hemagglutinin titres and, at high ECP doses, hemocyte motility [11], although ECP concentrations used in our study are likely lower than those used by Garreis et al. [11] making comparisons between both studies difficult. The chemoattractant experiment demonstrated that *P. marinus* ECP produced prior to exposure to hemocytes did not significantly induce greater hemocyte motility; indicating feedback from live *P. marinus* cells may be required to induce increased hemocyte motility even prior to physical contact. It should be noted that *P. marinus* ECP, *V. alginolyticus* and *V. alginolyticus* ECP are able to freely diffuse through the membrane possibly leading to relatively

homogenous concentrations in the well. In contrast, *P. marinus* cells settle to the bottom resulting in a gradient of any secreted products, which could induce directional migration.

Physiological and gene expression changes in hemocytes were investigated to gain a better understanding of the mechanisms in which *P. marinus* induces hemocyte motility. Samples treated with Rho-associated protein kinase (ROCK) inhibitor Y-27632 did not induce significant changes to the motility of hemocytes exposed to *P. marinus*. However, qPCR data indicates a significant upregulation in Rho GTP expression both 6 and 24 h following hemocyte exposure to live *P. marinus*. These results suggest that while *P. marinus* may regulate Rho GTP expression, ROCK, one of the downstream effectors, is not involved in the increase of hemocyte motility induced by *P. marinus*. In addition, PCA analysis indicates that Rho GTP along with RalB clustered separately from most of the other motility-related genes. Rho GTP is a major player in multiple pathways and therefore, may also be regulated for other processes. For example, in *C. gigas*, Rho has been suggested to function as an anti-apoptotic factor [21].

Conversely, the integrin inhibitor RGDS and Arp2/3 inhibitor CK666 significantly suppressed motility in hemocytes exposed to *P. marinus* compared to non-treated samples (*P. marinus* alone). RGDS is an integrin-specific adhesion inhibitor. Integrins are cell membrane-associated adhesion molecules that facilitate cell motility by binding with low affinity to grip the extracellular matrix. Cells can modify cell adhesion through changes in ligand binding affinity of integrins via intracellular signals, or by extracellular ligand-integrin binding to induce cytoskeletal changes [12]. Since RGDS negated motility increase caused by *P. marinus*, modification of integrins may be a possible mechanism in which *P. marinus* regulates *C. virginica* hemocyte motility. RGDS has been demonstrated to inhibit spreading of hemocytes in *Biomphalaria glabrata* and *C. gigas* [7,42]. Furthermore, the putative integrin binding site, Cg βGBP-1 in *C. gigas*, has been suggested to play a role in the interactions between hemocytes and pathogens [17] such as *V. splendidus* [9]. Similarly, *P. marinus* may be utilizing integrin to regulate *C. virginica* hemocyte motility.

Transcription levels of tetraspanin increased significantly in hemocytes exposed to *P. marinus* as compared to hemocytes exposed to beads. Tetraspanins have been suggested to affect cell motility via regulation of vesicular trafficking of associated integrins [25] and are involved in intercellular adhesion in the mammalian system [2]. In *Crassostrea ariakensis*, the tetraspanin Ca-TSP has been shown to be present in granules and affect hemocyte aggregation [28]. In parallel, FAK expression was significantly upregulated in all three treatments compared to the control with the greatest increase noted in hemocytes exposed to *P. marinus*. FAK can be activated by integrin and is suggested to be involved in focal adhesion contacts turnover in the mammalian system and loss of FAK expression is associated with a decrease in motility [10,37]. Altogether, these findings suggest that the integrin pathway may be modified by *P. marinus* during the infection process. PCA analysis further indicates FAK shifts to cluster (and is strongly correlated) with tetraspanin 24 h post exposure supporting the involvement of both of these genes in the same integrin pathway. RGDS inhibition of *P. marinus*-induced motility one hour post exposure and enhanced FAK and tetraspanin expression at 24 h post exposure but not at 6 h suggest that integrin in hemocytes is modulated by *P. marinus* during different stages of the infection process.

CK-666 is an inhibitor for the Arp2/3 complex [36]. Arp2 in conjunction with Arp3 forms a complex that nucleates actin polymerization [45] and has been suggested to play a role in directional migration of cells along gradients of fibronectin, laminin, or vitronectin [36]. Increased hemocyte motility caused by *P. marinus* was inhibited by CK-666 suggesting that the Arp2/3 complex and the upstream pathway may be modulated by *P. marinus*. Significant upregulation of Arp2 in hemocytes exposed to *P. marinus* or beads compared to hemocytes exposed to heat-killed *P. marinus* and the control was observed. Hemocytes exposed to *P. marinus* resulted in greater upregulation of Arp2

compared to hemocytes exposed to beads. Taken together, *P. marinus* regulates Arp2, but perhaps through a different mechanism compared to beads. The manipulation of host Arp2/3 by pathogens to assist in spread of infection has been previously reported [6]. For example, the bacteria *Shigella flexneri*, can modify host Arp2/3 to gain motility within the host cell by producing effectors that interact with Neural Wiskott-Aldrich Syndrome Protein (N-WASP) which subsequently recruits the Arp2/3 complex, while the bacteria *Listeria monocytogenes* produces a protein that mimics N-WASP to directly activate Arp2/3 [3,45].

CDC42, a GTPase, functions upstream of Arp2/3 and activates it via N-WASP [28,34]. Profilin, a protein that accelerates the conversion of ATP to ADP and enhances elongation of actin filaments has also been suggested to interact with CDC42 to activate WASP and the Arp2/3 complex [13,46,47]. RalB, a GTPase protein, can activate CDC42 and has been shown to affect the velocity of migrating cells [18,35]. CDC42 expression was significantly upregulated in hemocytes exposed to beads compared to the control. Both CDC42 and profilin expression was significantly greater in hemocytes exposed to beads compared to all other treatments. Interestingly, both CDC42 and profilin were significantly upregulated at 6 h post exposure but not at 24 h post exposure indicating increased expression of these genes were triggered relatively early after exposure to beads. RalB expression was significantly greater in all treatments compared to the control. Correlation analysis indicated Arp2 is significantly correlated with CDC42 ($R = 0.484$, $P = 0.007$) and profilin ($R = 0.540$, $P = 0.002$) and RalB ($R = 0.378$, $P = 0.036$) supporting the co-regulation of these genes in the Arp2/3 pathway. CDC42 and profilin in hemocytes exposed to beads was upregulated while hemocytes exposed to *P. marinus* lacked significant upregulation of these genes further suggesting a different mechanism of Arp2/3 activation may be employed by *P. marinus*. Interestingly, the bacteria *L. monocytogenes* has been shown to utilize Arp2/3 in its host for intracellular motility without profilin [27]. In mammalian cells, the bacteria *Salmonella enterica* was shown to produce effector molecules that induced actin-rich membrane ruffle formation via CDC42 activation. Similar to *L. monocytogenes* or *S. flexneri*, *P. marinus* may produce its own effector for Arp2/3. Lack of Arp2/3 upregulation by hemocytes exposed to heat-killed *P. marinus* further supports the idea that feedback from oyster hemocytes may lead to the production of an Arp2/3 effector molecule in live *P. marinus* cells although additional experiments are needed to validate this scenario.

Since CK-666 and RGDS suppressed motility to levels comparable to exposure to beads alone, other motility pathways induced by physical contact may still be present. Discriminant analysis showed that hemocytes exposed to the different treatments 6 h post-exposure were clustered separately from hemocytes alone suggesting all the treatments elicited a response in the expression of motility-related genes. It is striking to see, however, that hemocytes exposed to live *P. marinus* were diametrically opposed to those incubated with beads with hemocytes exposed to heat-killed *P. marinus* occupying middle grounds. These findings suggest that the change in gene expression is not a mere result of phagocytosis of particles, but rather a specific biological response to exposure to live (and to a much lesser extent dead) parasite cells. Interestingly, 24 h post exposure, hemocytes exposed to *P. marinus* clustered closer to control hemocytes indicating that they may have been returning to basal levels of motility-related gene expression. Taken together, these results indicate temporal regulation of multiple motility pathways by *P. marinus* and specifically, the likely involvement of integrin and Arp2/3 and the corresponding upstream pathways.

Cell motility is affected by *P. marinus* in a pathogen-specific manner. While *P. marinus* components such as ECP induced some increase in cell motility, *P. marinus* cells clearly induced the greatest increase in hemocyte motility both through direct contact and via secreted substances. Although *P. marinus* is readily phagocytosed by *C. virginica* hemocytes, intracellular degradation is evaded [20,43]. Our recent studies have described hemocytes associated with the mucus lining of pallial surfaces (epithelial tissues associated with gills, mantle, and

palps) of *C. virginica* and the migration of these hemocytes across the pallial epithelia towards the circulatory system [23,24]. Therefore, enhanced hemocyte motility elicited by *P. marinus* may increase the opportunities for parasite phagocytosis as well as transepithelial movement of hemocytes loaded with *P. marinus*, thereby assisting *P. marinus* in the initial infection process.

Suppression of *P. marinus*-induced motility by CK-666 and RGDS indicates the possible manipulation of integrins and Arp2/3 and their upstream effectors by *P. marinus*. Although many of the Arp2/3 upstream effectors were not upregulated in hemocytes exposed to *P. marinus* during our gene expression experiment, Arp2/3 expression was significantly upregulated in hemocytes exposed to *P. marinus* indicating *P. marinus* may act directly on Arp2/3, perhaps with the production of its own effectors as previously described in *L. monocytogenes* or *S. flexneri* [3,45]. Additional studies into the possible effector molecules responsible for the change in motility and upregulation of Arp2/3 may help elucidate how *P. marinus* manipulates host motility to gain entry and spread within *C. virginica*. Furthermore, studies with additional time points pairing physiological changes with the underlying molecular modifications may provide better insight into the kinetics of molecular changes that occur between the early interactions and later development of the disease.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2018.04.019.

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