2005

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GD Brown

SL Kaattari

Kimberly S. Reece
Virginia Institute of Marine Science

Journal of Shellfish Research, Vol. 24, No. 4, 1027–1033, 2005.

EFFECT OF HOMOGENATE FROM DIFFERENT OYSTER SPECIES ON *PERKINSUS MARINUS* PROLIFERATION AND SUBTILISIN GENE TRANSCRIPTION

GWYNNE D. BROWN, STEPHEN L. KAATTARI AND KIMBERLY S. REECE*
Virginia Institute of Marine Science, P. O. Box 1346, Gloucester Point, VA 23062

ABSTRACT The modulation of *Perkinsus marinus* proliferation and subtilisin gene transcription by host (oyster) tissue was examined. *Perkinsus marinus* cells were cultured for 4 weeks in media supplemented with extract from either one of four different *Crassostrea virginica* stocks or with extract from one of two other *Crassostrea* species, *C. ariakensis* and *C. gigas*. After 4 weeks in culture, we determined cell counts and relative subtilisin gene transcription levels using quantitative real-time polymerase chain reaction (qRTPCR). Cell proliferation and subtilisin gene transcription were significantly lower when *P. marinus* cells were grown in the presence of homogenate from any of the three oyster species than in unsupplemented media. *Perkinsus marinus* subtilisin gene transcription was also significantly lower in cells cultured in media supplemented with homogenate from either *C. ariakensis* or *C. gigas*, than in media containing extract from the native oyster host, *C. virginica.* Gene transcription levels among cells grown in media supplemented with homogenate from the different stocks of *C. virginica* were not significantly different from one another.

KEY WORDS: *Perkinsus marinus*, subtilisin, serine protease, real-time PCR, *Crassostrea virginica*, *Crassostrea gigas*, *Crassostrea ariakensis*

INTRODUCTION

Dermo disease in oysters is caused by the protozoan parasite *Perkinsus marinus.* In its native host, *Crassostrea virginica*, heavy infections of *P. marinus* result in significant tissue destruction throughout the animal (Mackin 1962) with subsequent mortalities occurring primarily during the second year of infection (Burreson & Ragone-Calvo 1996). *Perkinsus marinus* has also been detected in other molluscs, including *Boonea impressa* (White et al. 1987), *Mya arenaria* (Kotob et al. 1999), *Mercenaria mercenaria, Macoma mitchelli* and *Macoma balthica* (Coss et al. 2001). Any estuarine mollusc could likely serve as a reservoir for *P. marinus* (Perkins 1996), even though in the field, little to no pathogenesis has been observed in animals other than *C. virginica.*

The apparent resistance, or tolerance, of the closely related oyster species *Crassostrea ariakensis* and *Crassostrea gigas* to *Perkinsus marinus* infection has created interest in the possible introduction of diploid Asian oysters into the United States coastal waters of Virginia and Maryland. In *P. marinus* challenge studies comparing *C. ariakensis* or *C. gigas* to *C. virginica*, infections remained sublethal without significant physiologic effects in the Asian species, whereas weighted prevalence and mortality in *C. virginica* were high (Meyers et al. 1991, Barber & Mann 1994, Calvo et al. 2000, Calvo et al. 2001). Why differences in *P. marinus* infection levels and in pathogenicity occur among the different oyster species is not known, although researchers have discovered evidence suggesting a basis for differences in host specificity. Gauthier and Vasta (2002) found a higher rate of uptake of live *P. marinus* trophozoites by hemocytes from *C. virginica* in comparison with *C. gigas* hemocytes, suggesting that there is a difference in *P. marinus*-host recognition or a difference in antimicrobial activity among hemocytes of the different oyster species. Differences have also been observed in the protease profiles of extracellular products (ECP) produced from *P. marinus* cells grown in chemically defined media supplemented with homogenate from the different oyster species. Induction of lower molecular weight proteases was seen only from ECP of cells cultured in the presence of homogenate from the native oyster, *C. virginica* (MacIntyre et al. 2003).

Many pathogens, including *P. marinus*, experience rapid attenuation when cultured *in vitro* (Ford et al. 2002). Thus, it becomes difficult, if not impossible with axenic cultures to affect host dependent parasite differentiation, proliferation, or virulence factor transcription. Transcription of such virulence factors can be stage specific or present in cell types not commonly seen *in vitro* or may only be expressed in the presence of the host organism. For example, Bruchhaus et al. (2003) determined that only 8 of 20 genomic cysteine protease sequences found in *Entamoeba histolytica* were expressed in a cultured strain of cells. To overcome these difficulties and to produce conditions more comparable to those encountered *in vivo*, researchers have supplemented *P. marinus* growth media with oyster plasma or with extracts prepared from whole oysters (Gauthier & Vasta 2002, MacIntyre et al. 2003, Earnhart et al. 2004). *Perkinsus marinus* cells grown in oyster extract-supplemented media have a similar morphology to *P. marinus* cells observed in the host, with a greater number of tomont stages present than seen in cultures grown in defined media alone (MacIntyre et al. 2003, Earnhart et al. 2004). The rapid attenuation observed after isolation and subsequent culture of *P. marinus* cells was also reversed by passaging cultured cells through media supplemented with oyster homogenate prior to infection trials (Earnhart et al. 2004).

In this study, we investigated the effects that tissue homogenate from different oyster species had on *P. marinus* cell proliferation and the gene transcription of a recently identified *P. marinus* subtilisin serine protease gene (Brown & Reece 2003). Serine proteases present in the ECP of *P. marinus* cells grown *in vitro* have been implicated as virulence factors, possibly involved with suppression of host immune functions (Garreis et al. 1996, Tall et al. 1999) and with tissue degradation (La Peyre et al. 1996). Phylogenetic analysis of the two *P. marinus* subtilisin gene sequences characterized by Brown and Reece (2003) grouped both sequence types with subtilisin-like serine proteases from two important disease-causing protozoan parasites: *Toxoplasma gondii* and *Plasmodium falciparum.* Although the exact functions of the serine proteases from these parasites are not known, they have been identified as candidates for involvement with host cell invasion (Blackman et al. 1998, Barale et al. 1999, Hackett et al. 1999, Sajid et al. 2000, Miller et al. 2001). To determine whether extracts from

*Corresponding author. E-mail: kreece@vims.edu

different oyster species affected transcription of the *P. marinus* subtilisin gene and cell proliferation, we supplemented defined media (JL-ODRP-3) (La Peyre & Faisal 1997) with filtered homogenate from various *C. virginica* stocks, homogenate from *C. ariakensis* or homogenate from *C. gigas.* After cell counts, RNAs from the cells grown in the different media supplements were then used in quantitative real-time polymerase chain reactions (qRTPCR) with *P. marinus* subtilisin gene primers and hybridization probes.

MATERIALS AND METHODS

Oysters

Perkinsus-free *C. virginica* (CvWA) and *C. gigas* (Cg) oysters were obtained from Taylor Shellfish Farms (Shelton, Washington, USA). A stock of *C. virginica* originating from Tangier Sound (CvTg) Virginia and a *C. virginica* strain selectively bred for *Haplosporidium nelsoni* and *P. marinus* disease resistance (CROSBreed, designated CvXB) were collected from a deployment site on the Yeocomico River (Virginia). *Crassostrea ariakensis* oysters (Ca) were provided by Dr. Stan Allen of the Aquaculture Genetics and Breeding Technology Center at the Virginia Institute of Marine Science. Before homogenizing, oysters were maintained in 20-L tanks in 1.0-μm filtered York River water. Water was changed twice weekly and oysters were fed commercial algae (Reed Mariculture, San Jose, CA). All effluent was chlorinated before release. Native oysters were also collected from the Rappahannock River Virginia (CvR) the day before homogenization and maintained overnight at 4°C.

Supplementation of JL-ODRP-3 Media With Oyster Homogenate

Whole oysters were minced and homogenized individually using a glass Tenbrock homogenizer. Homogenate from two to three oysters from the same stock were pooled for each culture flask, to minimize oyster-to-oyster variation. Homogenate was spun at ×16,000*g* for 30 min at 14°C to 19°C in an ultracentrifuge (Sorvall, Kendro Laboratory Products, Newton, CT). The supernatant was filtered through 0.22-μm syringe filters (uStar LB; Costar, Corning Inc., Acton, Massachusetts) and the protein concentration was determined by a bicinchronic acid assay (Pierce, Rockford, Illinois). A pilot experiment was performed using 1.0 mg of filtered homogenate from either *C. gigas, C. ariakensis* or *C. virginica* supernatant, per mL of JL-ODRP-3 media. The pilot study indicated that further experiments should be performed using lower protein supplement concentrations. Previous research indicated an increase in survival rates for *P. marinus* cells grown in *C. ariakensis* extract supplemented media with protein concentrations of 0.11 mg mL^{-1} and 0.33 mg mL^{-1} (Earnhart et al. 2004). All subsequent experiments, therefore, used a final oyster homogenate concentration of 0.25 mg mL^{-1} in defined medium.

Perkinsus marinus Culture

The *P. marinus* isolate VA-2 (P-1) was used in all experiments. Initial seed density was 1×10^6 *P. marinus* cells mL^{-1} in 50 mL of media in 75 cm^2 flasks (Corning, Inc.), containing either unsupplemented media (JL-ODRP-3) or media supplemented with fresh filtered homogenate supernatant from the different oysters. A minimum of three flasks was used for each group. Cultures were incubated at 27°C for 4 weeks under humidified 95% air/5% CO_2. Cell counts were performed using a Neubauer haemacytometer and cell viability determined by neutral red uptake. For those cultures inoculated with 0.25 mg mL^{-1} of oyster homogenate, cell diameters were determined for a minimum of 100 cells from each culture. A "pellet volume" was also determined from the average cell volume $[(4/3)\pi r^3]$ and cell count for that culture to determine whether change in morphology, i.e., increase in cell size, created a similar or different total culture biomass among different treatment groups. Cells were pelleted by centrifugation, ×800*g* for 10 min at room temperature and either stored in RNALater buffer (Ambion Inc., Austin, TX) or immediately resuspended in TRIzol (Invitrogen, Carlsbad, CA). Two tailed *t*-tests for unequal sample sizes were run to determine whether the different treatment groups possessed significantly different cell counts.

Real-time PCR

Total RNA was isolated from each isolate using the TRIzol reagant system (Invitrogen Corporation) (Simms et al. 1993, Simms 1995). RNA was quantified spectrophotometrically. Initial and subsequent experiments (1.0 or 0.25 mg mL^{-1}, respectively protein supplement concentrations) comparing the effect of media supplementation with homogenate from different oyster species on subtilisin gene transcription used the subtilisin primer pair (forward primer "Sub for" - 5′ CTG CTA ACG CTG GCC AT 3′ and reverse primer "Sub back" - 5′ CAA TAT TAA CCA CAG AAC CGA TGT 3′) and subtilisin hybridization probes ("Sub2 Fl+" - 5′ TTC CTT CTA TGC TCT GCG TTG GC X 3′ and "Sub Anchor LC" - Red640 5′ CGA GTT CTT CGA CAC CGA CCT CGC C p 3′). Subtilisin primer/probe design was performed by TIB Molecular (Adelphia, NJ) using the *Perkinsus marinus* subtilisin gene sequences in GenBank (AY340222 – AY340234). Five hundred nanograms of total RNA were used in a 20 μL LightCycler RNA Master Hybridization Probe reaction with a final concentration of 4.25 μM MgO_2, 0.5 μM of each primer, 0.2 μM of the anchor probe and the Fl probe, and 7.5 μL of enzyme. The reaction was run on a LightCycler Instrument (Roche, Indianapolis, IN) using the conditions outlined in Table 1. Control reactions using the DNA Master Hybridization Probe reaction were also run to demonstrate that the product was not of DNA origin.

All experiments, except the pilot study, also used an actin primer pair (forward primer "Actin F" - 5′ TCG TTA TGG ATT CCG GTG AT 3′ and reverse primer "Actin R" - 5′ TCA AGT GCG ACG TAG GAC A 3′) and an actin probe set ("Actin FL" - 5′ TTG ACC GAA CGT GGT TAC ACA TTC X 3′ and "Actin Anchor LC" - Red640 5′ CTA CTA CGG CTG AGA GAG AGA TCG TCC p 3′) (TIB Molecular) (U84288). To minimize concentration calculation error caused by differences in subtilisin and actin PCR efficiency, standard curves were developed separately for subtilisin and actin PCR reactions. In each run, subtilisin gene transcription levels were normalized to the level of actin transcription for each particular sample to account for sample-to-sample variability, differences in spectrophotometry readings and pipetting error. Quantification analysis on the LightCycler used the second derivative maximum and arithmetic baseline adjustment. Two tailed *t*-tests for unequal sample sizes were run to determine whether differences observed in relative transcription levels among groups were significant. Pearson's correlation coefficient was determined to measure the strength of the relationship between cell count and the relative subtilisin gene transcription level and the strength of the relationship between cell count and the actin gene transcription level.

TABLE 1.

LightCycler reaction conditions for determination of relative subtilisin and actin gene transcription.

Program	Cycles	Temp °C	Time (s)	Temp Transition Rate	Acquisition Mode	Analysis Mode
RT	1	61	1200	20	None	
Denaturation	1	95	30	20	None	
Amplification		95	1	20	None	
	45	52	15	20	Single	Quantitative
		72	12	2	None	
Cooling	1	40	30	20	None	
Melting curve	1	95	5	20	None	Melting curve
		59	30	20	None	
		95	0	0.1	Continuous	

Cloning and Sequencing

LightCycler products were analyzed on 1.5% agarose gels to confirm that the expected product sizes were obtained. Actin and subtilisin LightCycler products were also reamplified with *Taq* polymerase (Invitrogen Corporation) to ligate products into the TA cloning vector pCR 2.1 (Invitrogen Corporation). Cloning and transformation into *Escherichia coli* INVαF′ followed the manufacturer's protocol. Clones containing inserts were cultured overnight in ×2 Yeast Tryptone (YT) media with 50 mg L^{-1} ampicillin. Plasmid DNA was extracted using Plasmid miniPrep Kit (Qiagen, Valencia, California) and sequenced using the Thermo Sequenase IR labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Corporation, Arlington Heights, Illinois) in conjunction with M13 forward and reverse labeled primers (LI-COR, Inc., Lincoln, Nebraska). Sequencing reactions were run on a LI-COR 4200 automated sequencer (LI-COR, Inc.).

RESULTS

Effect of Homogenate Supernatant on Cell Proliferation

In all studies, *P. marinus* cell counts were significantly higher in treatment groups lacking oyster homogenate than in cultures supplemented with extract from either *C. virginica, C. ariakensis* or *C. gigas.* At a supplementation dose of 1.0 mg mL^{-1}, cell counts where treatment groups contained extract from *C. virginica* (1.08×10^6 cells mL^{-1}) or *C. ariakensis* (0.5×10^6 cells mL^{-1}) were 8- to16-fold less, respectively, than cell counts of *P. marinus* grown in media alone, indicating little to no cell proliferation or in some cases even cell death. *Perkinsus marinus* cells were not viable in those cultures with *C. gigas* supplemented media.

Cell counts for *P. marinus* grown in media supplemented with only 0.25 mg mL^{-1} of *C. ariakensis* or *C. gigas* extract were 0.27×10^6 cells mL^{-1} and 0.36×10^6 cells mL^{-1}, respectively (Fig. 1a), significantly lower than those cultured in defined media alone (9.3×10^6 cells mL^{-1}; $P < 0.0001$) or those grown in media supplemented with homogenate from *C. virginica* (1.1×10^6 cells mL^{-1}; $P < 0.0001$). Viability, as assessed by neutral red uptake, was never greater than 67% for cultures supplemented with nonnative oyster homogenates. Cell pellet size was also significantly smaller ($P < 0.05$) for those cultures containing oyster supplement from either Asian oyster species compared with cells grown in defined media alone (Fig. 1b). Although average cell size was larger (6.0-μm diameter) in flasks supplemented with *C. gigas* homogenate than in flasks supplemented with *C. ariakensis* homogenate (5.0 μm), the difference in cell pellet size was not significant.

Counts for *P. marinus* cells grown in defined media (10.1×10^6 cells mL^{-1}) were also significantly higher ($P < 0.01$) than cell counts for *P. marinus* cultured in media supplemented with oyster extract from the different *C. virginica* stocks at a concentration of 0.25 mg mL^{-1} (Fig. 1a). There was some variability among *C.*

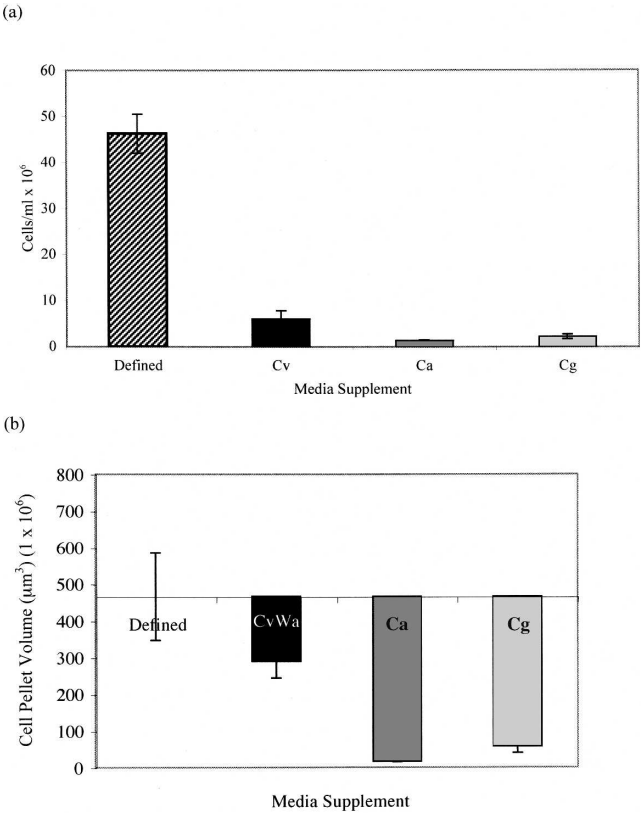

Figure 1. ***Perkinsus marinus.*** **(a) Cell counts among *P. marinus* cells grown in media supplemented with 0.25 mg mL^{-1} of either: *C. virginica* homogenate, *C. ariakensis* homogenate, or *C. gigas* homogenate, as compared with that of cells cultured in defined media alone. (b) Deviation of calculated cell pellet volume (average cell volume [(4/3)πr^3] × cell count) of *P. marinus* cells grown in media supplemented with 0.25mg mL^{-1} of either: *C. virginica* stock CvWa homogenate, *C. ariakensis* homogenate, or *C. gigas* homogenate, as compared with that of cells cultured in defined media alone. Standard error bars are shown for each treatment group.**

virginica cultures in cell count and viability at the end of the 4-week culture period with an insignificant increase in average cell numbers from initial inoculation density in some treatment groups (CvR – 1.1×10^6 cells mL^{-1} with 97% viability; CvWa – 2.1×10^6 cells mL^{-1} with 98% viability) and a decrease in cell numbers in other cultures from the initial inoculation density similar to that observed in *C. ariakensis* or *C. gigas* treatments (CvTg – 0.27×10^6 viable cells mL^{-1} with 60% viability). Cells from two of the three flasks supplemented with homogenate from CvXB oysters were not viable at the end of 4 weeks; therefore cell counts and subtilisin transcription could not be analyzed for this treatment group. Differences in cell size were not significant among cultures supplemented with homogenate from the three viable *C. virginica* stocks, although the average *P. marinus* cell size was slightly larger in the CvWa treatment group (6.4 μm) and the cell pellet size for that group was significantly greater ($P < 0.01$) than that observed for the CvR and CvTg supplemented flasks. CvWa was the only group that had a significantly ($P < 0.05$) larger cell size than those cells grown in defined media (4.5 μm). Cell pellet sizes, however, for cultures grown in defined media alone were still significantly greater than pellet sizes for cultures containing supplement from any *C. virginica* stock ($P < 0.05$), including CvWa (Fig. 1b). Because of variations observed in cell number and cell pellet size among cultures supplemented with different stocks of *C. virginica*, there were no significant differences overall in cell pellet size among native and nonnative treatment groups, however, there was a significant difference ($P < 0.01$) between CvWa supplemented cultures and Cg or Ca supplemented cultures.

Effect of Homogenate Supernatant on Subtilisin Transcription

Similar to cell counts, *P. marinus* subtilisin gene transcription levels in cells grown in media supplemented with supernatant from oyster homogenates were significantly lower than in the unsupplemented control media. In the pilot study, the detected number of transcripts was 2.6 times greater in cells grown in media alone compared with those cultured in the presence of *C. virginica* extract and 7 times greater than that detected in cells grown in the presence of *C. ariakensis* extract (Fig. 2). Subtilisin gene transcript levels in *P. marinus* cells cultured in the *C. ariakensis* supplemented media was significantly lower than in the cells cultured in *C. virginica* supplemented media ($P < 0.01$).

Transcription of the subtilisin gene was also depressed at the lower homogenate dosage, 0.25 mg mL^{-1}, in *P. marinus* cells grown in oyster-supplemented media as compared with that of cells grown in defined media alone. Transcription of the subtilisin gene was almost twice or one-third again as high in *P. marinus* cells grown in *C. virginica* supplemented media, regardless of stock, as that in cells grown in either *C. gigas* or *C. ariakensis* homogenate-supplemented media (Fig. 3). T-tests comparing normalized *P. marinus* subtilisin gene transcription levels in media supplemented with tissue from the Asian oysters to transcription levels in media supplemented with tissue from the native oyster *C. virginica* showed that the differences observed were significant ($P < 0.001$). No significant differences were found in normalized subtilisin gene transcription among *P. marinus* cultures grown in media supplemented with homogenate from different *C. virginica* oyster stocks (Fig. 3).

The Pearson correlation coefficient value between cell count and actin gene transcription was $r = 0.61$ (Fig. 4a). Coefficient values, however, between cell counts and actin gene transcription for only those treatment groups containing oyster supplement was $r = -0.55$. The Pearson correlation coefficient value between cell count and relative subtilisin gene transcription was $r = 0.82$ (Fig. 4b). This positive correlation was maintained, $r = 0.62$, with removal of treatment groups lacking oyster supplement.

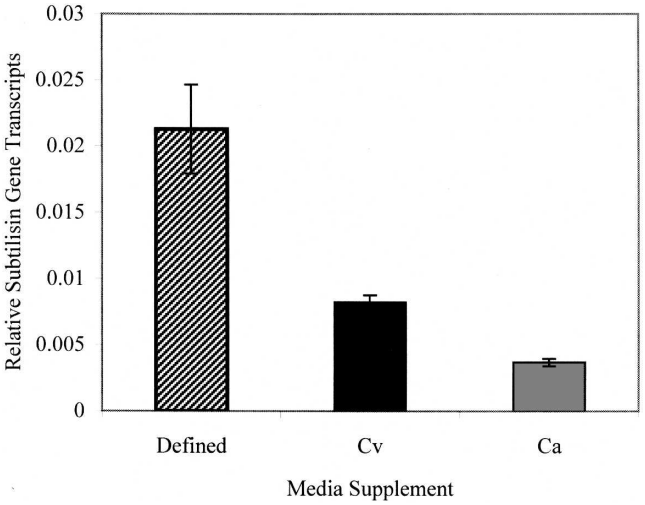

Figure 2. ***Perkinsus marinus.*** **Subtilisin gene transcription of *P. marinus* cells grown in defined media only, defined media supplemented with 1.0 mg mL^{-1} of *C. virginica* homogenate (Cv), and media supplemented with 1.0 mg mL^{-1} of *C. ariakensis* homogenate (Ca). Standard error bars are shown for each treatment group.**

Confirmation of Amplification Product Identities

LightCycler amplification products were all of the expected size and sequences of reamplified, cloned products were appropriately identified as *P. marinus* actin or serine protease gene sequences. In addition, no amplification products were detected in control amplification reactions lacking reverse transcriptase, demonstrating quantification of RNA, not DNA, in the LightCycler reactions.

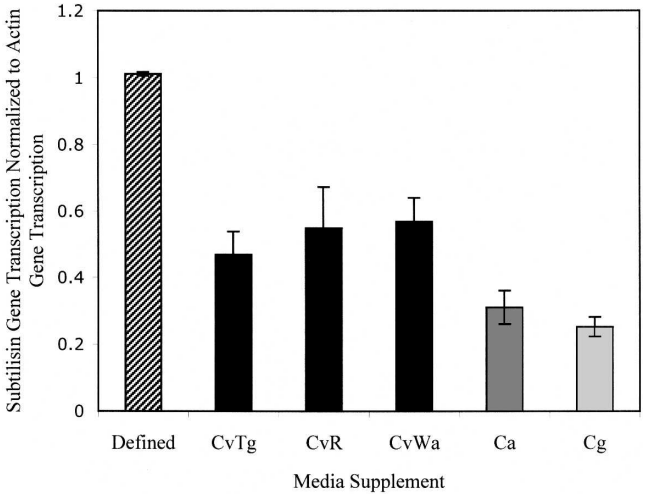

Figure 3. ***Perkinsus marinus.*** **Relative concentrations of subtilisin/actin gene transcription in *P. marinus* cells cultured in defined media supplemented with 0.25 mg mL^{-1} of oyster homogenate from *Crassostrea virginica* stocks CvTg, CvR, and CvWa and the non-native oysters *C. ariakensis* (Ca) and *C. gigas* (Cg). Standard error bars are shown for each treatment group.**

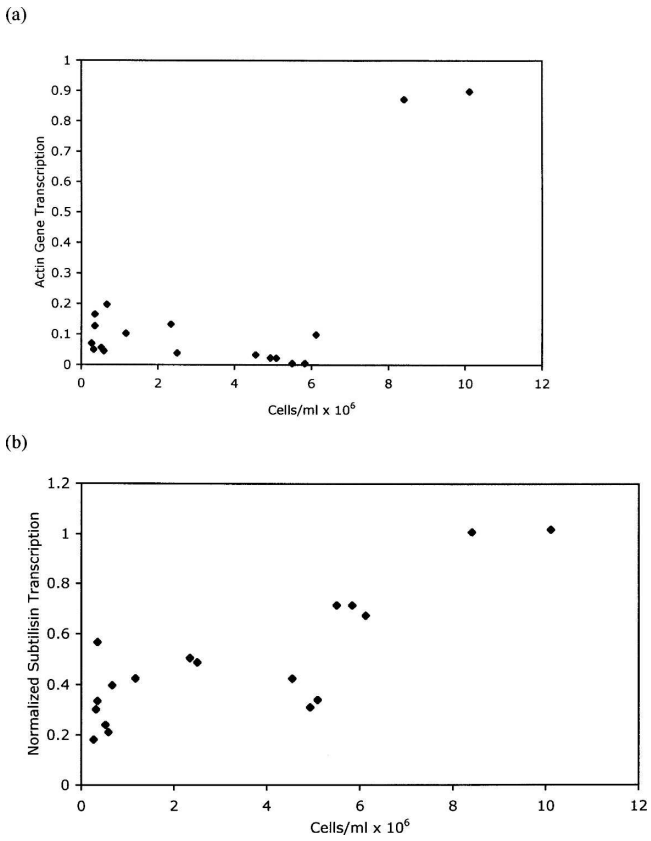

Figure 4. ***Perkinsus marinus.*** **(a) Correlation between cell proliferation and actin gene transcription levels. (b) Correlation between cell proliferation and relative subtilisin gene transcription levels.**

DISCUSSION

Media Supplementation With Homogenate From Different Oyster Species Modulates Perkinsus marinus *Cell Proliferation*

The lack of cell proliferation among *P. marinus* cells cultured in media supplemented with extract from oyster homogenate is similar to that observed by MacIntyre et al. (2003) and Earnhart et al. (2004), as well as to studies conducted using plasma from host oysters to supplement media (Gauthier & Vasta 2002). At the lower supplementation dose (0.25 mg mL^{-1} of oyster homogenate), after 4 weeks in culture, we observed either lower, or approximately the same, cell counts as that of the initial inoculation concentration.

Regardless of the supplement concentration, cell proliferation was affected by the presence of oyster tissue homogenate. *In vivo*, *P. marinus* appears to decrease its growth rate, or increase its doubling time, at densities higher than 10^4 cells g^{-1} dry weight of oyster (Saunders et al. 1993). Increase in doubling times with an increase in cell density is common for protozoa and may be caused by a decrease in nutrients available for growth with a decrease in the availability of host nutrients (Choi et al. 1989). Saunders et al. (1993) observed that many infected populations of oysters survive over the summer with parasite burdens within a few doublings of lethal limits and suggested epizootics could be caused by environmental factors tipping the nutrient scale in favor of increased *P. marinus* cell proliferation. In this study, however, exposure of *P. marinus* cells to host products significantly decreased proliferation, even in the presence of a nutrient-rich media. This would suggest that the host products were responsible for the inhibition of cell proliferation and in some instances, cell death.

Gauthier and Vasta (2002) found that cell proliferation was inhibited in *P. marinus* cultures supplemented with plasma from heavily infected *C. virginica* oysters more than in those containing plasma from uninfected host oysters. Similarly, cultures supplemented with extract from the naïve CvWa used in this study had higher cell counts than those supplemented with extract from the previously exposed *C. virginica* stocks. In contrast, plasma from the nonnative *C. gigas* or *C. rivularis* (*C. ariakensis*) has been found to enhance proliferation (Gauthier & Vasta 2002). In our experiment, however, supplementing cultures with whole homogenate from *C. gigas* or *C. ariakensis* decreased cell proliferation and resulted in significant parasite death. Parasite mortality was also observed in the three cultures that were supplemented with homogenate from the *C. virginica* stock, CvXB, selectively bred over several generations for *Haplosporidium nelsoni* and subsequently, starting in the 1990s, for *P. marinus* resistance. Viability in these cultures was extremely low, lacking any replicates or enough RNA to conduct the subtilisin transcription experiments.

It is still too early in this research to draw any strong conclusions regarding the nature of the inhibitory effects of oyster homogenate on parasite cell proliferation, either from native or nonnative oysters. It is possible that inhibition of *P. marinus* cell proliferation could be controlled by feedback mechanisms modulated by the parasite itself. When starting clonal cultures, "feeder layers" containing proliferating *P. marinus* cells, or significant amounts of "culture spent" media were required to stimulate cell division and subsequent culture growth (Gauthier & Vasta 1993, Bushek et al. 2000). Whether this mechanism occurs at higher densities is uncertain. The leveling off that occurs in growth curves of cultured cells (La Peyre 1996) and the increase in doubling time observed *in vivo* (Saunders et al. 1993), may be affected more by lack of nutrients or by host signals than by a parasite feedback loop. The results produced here, however, and those reported from previous studies (MacIntyre et al. 2003, Earnhart et al. 2004), would suggest that inhibition is caused by factors produced by the oyster. Exact mechanisms are still unknown, and we used whole oyster homogenate to supplement the culture media. Therefore, the parasite was exposed to a variety of proteases and potential inhibitors not encountered in homogenate free media and probably not encountered in a healthy intact oyster. It is likely that a complicated interaction exists between the host, the parasite and the environment, where different host products and parasite products are constantly being regulated as the infection progresses, cell densities change, nutrients decrease and the environmental conditions fluctuate.

Subtilisin Gene Transcription

In this study, subtilisin gene transcription by *P. marinus* was suppressed by the presence of oyster homogenate in the media in comparison with control flasks containing defined media alone. As stated earlier, this could be because of inhibitory effects of the host product. Earnhart has observed (personal communication) decreases in protease activity of extracellular products from *P. marinus* cells grown in the presence of *C. virginica* whole homogenate compared with the proteolytic activity of those cells cultured in fully defined media. Taken together with the results of this study it will be interesting to determine if host products may be able to directly affect parasite protease activity and the transcription of the

genes encoding these enzymes. Relative subtilisin gene transcription in the current study was also significantly higher in *P. marinus* cells grown in media supplemented with homogenate from the parasite's natural host species than in cells cultured in the presence of either *C. ariakensis* or *C. gigas* homogenate (Fig. 3). Whether this is caused by host specificity, or greater suppression by the tolerant oyster species, or a combination of both is unknown. The significantly lower subtilisin gene transcription seen in the tolerant oysters species, and lack of any significant difference among subtilisin gene transcription among the *C. virginica* stocks, even though there were some differences in cell proliferation, could indicate that the host species affects transcription of the subtilisin gene in the parasite.

Induced subtilisin gene transcription in *P. marinus* cells grown in media supplemented with whole homogenate from *C. virginica,* compared with cells grown in defined media alone or cultured in media supplemented with homogenate from the tolerant species, *C. ariakensis* or *C. gigas,* would have been more indicative of host specificity versus host suppression of transcription. Unfortunately we did not see this using supernatant from whole oyster homogenate. C. Earnhart (personal communication) has found increased proteolytic activity in ECP from cultures supplemented with increasing concentrations of gill/mantle tissue, with comparable activity at the highest dose concentration used (1.50 mg mL^{-1}) to that seen in defined media. Supplementation doses this high, however, are not practical using extract from whole oyster homogenate; because death of the parasite occurs when whole homogenate from the tolerant species is used and evidently can also occur with some stocks of the native oyster, as occurred here with CvXB supplemented cultures. Future work using different tissues/organs from these three *Crassostrea* oyster species, rather than whole homogenate, and examining gene transcription at different supplementation concentrations and at different time intervals, should help us to understand the suppression/induction model for transcription of the *P. marinus* subtilisin gene. Perhaps we will be able to determine whether a particular tissue type induces subtilisin gene transcription, or whether a particular tissue type from a tolerant oyster species causes greater suppression, narrowing down the number of candidate genes that affect *P. marinus* resistance in oysters.

We observed a positive correlation ($r = 0.82$) (Fig. 4b) between cell count and subtilisin gene transcription in this study. Although the actin gene transcription correlation with cell count was also positive, the correlation was not as strong ($r = 0.61$) and was not supported by those treatment groups containing extracts from oyster homogenate ($r = -0.55$), whereas the positive correlation between cell counts and subtilisin gene transcription ($r = 0.62$) was retained after removal of the treatment groups with unsupplemented media. The potential for differential actin gene transcription under varying culture conditions is a concern when comparing results from treatment groups containing oyster supplement to treatment groups lacking any oyster extract (i.e., media alone). However, the use of the actin gene to normalize subtilisin gene transcription seems to be justified for this study when comparing those samples with media containing oyster extract. The difficulties in selecting a reporter gene for normalization are well documented (Bustin 2004). Future work may want to examine other housekeeping genes or ribosomal RNA genes as candidates for normalizing target gene transcription to determine if they are more appropriate.

The positive correlation observed between cell count and subtilisin gene transcription could support claims that the subtilisin gene is a virulence factor, however, little inference should be made at this point. The same factors suppressing cell division in the oyster-supplemented cultures may, or may not, be suppressing subtilisin gene transcription. In addition, cytotoxic effects caused by cell death may be responsible for the decrease observed in subtilisin gene transcription levels in cells cultured in media supplemented with extract from oysters. Viability was much lower in those cultures supplemented with *C. ariakensis* or *C. gigas* extract than in cultures supplemented with *C. virginica* extract. This does not, however, explain why transcription was significantly lower in *C. virginica* supplemented cultures that had greater than 98% viability as compared with cultures with unsupplemented media, nor can this explain why *C. virginica* supplemented cultures with low viability (CvTg-60%) had similar transcription levels to other *C. virginica* supplemented cultures.

CONCLUSIONS

Increased interest in serine protease genes of pathogenic organisms has developed because of the importance of serine proteases in parasite evasion of host defense mechanisms (Chaudhuri et al. 1989), invasion of host tissue (Banyal et al. 1981, Blackman et al. 1998, Hackett et al. 1999), parasite metabolism and parasite growth (McKerrow et al. 1993). In addition, variations in expression levels of some protease genes correlate with pathogenicity (Ramamoorthy et al. 1992, Tanaka et al. 1994, Gonzalez-Aseguinolaza et al. 1997, Blackman et al. 1998). This study demonstrates that *P. marinus* cell proliferation and subtilisin gene transcription is suppressed by the presence of extract from whole oyster homogenates compared with growth and gene transcription in defined media alone. In addition, differential transcription of a *P. marinus* subtilisin gene occurs in media supplemented with homogenate from closely related oyster species, with the highest level of transcription observed in the host species, *C. virginica*, regardless of the stock. Subtilisin gene transcription was also correlated with cell counts. Continued work examining the temporal, tissue-specific and stage-specific differences in subtilisin gene transcription among the tolerant and susceptible oysters should increase our knowledge of the role this potentially important gene and its encoded product may play in *P. marinus* infection.

ACKNOWLEDGMENTS

The authors thank V. Encomio for supplying the Yeocomico deployed *Crassostrea virginica* oysters, Dr. S. K. Allen of the Aquaculture Genetics and Breeding Tech Center at VIMS for providing *Crassostrea ariakensis* and Dr. E. M. Burreson's lab for supplying the Rappahannock River oysters. This research was sponsored by the NOAA office of Sea Grant, United States Department of Commerce, under Grant No. NA16RG1697 to the Virginia Graduate Marine Science Consortium and Virginia Sea Grant College Program. VIMS contribution #2703.

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