ABSTRACT

Title of Dissertation: HOST PREFERENCE OF *PERKINSUS* SPECIES: EPIZOOTIOLOGICAL, ENVIRONMENTAL, AND MOLECULAR ASPECTS

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Perkinsus species are protistan parasites of mollusks. In Chesapeake Bay, *P. marinus*, *P. chesapeaki*, *P. andrewsi*, and a *Perkinsus* isolate from the hard clam *Mercenaria mercenaria* [*Perkinsus* sp. (*M. mercenaria*)] are sympatric.

In vitro experiments by others suggest a preference of *P. marinus* for the eastern oyster *Crassostrea virginica*. Studies conducted in this dissertation on the distribution of *Perkinsus* species in *C. virginica* and *M. mercenaria* from Virginia to Maine using PCR-based detection assays provided further evidence for a host preference of *P. marinus*. While *P. marinus* was the most prevalent species in *C. virginica*, its prevalence was significantly lower in *M. mercenaria*.

Interestingly, the assay designed to be specific for *Perkinsus* sp. (*M. mercenaria*) also amplified *P. andrewsi*. Characterization of the rRNA gene loci of both *Perkinsus* species revealed the presence of a second rRNA gene unit in *P. andrewsi* with high percent sequence identity to the unit of *Perkinsus* sp. (*M. mercenaria*), explaining the cross-amplification. Furthermore, DNA samples of *M. mercenaria* inhibited PCR amplification, which was overcome by adding bovine serum albumin and dimethyl sulfoxide to the PCR reaction mixture.

P. marinus resides in oyster hemocytes scavenging and/or inhibiting the production of reactive oxygen species (ROS) usually generated by oyster hemocytes. ROS scavenging enzymes include superoxide dismutases (SODs) and peroxidases such as catalases. SODs have been characterized in *P. marinus*, but peroxidases have not been detected. Results from the present study suggest that, while lacking catalase, *P. marinus* has ascorbate dependent peroxidases usually found in plants. Alternatively, *P. marinus* may suppress the production of ROSs by enzymes such as phosphatases. A secreted acid phosphatase activity reported earlier in *P. marinus* was further characterized in the present study, and its purification attempted. Furthermore, a search of a genome database yielded several phosphatase-like genes, including a putative protein phosphatase 2C predicted to be secreted. Further analysis could neither confirm its secretion nor its involvement in host preference.

However, the approaches implemented throughout this research represent a strategy for processing additional phosphatase and other gene sequences

identified in genome databases that will further the understanding of the biology of *Perkinsus* species.

HOST PREFERENCE OF *PERKINSUS* SPECIES: EPIZOOTIOLOGICAL, ENVIRONMENTAL, AND MOLECULAR ASPECTS

By

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DEDICATION

Für Klara

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	amino acids
ACP	acid phosphatase
AMP	antimicrobial peptide
APX	ascorbate peroxidase
ASA	ascorbic acid
ASC	ascorbate
BLAST	basic local alignment search tool
BLASTN	nucleotide-nucleotide BLAST
BLASTP	protein-protein BLAST
BLASTX	translated query vs. protein database BLAST
BSA	bovine serum albumin
CAT	catalase
CDD	conserved domain database
cDNA	complementary DNA
CDS	coding sequence
Cl^-	chloride
COOP	cooperative observer network
CPX	catalase-peroxidase
CTAB	cetyltrimethylammonium bromide
DAPI	4',6'-diamidino-2-phenylindole
DHA	dehydroascorbate

DHAR	dehydroascorbate reductase
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSP	dual specificity phosphatase
FBS	fetal bovine serum
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMBOSS	The European Molecular Biology Open Software Suite
Ek/LIC	enterokinase ligation independent cloning
EST	expressed sequence tag
EtBr	ethidium bromide
FTM	fluid thioglycollate medium
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulfide
GSX	glutathione peroxidase
H_2O_2	hydrogen peroxide
HAM	Ham’s nutrient mixture F12
HAD	haloacid dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOCl	hypochlorous acid

HRP	horseradish peroxidase
ICS	intercluster spacer
IgG	immunoglobulin G
IGS	intergenic spacer
IPTG	isopropyl 1-thio-D-galactopyranoside
ITS	internal transcribed spacer
LSU	large subunit
mACP	membrane-bound ACP
MDHA	monodehydroascorbate
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NTS	non transcribed spacer
O_2	oxygen
$^{\bullet}O_2^-$	superoxide
OCl^-	hypochlorite
$^{\bullet}OH$	hydroxyl radical
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline (20 mM phosphate buffer, 150 mM NaCl, pH 7.2)
PBST	PBS with 0.05% (v/v) Tween 20
PCR	polymerase chain reaction
PKC	protein kinase C
PMSF	phenylmethylsulphonylfluoride

pNP	p-nitrophenol
pNPP	disodium-p-nitrophenol phosphate
PP	serine/threonine protein phosphatase
PP2C	protein phosphatase 2C
PPM	serine/threonine protein phosphatase M
PPP	serine/threonine protein phosphatase P
PTP	phosphotyrosine phosphatase
PVDF	polyvinylidene difluoride
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RNA	ribonucleic acid
rPmPP2C2	recombinant expressed *Perkinsus marinus* PP2C2
ROS	reactive oxygen species
rRNA	ribosomal RNA
sACP	secreted ACP
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
SSU	small subunit
TBLASTN	protein-translated nucleotide BLAST
TIGR	The Institute for Genomic Research
TLR	Toll-like receptor
U	unit
UTR	untranslated region

COMMON AND LATIN NAMES OF HOSTS OF *PERKINSUS* SPECIES

Atlantic ribbed mussel	*Geukensia demissa*
Australian blacklip abalone	*Haliotis ruber*
Baltic tellin or macoma clam	*Macoma balthica*
bent mussel or hooked mussel	*Ischadium recurvum,*
eastern oyster or American oyster	*Crassostrea virginica*
European clam	*Ruditapes decussatus*
flat oyster	*Ostrea edulis*
Japanese Manila clam	*Venerupis philippinarum*
Japanese scallop	*Patinopecten yessoensis*
Matagorda macoma or Mitchell macoma	*Macoma mitchelli*
northern quahog, hard clam, little-neck clam, or cherrystone clam	*Mercenaria mercenaria*
soft shell clam or soft clam	*Mya arenaria*
stout razor clam	*Tagelus plebeius*

CHAPTER 1

INTRODUCTION

Overview of *Perkinsus* species

Perkinsus species are protistan parasites of mollusks and often associated with disease in these invertebrates. *P. marinus*, the first species described, has been associated with mass mortalities of the eastern oyster, *Crassostrea virginica*, along the Gulf of Mexico and Atlantic coast.

P. marinus was initially classified as a fungus within the genus *Dermocystidium* (*D. marinum*; Mackin et al. 1950). Subsequent ultrastructural studies on its zoospore stage revealed morphological characteristics that resemble those of apicomplexan parasites, leading to the establishment of a new genus *Perkinsus* within the phylum Apicomplexa, and the renaming of *D. marinum* as *P. marinus* (Levine 1978). Molecular phylogenies, however, provided evidence that questioned the inclusion of *Perkinsus* species within the Apicomplexa (Siddall et al. 1997). More recently, taxonomic affinities of *P. marinus* with *Parvilucifera infectans*, a protistan parasite of dinoflagellates, led Norén et al. (1999) to establish the new phylum Perkinsozoa as a sister-group of the *Apicomplexa* that includes the genera *Perkinsus*, *Parvilucifera*, and *Cryptophagus* (Brugerolle 2002, Norén et al. 1999). However, further molecular phylogenies based on protein (actin, tubulins, and heat shock protein 90) and

small subunit (SSU) ribosomal ribonucleic acid (rRNA) gene sequences suggest that the Perkinsozoa are the earliest divergent group from the dinoflagellate lineage (Kuvardina et al. 2002, Leander and Keeling 2004, Saldarriaga et al. 2003).

The designation of *Perkinsus* species has traditionally been based on host species, type location, and morphology. More recently, sequences of rRNA genes and intergenic regions have been used to support new species designations, and question the validity of currently accepted species (Burreson et al. 2005, Coss et al. 2001b, Dungan et al. 2002, Kotob et al. 1999a, Murrell et al. 2002).

In addition to *P. marinus*, six *Perkinsus* species have been described that have been isolated from different mollusks: *P. olseni* (syn. *P. atlanticus*) from the Australian blacklip abalone, *Haliotis ruber* (Lester and Davis 1981), and the European clam, *Ruditapes decussatus* (Azevedo 1989), *P. qugwadi* from the Japanese scallop*, Patinopecten yessoensis* (Blackbourn et al. 1998), *P. chesapeaki* from the soft shell clam, *Mya arenaria* (McLaughlin et al. 2000), *P. andrewsi* from the Baltic clam, *Macoma balthica* (Coss et al. 2001b), *P. mediterraneus* from the flat oyster, *Ostrea edulis* (Casas et al. 2004), and *P. honshuensis* from the Japanese Manila clam, *Venerupis philippinarum* (Dungan and Reece 2006). The species designations of *P. chesapeaki*, *P. mediterraneus*, and *P. honshuensis* were based on morphology, type host, and type location, and have been supported by rRNA gene and intergenic spacer sequences (Casas et al. 2004, Dungan and Reece 2006, McLaughlin et al. 2000). *P. olseni*

and *P. atlanticus* were considered distinct species based on location (Azevedo 1989, Lester and Davis 1981), however, sequence analysis of the non-transcribed spacer (NTS) of the rRNA gene unit led to their synonymization (Lester and Davis 1981). The species designation of *P. andrewsi* was based on rRNA gene and intergenic sequences obtained from a clonal culture. Morphological characteristics of this isolate were considered insufficient to support its species designations (Coss et al. 2001a, Coss et al. 2001b).

Transmission of *Perkinsus* species infections is believed to take place through trophozoites that are released by moribund bivalves into the environment and filtered by healthy neighboring bivalves (Andrews 1996). Once ingested, the trophozoite is phagocytosed by hemocytes located in the gut epithelium (Mackin 1951, Perkins 1976), mantle and gills (Allam and Ford 2002). Phagocytosed *P. marinus* trophozoites reside in a phagosome-like structure, where they proliferate by multiple fission and/or budding. Infected hemocytes eventually lyse and the released trophozoites are phagocytosed by other hemocytes, which migrate throughout the tissues and disseminate the parasite (Mackin 1951, Perkins 1976). If released into the water column, mature trophozoites enlarge to become prezoosporangia, which produce a large numbers of biflagellated zoospores (Perkins 1996) (Figure 1). These motile zoospores presumably give rise to trophozoites once they infect oyster tissue, but the mechanism of infection is unknown (Andrews 1996).

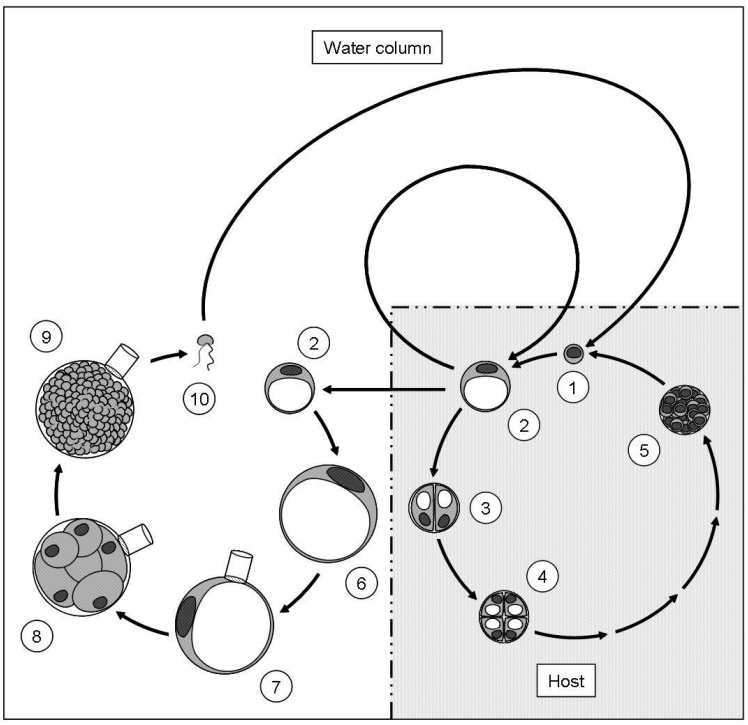


Figure 1. Lifecycle of *Perkinsus* species. 1 - 5: Development in the host. Trophozoites reside either intracellular in host hemocytes or are extracellular and multiply by multiple fission (3 - 5) and/or budding. 6 - 10: Zoosporulation in the water column. 1: immature trophozoite; 2: mature trophozoite; 3. two-cell stage; 4 four-cell stage; 5: 64-cell stage; 6: enlarged trophozoite; 7, 8: prezoosporangia; 9: zoosporangium; 10: biflagellated zoospore. Modified from Perkins (1996).

Distribution of *Perkinsus* species along the Atlantic coast of North America

P. marinus was the first *Perkinsus* species described. Its occurrence was first reported in 1948 in *C. virginica* in the Gulf of Mexico (Mackin et al. 1950). In subsequent years, *Perkinsus* infections were found discontinuously in most regions of the Gulf of Mexico and along the Atlantic Coast of the USA, allowing the distribution range to be expanded from Tabasco, Mexico, to Chesapeake Bay, USA in the North (Burreson et al. 1994, Ford 1996, Soniat 1996) (Figure 2). During the 1980s and 1990s, however, a northward expansion of the distribution

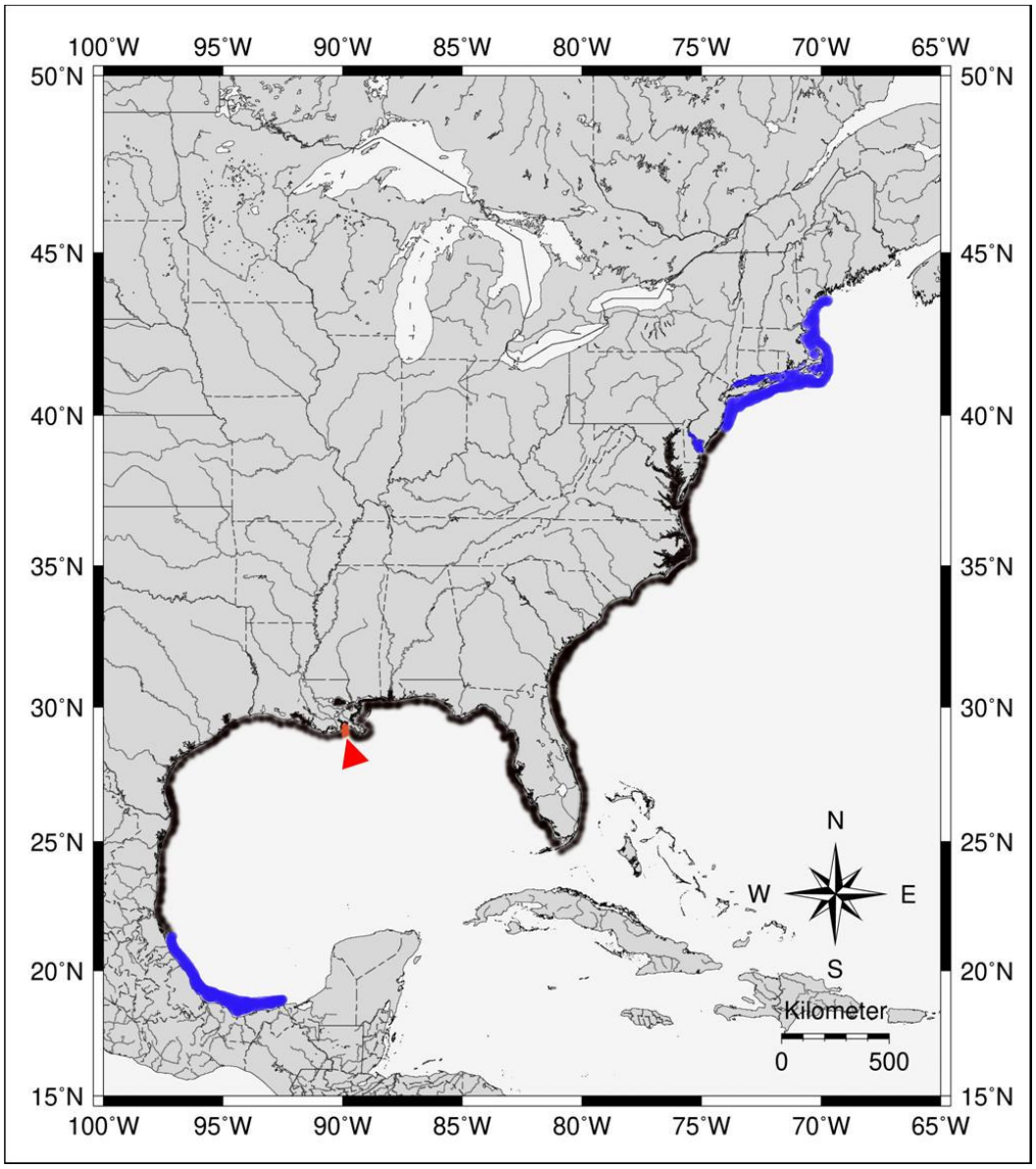


Figure 2. Distribution of *Perkinsus* species along the Atlantic coast of North America. Red arrow head: first description of *P. marinus* in 1948; (■): distribution of *Perkinsus* species in 1988; (■): distribution of *Perkinsus* species in 1996 (Burreson et al. 1994, Ford 1996, Soniat 1996). The map was generated with the Generic Mapping Tools, v.4.1.4 (Smith and Wessel 1990, Wessel and Smith 1998).

of *Perkinsus* species was observed, with infected *C. virginica* found as far north as Maine, USA (Ford 1996) (Figure 2).

Most studies on the distribution and epizootiology of *Perkinsus* species used the fluid thioglycollate medium (FTM) assay developed by Ray (1952) to

detect *Perkinsus* species. This assay is simple and inexpensive, but does not discriminate between *Perkinsus* species. Until 2000, *P. marinus* was the only accepted *Perkinsus* species along the Atlantic coast of North America. *Perkinsus* species in other bivalves were reported, but not further identified (Perkins 1988). Hence any *Perkinsus* infection in *C. virginica* has been attributed to this species. However, in 2000 and shortly thereafter, two additional *Perkinsus* species, *P. andrewsi* and *P. chesapeaki*, initially isolated from *M. balthica* and *M. arenaria*, respectively, were described in Chesapeake Bay, USA (Coss et al. 2001b, McLaughlin et al. 2000). In addition to *M. balthica*, its type host, *P. andrewsi* was detected in other clam species, and in *C. virginica* (Coss et al. 2001b). Similarly, *P. marinus* was found in the clams *M. balthica*, *M. mitchelli*, *Mercenaria mercenaria,* and *M. arenaria* (Coss et al. 2001b, McLaughlin et al. 2000). In addition, individual oyster and clam specimens were found to harbor multiple *Perkinsus* species (Coss et al. 2001b). These findings raise the question whether all *Perkinsus* infections detected in oysters with the FTM assay truly represent *P. marinus* infections.

Species-specific diagnostic assays that are based on the polymerase chain reaction (PCR) technology have been developed for *P. marinus*, *P. olseni,* and *P. andrewsi* (Coss et al. 2001b, de la Herrán et al. 2000, Marsh et al. 1995, Robledo et al. 1998, Yarnall et al. 2000). In addition, a “generic” PCR-based assay was developed based on conserved sequences of the intergenic region of the rRNA gene cluster that links the 5S gene and the SSU gene from *P. marinus, P. andrewsi*, and *P. olseni* (Robledo et al. 2002). Whether this assay detects the

other described *Perkinsus* species has not been confirmed. Nevertheless, with these tools available, it is now feasible to address the distribution of *P. marinus*, *P. andrewsi*, and *P. olseni*, the host range of these *Perkinsus* species, their host specificity and host preference, and to identify new *Perkinsus* species.

Impact of *Perkinsus* species on the shellfishery and the environment of Chesapeake Bay

P. marinus is a recognized pathogen of the eastern oyster *C. virginica* that caused widespread mortalities in both natural and farmed populations. Prior to 1980 *Perkinsus* species infections were prevalent in high salinity areas in lower Chesapeake Bay (Burreson and Ragone Calvo 1996). In these endemic areas oyster mortalities were observed that did not significantly affect the oyster fishery. However, due to consecutive drought years and warm winters in the mid to late 1980s and due to movement of infected oysters, *Perkinsus* infections were spread throughout Chesapeake Bay and are now also established in low salinity areas that are sources of oyster seed stock (Burreson and Ragone Calvo 1996, Krantz and Jordan 1996). As a result of the disease and continued over fishing, *C. virginica* populations in Chesapeake Bay drastically declined in the late 1950 and remained low (Burreson and Ragone Calvo 1996). Consequently, annual landings of *C. virginica* in Chesapeake Bay declined over the last five decades from approximately 16,000 metric tons in the 1950s to roughly 400 metric tons in 2005 (The NOAA National Marine Fisheries Service (NMFS) landings database; http://www.st.nmfs.gov). The depletion of oyster beds has also environmental consequences. Oysters are considered a keystone species in Chesapeake Bay

and form reefs that are used as habitats by a variety of organisms (Kennedy 1991). Furthermore, oysters are efficient suspension-feeders, and it has been proposed that in the late 1800s, the oyster population in Chesapeake Bay filtered the entire water body of the Bay in less than a week (Kennedy 1996, Newell 1988). In contrast, it has been estimated that it takes more than 46 weeks for the current oyster population to filter the Bay (Kennedy 1996). Thus, due to the depletion of oyster beds not only habitat has been lost, but also the Bay's food web may have shifted from a benthic food web to a pelagic dominated food web (Kennedy 1996, Newell 1988).

Host range, host specificity, and host preference

The host range of a parasite species is defined as the total number of host species that have been reported to be parasitized by that particular species (Lymbery 1989). Host specificity of a parasite species is commonly used to express the extent to which a parasite species is restricted in the number of host species used (Lymbery 1989, Rohde 1980). To measure the degree of host specificity of a parasite, not only the number of host species, but also the prevalence and/or intensity/density of infection, are taken into account (Rohde 1980). The term host preference describes the use of a particular host species by a parasite species and takes into consideration the prevalence of the parasite and the availability of the host species (Lymbery 1989). Thus, if two host species that are infected by a parasite species are equally available to this parasite, the host species that is infected more frequently is the preferred host species of this

parasite. It is important to note that host preference does not imply active host selection by the parasite (Lymbery 1989).

Host specificity and host preference express the success of a parasite to infect a host species and establish itself in or on the host. For endoparasites, such as *Perkinsus* species, success of infection is dependent on the ability of the parasite to find or be found by the host, to gain entry to the host (either by active or passive invasion), and, once inside the host, to evade host defense mechanisms. To establish itself and proliferate, the parasite has to have access to nutrients and other resources.

Internal defense mechanisms of bivalves

Perkinsus species are endoparasites of mollusks. Like other invertebrates, mollusks do not possess acquired immunity. Their defense mechanisms rely on innate immune responses that consist of humoral and cellular components and include antimicrobial peptides (AMPs), lysosomal enzymes, lectins, and Toll-like receptors (TLRs).

AMPs have been reported from mussels, scallops and oyster species (Charlet et al. 1996, Hubert et al. 1996, Mitta et al. 1999, Seo et al. 2005, Zhao et al. 2007). While four groups of AMPs, mytilins, myticins, mytymicins, and defensin-like molecules, have been described in *Mytilus* species, in *Crassostrea* species, up to date only defensin-like molecules have been reported (Seo et al. 2005). However their role in defense against *Perkinsus* infection has not been established.

Lysosomal enzyme activities are found intrahemocytically and in the hemolymph. It is thought that these enzymes are released by degranulation of the hemocytes (Anderson 1996 and references therein). The most studied lysosomal enzyme is lysozyme that has bactericidal activity. Indirect evidence suggests that it might play a role in host defense against *Perkinsus* species. La Peyre et al. (1989) reported a positive correlation between lysozyme concentration and *C. virginica* survival. Furthermore, parasitized oysters from low salinity regions from the James River (MD) have elevated lysozyme levels and higher survival rates compared to oysters from high salinity areas (Chu et al. 1993, Chu and La Peyre 1993b). However, because *P. marinus* infection density increases with salinity, the effect of lysozyme as an anti-*Perkinsus* agent is difficult to assess from these studies (Anderson 1996). In addition to lysozyme, acid phosphatase (ACP), aminopeptidase, β-glucuronidase, and lipase have been found in the hemolymph of *C. virginica*, but their role in defense against *Perkinsus* species is unclear (Anderson 1996).

Lectins play an important role in the recognition steps of the immune response in invertebrates. Lectins have been found in many bivalves including oysters. It has been demonstrated that they possess opsonic properties and enhance the phagocytic ability of bivalves. In *C. virginica* hemocyte-bound and soluble lectins have been described that bind galactose and other sugars (Vasta et al. 1984, Vasta et al. 1982). *C. gigas*, which is not a natural host for *P. marinus*, possesses lectins specific for sialic acids (Hardy et al. 1977).

TLRs are pattern recognition receptors involved in innate immunity. In mollusks, TLRs have been described from the scallop *Chlamys farreri* (Qiu et al. 2007), but not been found in *C. virginica* or *M. mercenaria*.

Mechanisms of entry, intracellular survival, and proliferation of *Perkinsus* species

Any factor that contributes to successful transmission, entry, survival, and proliferation of an endoparasite will also influence its host specificity and determine its host range. Most studies on parasite entry, intracellular survival, and proliferation of a *Perkinsus* species were focused on *P. marinus* and are summarized below.

Preliminary results from studies on *P. marinus* cell surface carbohydrates suggest that galactose and other sugars are present (Sen and Vasta, unpublished data). Hence, because exposed sugars on the *P. marinus* trophozoite surface correspond to the ligands for *C. virginica* hemocyte and humoral lectins, it was proposed that the entry of *P. marinus* into the *C. virginica* hemocyte is mediated by the host's non-self recognition factors. Interestingly, similar studies revealed differences in the cell surface carbohydrate composition between *Perkinsus* species (Saito and Vasta, unpublished data) suggesting, that these *Perkinsus* species may be phagocytosed at different rates depending on the host lectin repertory. Indeed, Gauthier and Vasta (2002) observed in *in vitro* experiments that *P. marinus* trophozoites were phagocytosed by *C. virginica* hemocytes at a higher rate than by those of *C. gigas* that is not a natural host for *P. marinus*.

In vitro studies on the proliferation of *P. marinus* trophozoites exposed to plasma of clams (*M. mercenaria*, and *Anadara ovalis*), mussels (*Mytilus edulis* and *Geukensia demissa*), and oysters (*C. virginica*, *C. gigas*, and *C. ariakensis* [syn. *C. rivularis*]) also show that *P. marinus* is the least inhibited by plasma of naïve *C. virginica*, its type host (Anderson 2001, Gauthier and Vasta 2002). These observations suggest that, among the bivalves tested, *C. virginica* is the most suitable host to *P. marinus*. Therefore, even though the observation that *P. marinus, P. olseni*, and *P. andrewsi* can be found in multiple mollusks hosts (Azevedo 1989, Coss et al. 2001b , Lester and Davis 1981) suggests a lack of strict host-specificity, *P. marinus* may exhibit host preference.

After phagocytosis, *P. marinus* resides in a phagosome-like structure and proliferates. Upon phagocytosis by *C. virginica* hemocytes, biotic or abiotic particles usually reside in a phagocytic vacuole that is rapidly acidified (Beaven and Paynter 1999), becomes rich in reactive oxygen species (ROS), and receives lysosomal enzymes that kill and degrade the particles (Adema et al. 1991, Anderson et al. 1995, Cheng 1975). The oxidative burst that is normally observed in oyster hemocytes, however, does not take place upon phagocytosis of live *P. marinus* trophozoites or live trophozoites together with zymosan, which is prepared from yeast cell walls and consists of protein carbohydrate complexes. In contrast, killed trophozoites or zymosan alone elicit ROS production (Anderson 1999, Volety and Chu 1995). Live *P. marinus* trophozoites also show relatively high resistance to superoxide ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2) (Schott et al. 2003a). These findings together suggest that *Perkinsus* trophozoites actively

scavenge ROS through the production of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and other peroxidases, and/or express enzymes that inhibit the production of ROS such as ACPs (reviewed in Anderson 1996).

Other factors that may contribute to the intracellular survival and to the pathogenicity of *Perkinsus* species are other excreted products such as proteases (reviewed in Anderson 1996).

The antioxidant pathway of *Perkinsus* species

ROS production is initiated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that generates $^{\bullet}O_2^-$. The $^{\bullet}O_2^-$ is dismutated either spontaneously, or enzymatically, to H_2O_2. The H_2O_2 in turn is enzymatically reduced to H_2O. Both, $^{\bullet}O_2^-$ and H_2O_2 are relatively long-lived molecules and can generate hydroxyl radicals ($^{\bullet}OH$) by either reacting with each other (Haber-Weiss reaction), or by reactions of H_2O_2 with metal ions (e.g., with Fe^{2+}, Fenton reaction). The latter reaction also produces hydroxyl anions (OH^-). $^{\bullet}OH$ is considered to be the most toxic form of oxygen. H_2O_2 also serves as a substrate to myeloperoxidases (MPOs) that produce hypochlorous acid (HOCl) in the presence of chloride. Hypochlorite (OCl^-) in turn can react with H_2O_2 (Steinbeck et al. 1992). The product of this reaction is a singlet oxygen that reacts with H_2O to generate $^{\bullet}OH$, oxygen, and $^{\bullet}O_2^-$ (Wentworth et al. 2002) (Figure 3).

Most eukaryotic organisms remove $^{\bullet}O_2^-$ enzymatically by SODs. The produced H_2O_2 is removed by CAT that generates H_2O and oxygen and

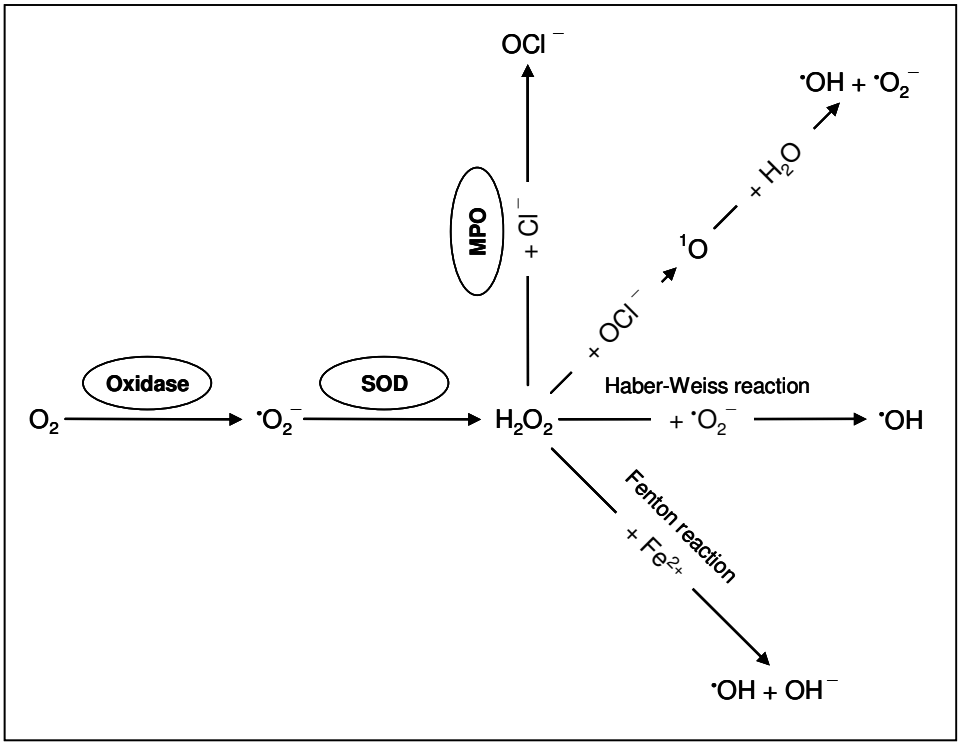


Figure 3. Production of reactive oxygen species. For detailed description see text. Cl^-: chloride; H_2O_2: hydrogen peroxide; MPO: myeloperoxidase; O_2: oxygen; ^1O: singlet oxygen; $^\bullet O_2^-$: superoxide; OCl^-: Hypochlorite; OH^-: hydroxyl anions; $^\bullet OH$: hydroxyl radicals; SOD: superoxide dismutase.

glutathione peroxidase (GSX) that, in addition to H_2O_2, can utilize a variety of peroxides as substrates. GSX oxidizes glutathione that in turn is reduced by the glutathione reductase (GR) using NADPH (reviewed in Splettstoesser and Schuff-Werner 2002).

Studies on the antioxidant pathway in *P. marinus* showed that the parasite possesses at least two distinct iron type SODs, one of which is upregulated by iron-starvation or upon exposure of cultured cell to host serum (Ahmed et al. 2003, Gauthier 1998, Schott and Vasta 2003, Schott et al. 2003b, Wright et al. 2002). Other antioxidant enzymes of *P. marinus* are yet to be fully characterized. However, *P. marinus* apparently lacks CAT (Schott et al. 2003a, Chapter 4), as

well as GPX-like activities (Schott and Vasta, unpublished). Instead, it possesses an ascorbate peroxidase (APX)-like activity (Schott et al. 2003a, Chapter 4).

Phosphatases of *Perkinsus* species

Phosphatases are a heterologous group of enzymes that catalyze the hydrolysis of phosphomonoesters. They have been classified based on physiological criteria (e.g. ACPs and alkaline phosphatases), substrate specificity [e.g. non-specific phosphatases, serine/threonine protein phosphatases (PPs), phosphotyrosine phosphatases (PTPs), and dual specificity phosphatases (DSPs)], or sequence similarities [e.g. histidine ACPs, serine/threonine protein phosphatase P (PPP) and serine/threonine protein phosphatase M (PPM) families].

Phosphatases are involved in the regulation of various biological processes, including cell cycle regulation, signal transduction, nutrition, and modulation of host/pathogen interactions. Phosphatases suggested to be involved in host/pathogen interactions include ACPs, PPs of the types 2A, 2B, and 2C, and PTPs (Fox et al. 2001, Reilly et al. 1996, Remaley et al. 1985b, Saha et al. 1985, Sue-A-Quan et al. 1995, Takekawa et al. 1998, Warmka et al. 2001, Yamamori et al. 2000, Archambaud et al. 2005, Gilbert et al. 2007).

In *P. marinus*, intracellular and extracellular ACP activity has been reported (Volety and Chu 1995, 1997). Attempts to localize ACP activity by transmission electron microscopy revealed activity around the nucleus, which led to the conclusion that phosphatases might be involved in cell cycle regulation.

However, the histological staining protocol used would detect most phosphatase activities, and lacks specificity for the enzyme of interest (e.g. a secreted ACP). Later, Chu et al. (1998b) reported that more virulent strains have higher ACP activity than less virulent strains. Beyond that, ACP activities in *P. marinus* have not been further characterized, and their function in intracellular survival, either by mediating defense against host responses, or nutrient acquisition, is unclear.

Among the protein phosphatases, only the sequence of a PP1-like gene has been reported (Coss and Vasta unpublished), that appears to be constitutively expressed (Gauthier 1998).

Perkinsus marinus as a model organism for host-parasite interactions

P. marinus exhibits unique features that make it an attractive model with which to study the molecular mechanisms of intracellular survival. The parasite can be grown and maintained in fully defined medium without host components (Gauthier and Vasta 1995), and is non-pathogenic for humans. It can be cultured in large volumes (>1 liter) with a 24 h doubling time, and reaching 10^8 cells/ml in seven days. Its host, *C. virginica*, is readily available from various sources, and can be easily maintained in the laboratory.

Oyster hemocytes and other cells can be isolated and maintained as *in vitro* primary cultures for several weeks. Over the years our laboratory has developed numerous molecular tools and resources that include complementary deoxyribonucleic acid (cDNA) and genomic libraries, and yeast- and bacteria-based complementation assays. Furthermore, expressed sequence tags (ESTs) are available, that are generated within a functional genomics project in

collaboration with J. Kissinger at the University of Georgia, and through the *P. marinus* genome sequencing project in collaboration with The Institute for Genomic Research (TIGR). Furthermore, we have additional *Perkinsus* species and isolates in culture that enable us to pursue comparative studies.

Project goals and rational

Host specificity and host preference of an intracellular parasite is determined by a variety of factors, including factors related to host- and host cell-recognition as well as intracellular survival. Trophozoites of *Perkinsus* species reside and proliferate in a phagocytic vacuole within host hemocytes, suggesting that they have developed mechanisms to protect themselves from host responses such as oxidative burst, and through this vacuole they acquire nutrients (Perkins 1988, 1996). *P. marinus* is known to actively suppress the oxidative burst of its host (Anderson 1999, Volety and Chu 1995). It has been proposed that it rapidly scavenges the host derived ROSs using its antioxidant enzymes (Anderson 1996, Schott and Vasta 2003). *P. marinus* possesses at least two iron type SODs that have been extensively characterized (Ahmed et al. 2003, Schott and Vasta 2003, Schott et al. 2003b, Wright et al. 2002). Our knowledge on other enzymes downstream in the antioxidant pathway, however, is limited. Alternatively, *Perkinsus* species may inhibit the production of ROSs. Phosphatases, including PPs and membrane-bound and extracellular ACPs, have been proposed to contribute to intracellular survival of protistan parasites and prokaryotic pathogens, either by modulating the host immune responses

(including the inhibition of the production of ROS) and/or by providing nutrients such as soluble inorganic phosphates.

The aim of my dissertation was to assess the distribution and prevalences of several *Perkinsus* species in the eastern oyster, *C. virginica* and the hard clam, *M. mercenaria*, as a measure of host preference. Furthermore, molecular mechanisms (i.e. components of the antioxidant pathway, and phosphatases) that may contribute to the intracellular survival and thus host preference of *P. marinus* were to be elucidated.

Research objectives

This dissertation work describes: (1) the further characterization of the rRNA loci of *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*); (2) the assessment of infection prevalence of *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) along the Northeast Atlantic Coast of the USA and Canada in *C. virginica* and *M. mercenaria*; (3) the search for antioxidant enzymes downstream of SODs (i.e. CAT); and (4) the partial purification and characterization of secreted ACP activities of *P. marinus*, the gene characterization of a protein phosphatase 2C (PP2C) of *P. marinus*, and the preliminary characterization of the expression patterns of PP2C upon exposure of *P. marinus* trophozoites to serum of *C. virginica* and *M. mercenaria*.

CHAPTER 2

MOLECULAR CHARACTERIZATION OF THE rRNA GENE LOCUS OF *PERKINSUS ANDREWSI* AND A RELATED *PERKINSUS* ISOLATE [1]

SUMMARY

Perkinsus species are parasitic protists of mollusks, currently classified within the Perkinsozoa, a recently established phylum that is the earliest divergent sister group to the dinoflagellate lineage. Ribosomal RNA genes and their intergenic spacers have been used to support the taxonomy of *Perkinsus* species, the description of new species, and to develop molecular probes for their detection and identification. Recently, the partial sequence of the rRNA locus of two *Perkinsus* isolates from the baltic clam (*M. balthica*) and the hard clam (*M. mercenaria*) was described. The rRNA genes and intergenic spacers of the *Perkinsus* isolates differed from those described in the currently accepted

[1] The majority of this chapter was published in Pecher et al. (2004), with the exception of the characterization of the rRNA gene locus of the related *Perkinsus* isolate, and is presented in amended form consistent with the policy of Blackwell Publishing on the fair use of reprinting articles of an author in volumes of which the author is an editor or co-editor.

species to a degree that led to designation of the *M. balthica* isolate as *P. andrewsi* n. sp. The *M. mercenaria* isolate has not been formally described as a *Perkinsus* species and therefore is hereafter referred to as *Perkinsus* sp. (*M. mercenaria*). Interestingly, a PCR-based assay developed for *Perkinsus* sp. (*M. mercenaria*) cross-amplified *P. andrewsi* genomic deoxyribonucleic acid (DNA), despite marked sequence differences. This chapter describes the further characterization of the rRNA gene locus of the two isolates with the aim of developing specific assays for each of the isolates. The present study led to the identification of an additional rRNA gene unit (rRNA-B) in the *P. andrewsi* hapantotype. Except for the 5.8S gene, all regions of the rRNA-B gene unit exhibited sequence differences from that initially described (rRNA-A). Each rRNA gene unit was arranged in a "head to tail" tandem repeat. This is the first report of an additional distinct rRNA gene unit in a *Perkinsus* species. *Perkinsus* sp. (*M. mercenaria*) appears to have only one rRNA gene unit that shares high percent sequence identity with the rRNA-B gene unit of *P. andrewsi*, suggesting that *Perkinsus* sp. (*M. mercenaria*) is a *P. andrewsi* variant.

INTRODUCTION

Perkinsus species (Perkinsozoa, Perkinsea) (Norén et al. 1999) are protistan parasites of mollusks. Infections often result in mass mortalities in cultured and natural mollusk populations. *P. marinus* was initially classified as a fungus within the genus *Dermocystidium* (*D. marinus;* Mackin et al. 1950). Taxonomic affinities of *P. marinus* with *Parvilucifera infectans*, a protistan

parasite of dinoflagellates, led Norén et al. (1999) to establish the new phylum Perkinsozoa that now includes the genera *Perkinsus*, *Parvilucifera*, and *Cryptophagus* (Brugerolle 2002, Norén et al. 1999). Based on protein (actin, tubulins, and heat shock proteins) and SSU rRNA gene phylogenies it has been proposed that the Perkinsozoa form the earliest divergent group from the Dinozoa (Kuvardina et al. 2002, Saldarriaga et al. 2003).

Initially, description of *Perkinsus* species was based on ultrastructure, type host, and type host location. The development of *in vitro* culture methods (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, La Peyre et al. 1993) allowed molecular characterization of *Perkinsus* species and isolates. Sequences of rRNA genes and intergenic regions in particular have significantly contributed to the taxonomic characterization of *Perkinsus* species (Fong et al. 1993, Goggin 1994, Goggin and Barker 1993), to support new species designations (Coss et al. 2001b, Kotob et al. 1999a), and to develop molecular probes for their detection and identification (Abollo et al. 2006, Coss et al. 2001b, de la Herrán et al. 2000, Elandalloussi et al. 2004, Gauthier et al. 2006, Marsh et al. 1995, Penna et al. 2001, Robledo et al. 1999, Robledo et al. 2000, Robledo et al. 2002, Robledo et al. 1998, Yarnall et al. 2000).

The organization of the rRNA gene cluster of *Perkinsus* species differs from that of most eukaryotes described so far. Unlike to the typical eukaryotic rRNA gene cluster that does not contain the 5S rRNA gene, the 5S rRNA gene is linked to the other rRNA genes and intergenic spacers in *Perkinsus* species (Coss 2000, Coss et al. 2001b, de la Herrán et al. 2000, Marsh et al. 1995). This

was corroborated by the characterization of the complete rRNA gene units of *P. olseni* and *P. marinus* (Robledo et al. 2002, Robledo and Vasta unpublished). In these species, the rRNA gene unit consists of the following regions: 5S rRNA gene, intergenic spacer (IGS), SSU, internal transcribed spacer (ITS) 1, 5.8S rRNA gene, ITS2, and large subunit (LSU). Within a rRNA gene cluster, the rRNA gene units are arranged as "head to tail" tandem repeats that are separated by intercluster spacers (ICS) (de la Herrán et al. 2000, Robledo et al. 2002). A similar rRNA gene organization was described for some fungi and algae (reviewed by Kawai et al. 1995), and the apicomplexan parasite *Toxoplasma* (Guay et al. 1992). However, in other Apicomplexa, such as *Plasmodium*, *Babesia*, *Theileria*, and *Cryptosporidium*, instead of the typical tandem organization of multiple rRNA gene units, these (from one to ten copies) are unlinked, and further, the 5S rRNA gene is unlinked to the other rRNA genes of each rRNA unit. In some species of *Plasmodium*, *Cryptosporidium*, and *Babesia* a higher level of complexity of the rRNA genes has been described: the multiple rRNA gene units may not be identical, and up to seven distinct rRNA units have been identified (Dame and McCutchan 1983, Gardner et al. 2002, Le Blancq et al. 1997, Reddy et al. 1991). In *Pl. falciparum*, four rRNA gene units (A, B, C, and D) have been reported, which transcribe at least two life stage-specifically expressed rRNA types (A and S) (Gunderson et al. 1987). Recently, a fifth rRNA gene unit has been identified that is almost identical to the S-type rRNAs in the SSU and 5.8S gene sequences, but differs significantly in the LSU gene sequence from the LSU sequences of the A- and S-type rRNAs (Gardner et

al. 2002). The presence of two distinct SSU and LSU gene sequences was also reported for dinoflagellate species within the genus *Alexandrium* (Scholin et al. 1993, Scholin et al. 1994, Yeung et al. 1996).

In Chesapeake Bay, three *Perkinsus* species, *P. marinus*, *P. chesapeaki,* and *P. andrewsi*, and a *Perkinsus* isolate from the hard clam *M. mercenaria* [*Perkinsus* sp. (*M. mercenaria*)] are sympatric. The heterospecificity of *P. andrewsi* and *P. chesapeaki* has been controversial and synonymization has been suggested (Burreson et al. 2005). According to the original description, *P. chesapeaki* is a distinct morphotype (McLaughlin et al. 2000). In contrast, *P. andrewsi* cannot be distinguished from other *Perkinsus* species based on morphology, but sequences of the rRNA genes and intergenic regions of *P. andrewsi* differ from other *Perkinsus* species (Coss et al. 2001a, Coss et al. 2001b). The *Perkinsus* isolate that was analyzed to clarify the relationship of *P. andrewsi* and *P. chesapeaki* has been designated as *P. chesapeaki* because it has been isolated from the type host of *P. chesapeaki*. However, this isolate appears to be morphologically identical to *P. andrewsi* and thus may not be *P. chesapeaki*. Therefore, for the present study, I consider *P. andrewsi* and *P. chesapeaki* as distinct *Perkinsus* species.

With the ability to culture *Perkinsus* species, the development of specific molecular probes became feasible (Marsh et al. 1995). Currently, PCR-based assays have been developed that are specific for the *Perkinsus* genus (Robledo et al. 2002), and for the individual species *P. marinus* (Gauthier et al. 2006, Marsh et al. 1995, Yarnall et al. 2000), *P. olseni* (syn. *P. atlanticus*) (de la Herrán

et al. 2000, Robledo et al. 2000), *P. andrewsi* (Coss et al. 2001b), and *Perkinsus* sp. (*M. mercenaria*) (Coss 2000). In addition, modified PCR-based assays have been established, including a multiplex PCR assay detecting *P. marinus* and *Haplosporidium* species (Penna et al. 2001) and assays that can distinguish between *P. marinus*, *P. olseni*, *P. mediterraneus,* and *P. andrewsi/P. chesapeaki* or *P. marinus* and *P. olseni* (Abollo et al. 2006, Elandalloussi et al. 2004). No specific PCR assay for *P. chesapeaki* is currently available. Its development has been hampered by the lack of a *bona fide* clonal *P. chesapeaki* culture. The original *P. chesapeaki* type culture has never been deposited in a public repository, and apparently has been lost (Burreson et al. 2005).

The species-specific assays developed for *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) are based on the IGS sequences. Surprisingly however, the assay initially developed for *Perkinsus* sp. (*M. mercenaria*) cross-amplified *P. andrewsi* genomic DNA, despite substantial sequence differences within the IGS regions of the described rRNA gene units based on which the primers have been designed (Coss 2000; Robledo and Vasta, unpublished data). One explanation of this finding is the presence of sequence polymorphisms within the IGS sequence of *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*).

Accordingly, cloning and sequencing of the complete IGS of both *Perkinsus* isolates was attempted which led to the discovery of an additional rRNA gene unit (referred to as rRNA-B) in the *P. andrewsi* genome that is distinct from the originally identified rRNA gene unit (referred to as r-RNA-A). This

chapter describes the characterization of the complete rRNA gene units of *P. andrewsi* (rRNA-A and rRNA-B) and *Perkinsus* sp. (*M. mercenaria*).

MATERIALS AND METHODS

Cell cultures

Cell culture reagents were purchased from Sigma Aldrich (St. Louis, MO), unless indicated otherwise. A *P. andrewsi* clonal culture that has been subcultured from the hapantotype (PAND-A8-4a, ATCC 50807; Coss et al. 2001b) and a *Perkinsus* sp. (*M. mercenaria*) clonal culture (Coss 2000) were maintained at 28 °C in DMEM:HAM's F12 (1:2) medium (for composition see Table 1) that has been adjusted to a pH of 6.6 (Gauthier and Vasta 1995).

Table 1. Composition of DMEM:HAM's F12 (1:2) culture medium used to culture *Perkinsus* species (Gauthier and Vasta 1995).

5 mg/ml Dulbecco' modified Eagle's medium (DMEM)
10 mg/ml Ham's F12 nutrients mixture (HAM's F12)
15 mg/ml artificial seawater (ASW; Instant Ocean, Mentor, OH)
50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
3.6 mM sodium bicarbonate
100U/ml penicillin G
100U/ml streptomycin sulfate
5% fetal bovine serum (FBS)

Isolation of DNA

For PCR, cloning and sequencing, DNA was extracted from log-phase *Perkinsus* cultures using the DNeasy Tissue kit from QIAGEN (QIAGEN, Valencia, CA), following the manufacturer's instructions. For Southern analysis, approximately $3.0x10^6$ *Perkinsus* cells of log phase cultures were lysed overnight at 55 °C in sucrose lysis buffer (150 mM NaCl, 15 mM Na citrate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v), sodium dodecyl sulfate (SDS), 27% (w/v) sucrose) containing 200 µg/ml proteinase K (Green 1997, Pecher et al. 2004). High molecular weight genomic DNA was subsequently extracted with phenol:chloroform:isoamyl alcohol, isopropanol precipitated, washed with 70% (v/v) ethanol and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) (Green 1997). DNA samples were treated with RNAse A (1mg/ml) and ethanol precipitated.

PCR amplification, cloning, and sequencing

Genes and intergenic regions of the rRNA gene unit were amplified by PCR with different primer sets. Primers used are listed in Table 2, and their relative positions within the rRNA gene units are shown in Figure 4.

Each PCR reaction mixture consisted of 5 mU Takara *Ex Taq*™ DNA polymerase (TaKaRa Bio Inc., Shiga, Japan), the proprietary Ex Taq™ reaction buffer (contains 2 mM Mg^{2+} at final concentration, TaKaRa Bio Inc.), 200 µM of each deoxyribonucleotide triphosphate (dNTP), 10 pmole of each primer, and 50 ng of genomic DNA. In negative PCR controls, the DNA template was substituted by double distilled, sterile H_2O.

Table 2. List of PCR primers that were used to amplify the rRNA genes and intergenic regions of *Perkinsus andrewsi* and to generate probes for Southern analysis.

Primer	Sequence (5 '- 3')	Specificity	Reference
M5	AAC CAT CCC GAC TAC CAT CTG G	IGS-B	Pecher et al. 2004/ present study
M6	TTC ATC ACT TGA GTT GCG	IGS-B	Pecher et al. 2004/ present study
M8	GCG AAA ATT GAC TTT CAG GTC G	IGS-B	Pecher et al. 2004/ present study
NTS1	AAG TCC TTA GGG TGC TGC	SSU	Robledo et al. 1999
NTS2	ATG AGC CAT TCG CAG TTT CGC C	SSU	Robledo et al. 1999
NTS6	ATT GTG TAA CCA CCC CAG GC	IGS-A	Coss et al. 2001
NTS7	AAG TCG AAT TGG AGG CGT GGT GAC	IGS-A	Coss et al. 2001
PER1	TAG TAC CCG CTC ATY GTG G	IGS	Robledo et al. 2002
PER2	TGC AAT GCT TGC GAG CT	IGS	Robledo et al. 2002
PMITS3	GGT AAT CTC ACA CAC ATC AGG C	LSU	Pecher et al. 2004/ present study
UPRA	AAC CTG GTT GAT CCT GCC AGT	SSU	Medlin et al. 1988
UPRB	TGA TCC TTC TGC AGG TTC ACC TAC	SSU	Medlin et al. 1988
UPRC	GTA GGT GAA CCT GCA GAA GGA TCA	SSU	Pecher et al. 2004/ present study

The PCR amplification of the complete IGS using the primers NTS1 and NTS2 was performed under conditions that have been optimized for the amplification of the IGS of *P. marinus* (Robledo et al. 1999). Briefly, the cycling conditions were as follows: 94 °C for 4 min, followed by 35 cycles of 91 °C for 1 min, 50 °C for 30 sec with an extension of 1 sec per cycle, 72 °C for 1 min, with a final extension at 72 °C for 10 min. To obtain complete rRNA gene unit sequences, three different amplicons containing rRNA genes and intergenic regions were generated by PCR. Partial IGS and the complete SSU were amplified using the primers M8 (Pecher et al. 2004, this chapter), and UPRB from

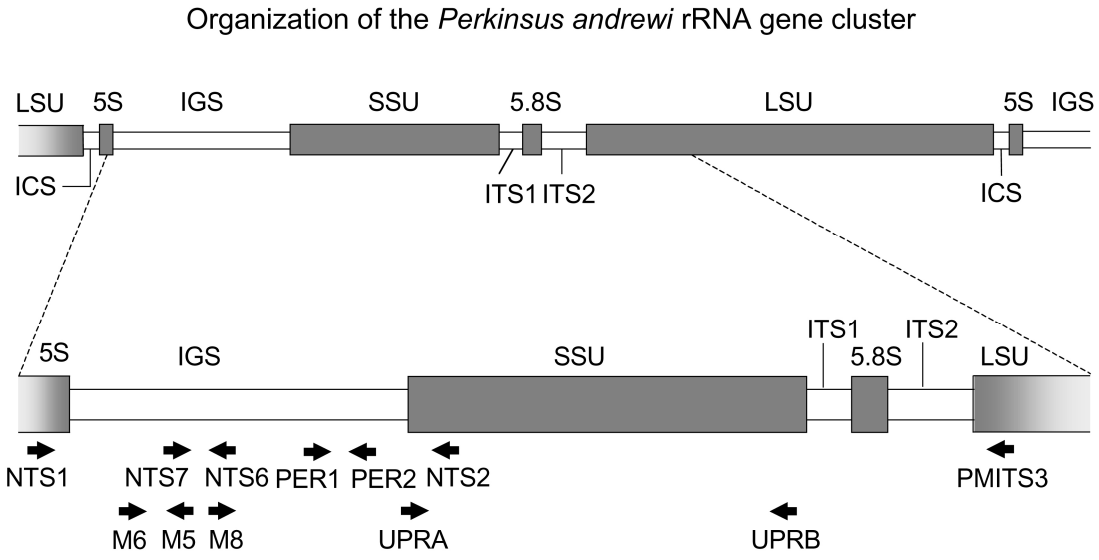


Figure 4. Localization of primers (arrows) used for the amplification of rRNA genes and intergenic regions of *Perkinsus andrewsi.* ICS: intercluster spacer; IGS: intergenic spacer; ITS: internal transcribed spacer; LSU: large subunit; SSU: small subunit.

(Medlin et al. 1988), using the cycling conditions described above for the amplification of the complete IGS. Amplicons containing partial SSU, the ITS1-5.8S-ITS2 region, the LSU-ICS-5S and partial IGS were generated using the primers UPRC (present study) and M5 (Coss 2000). Partial IGS, the complete SSU, and the complete ITS1-5.8S-ITS2 region were amplified using the IGS specific primers NTS7 (Coss et al. 2001b) and M8 and the primer PMITS3 (Pecher et al. 2004, this chapter). Partial IGS, the complete SSU, the ITS1-5.8S-ITS2 region, and the LSU-ICS-5S region were amplified using the primers NTS7, M8, M5 and UPRA (Medlin et al. 1988). The cycling conditions for these PCR amplifications were 96 °C for 3 min, followed by 30 cycles of 96 °C for 1 min, 58 °C for 1 min, 72 °C for 20 min, with a final extension at 72 °C for 20 min.

PCR products were separated by electrophoresis using 1% agarose gels and visualized by staining with ethidium bromide (EtBr) (0.5 µg/ml). Amplicons were gel-purified using the QIAquick Gel Extraction kit (QIAGEN). Purified amplicons were cloned using the Promega pGEMII vector system (Promega Corporation, Madison, WI). To characterize the rRNA gene units of *P. andrewsi* clones were sequenced using additional internal primers as follows: (a) three clones obtained from 10 PCR amplifications containing the IGS, (b) eight clones obtained from two PCR amplifications containing partial sequence of the IGS and the complete SSU region, (c) two clones from two PCR amplifications containing partial sequence of the IGS and the complete SSU-ITS1-5.8S-ITS2 region, and (d) one clone from one PCR amplification containing partial sequence of the IGS and the complete SSU-ITS1-5.8S-ITS2-LSU-ICS-5S region.

To complete the characterization of the rRNA gene unit of *Perkinsus* sp. (*M. mercenaria*), the LSU-ICS-5S region was sequenced with additional internal primers from three clones containing partial SSU, the ITS1-5.8S-ITS2-LSU-ICS-5S region, and partial IGS. The clones were obtained from three independent PCR amplifications.

To assess the potential intraclonal IGS variability in *P. andrewsi*, IGS amplicons were generated in five independent PCR amplifications using the primers NTS1 and NTS2 (Robledo et al. 1999) under the following optimized PCR conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 65 °C for 45 s, 72 °C for 1 min, with a final extension at 72 °C for 10 min. PCR

products were size-separated, gel-purified, cloned, and sequenced as described above. For each amplicon, at least nine clones were sequenced.

Sequencing services were provided by the Bioanalytical Services Laboratory at the Center of Marine Biotechnology, Baltimore, MD.

For comparison, rRNA sequences of the following *Perkinsus* species were obtained from GenBank™: *P. andrewsi* (accession number AF102171), *Perkinsus* sp. (*M. mercenaria*) (deposited as *Perkinsus* sp. CCA2001; AF252288), *P. marinus* (AF497479), *P. olseni* (syn. *P. atlanticus*; AF140295), *P. chesapeaki* (isolate G117; AF042707 and AF091541), *Perkinsus* species isolated from *M. arenaria* (ATCC 50864, deposited as *P. chesapeaki*; AF440464, AF440465, AF440466, and AF440467), and *T. plebeius* (ATCC 50866, deposited as *P. chesapeaki*; AF440468, AF440469, AF440470, and AF440471). Sequences were pair-wise aligned using the Needle-Wunsch algorithm within The European Molecular Biology Open Software Suite (EMBOSS) (v.4.0.0; Rice et al. 2000) with default parameters. A similarity search for the *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) ICS sequences was performed using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTN) (Altschul et al. 1990).

Assessment of IGS representation

To assess the relative representation of the two IGSs identified, serially diluted *P. andrewsi* genomic DNA was amplified with the primers NTS1 and NTS2 (Robledo et al. 1999) using the optimized PCR conditions. The PCR products were separated and visualized as described above. Gel photographs

(Polaroid black and white instant pack film 667, Polaroid, Waltham, MA) were scanned at high resolution (1,200 dpi). The intensity of amplicons on the digital images was analyzed using the software NIH Image 1.62 (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Because the intensity of EtBr-stained amplicons is proportional to their length (Sambrook and Russell 2001, Tanabe et al. 2002), intensity values for smaller amplicons were normalized to the length of the largest amplicon, by calculating the ratio of the size of the largest amplicon to the size of the amplicon of interest.

Single-cell PCR

A *P. andrewsi* cell suspension was incubated in FTM for one week (Ray 1952). During the incubation in FTM, *Perkinsus* species trophozoites enlarge and arrest in the prezoosporagium stage. To obtain single cells, 5 µl of the FTM treated culture was diluted 10-fold in distilled water. This cell suspension was further serially diluted in 50 µl of distilled water in a 96-well plate until wells with single cells could be identified under an inverted microscope. The entire content of wells containing single cells was transferred to a 0.5 ml thin walled microcentrifuge tube and frozen at -20 °C. The samples were thawed, dried in a speed vacuum concentrator, resuspended in distilled water to a final volume of 22 µl, boiled for 3 min, and the IGS amplified as described above. The PCR products were tested for the presence of *P. andrewsi* IGS sequences by PCR, using primers specific for the IGS-A (NTS6 and NTS7) (Coss et al. 2001b) and IGS-B (M5; present study) in combination with an universal primers for amplification of the IGS (NTS1) (Robledo et al. 1999) (Table 1).

Southern analysis

Four microgram of high molecular weight *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) genomic DNA were digested with the restriction enzymes *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I (New England BioLabs Inc., Beverly, MA, Promega, Invitrogen Corp. Carlsbad, CA) following the manufacturers' recommendations. Controls consisted of plasmids containing the IGS-A and IGS-B of *P. andrewsi* (200 ng and 20 ng or 15 ng, respectively), and uncut *P. marinus* genomic DNA (4 µg). DNA digests and control samples were fractionated on a 0.8% agarose gels, stained with EtBr, and transferred onto Nylon membranes (Hybond XL, Amersham Biosciences Corp., Piscataway, NJ) by capillary blotting (Sambrook and Russell 2001). Two gels were transferred to separate membranes. One membrane was processed for detection using radio-labeled probes, and the second membrane with the North2South® Direct HRP Labeling and Detection kit from Pierce (Rockford, IL) following the manufacturer's recommendations. To process the membrane for detection using radio-labeled probes, it was hybridized in an aqueous prehybridization/ hybridization solution (450 mM NaCl, 45 mM Na citrate, 2 mg/l Ficoll 400, 2 mg/l polyvinylpyrrolidone, 2 mg/l bovine serum albumin, 0.1% (w/v) SDS, 100 µg/ml salmon sperm DNA) at 68 °C overnight (Brown 1993), with either an IGS-A specific probe (probe A, 290 bp), an IGS-B specific probe (probe B, 305 bp), or a probe specific to *Perkinsus* species IGSs (probe PER, 315 bp). The membrane processed with the North2South® Direct HRP Labeling and Detection kit from Pierce was prehybridized in hybridization buffer provided with the kit for 30 min at 55 °C and

hybridized in the same buffer for 3 h at 55 °C with either probe A or probe B. After hybridization, the membrane was washed three times with low stringency buffer (2x SSC, 0.1% SDS (w/v)) at 55 °C followed by higher stringency washes (0.2x SSC, 0.1% SDS (w/v) and 0.1x SSC, 0.1% SDS (w/v) respectively) at 55°C. Both membranes were stripped two to three times by boiling in 0.1% (w/v) SDS between hybridizations.

Probes A and PER were generated by PCR from a plasmid containing the IGS-A of *P. andrewsi* using primers specific for the IGS-A (NTS6 and NTS7) and primers specific to *Perkinsus* species (PER1 and PER2; Robledo et al. 2002). Probe B was generated by PCR from a plasmid containing the IGS-B of *P. andrewsi* using IGS-B specific primers (M5 and M6; Pecher et al. 2004, this chapter). Probes A and B were either labeled with [α^{32}P] dCTP using *redi*prime™ II (Amersham Biosciences Corp.) or conjugated to a heat stable horseradish peroxidase (HRP) using the North2South® Direct HRP Labeling and Detection kit from Pierce following the manufacturer's recommendations. Probe PER was only labeled with [α^{32}P] dCTP.

Autoradiographic film (Kodak X-Omat Blue XB-1, Rochester, NY) was exposed to the nylon membrane hybridized with radio-labeled probes for 30 min at room temperature after hybridization with probe A, 3 h at -80 °C after hybridization with probe B, and 1 h at room temperature after hybridization with probe PER. To demonstrate specificity of the probes, exposures were extended to 16 - 18 h at -80 °C. The membrane that was hybridized with HRP-conjugated probes was incubated for 5 min in the North2South substrate reagents provided

with the kit, and exposed to autoradiographic film for 1 min at room temperature after hybridization with probe A, and 5 min after hybridization with probe B.

RESULTS

Sequence of the rRNA locus

PCR amplification of *P. andrewsi* DNA using primers that flank the IGS region yielded a prominent amplicon of the expected size (1,670 bp) and a smaller additional product (1,540 bp) that co-migrated with the amplicon obtained from PCR amplification of *Perkinsus* sp. (*M. mercenaria*) DNA (Figure 5).

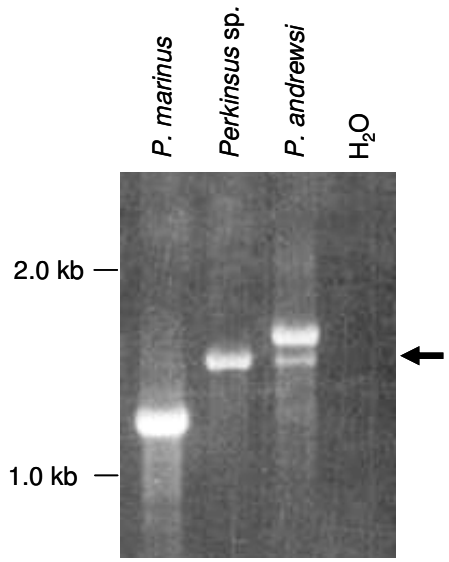


Figure 5. Agarose gel electrophoresis of PCR-amplified products with IGS primers. H_2O: negative PCR control; *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*) DNA. Note the additional 1.5 kb amplicon (arrow) in *P. andrewsi*, which corresponds to the later identified intergenic spacer of the rRNA-B gene unit. It co-migrates with the amplicon of *Perkinsus* sp. (*M. mercenaria*).

Sequence analysis of the smaller *P. andrewsi* amplicon revealed sequences of the 5S and SSU rRNA in the 3' and 5' ends, respectively, suggesting that this sequence represented an IGS. Alignment analysis showed that this IGS sequence (hereafter IGS-B) showed 71.3% identity to the previously described *P. andrewsi* IGS sequence (Coss et al. 2001b; hereafter IGS-A).

PCR amplification of *P. andrewsi* genomic DNA using primers based on the IGS-B (primer M8) and primers based on conserved regions in the 3' end of the SSU (primer UPRB) and the 5' end of the LSU (primer PMITS3) resulted in amplicons of 2,522 bp and 3,274 bp, respectively. Sequence analysis showed that both amplicons contained part of the IGS-B and a complete SSU rRNA sequence. In addition, the 3,274 bp amplicon contained an ITS1-5.8S-ITS2 sequence and sequences present in the 5' end of the LSU. Sequence analysis of clones that contained the IGS-B sequence and the SSU-ITS-1-5.8S-ITS2 region showed that, except for the 5.8S gene, all sequences of the rRNA genes and intergenic regions associated with the IGS-B differed from the initially described *P. andrewsi* rRNA genes and intergenic regions. This demonstrated contiguity of the newly described sequences.

To isolate and characterize the LSU, the 5S, and the ICS of both rRNA gene units of *P. andrewsi*, amplicons were generated by PCR using primers specific for the IGS-A (primer NTS6), IGS-B (primer M5), and a primer based in a conserved region of the 5' end of the SSU (primer UPRA) (Figure 4). The obtained amplicons were 7,042 bp and 6,881 bp long, respectively. Both amplicons contained a partial IGS sequence, the complete SSU, the ITS1-5.8-

ITS2 region, the complete LSU, and a region consisting of the ICS and the 5S that were contiguous with the LSU and the IGS, respectively. The LSU sequences of both gene units were 3,528 bp long and showed 99.0% identity. The 5S-A and B sequences were 121 bp long and to 99.2% identical. The ICSs of the rRNA-A and -B units differed both in size and sequence. The ICS-A (124 bp) and the ICS-B (109 bp) differed in 47 positions (37.9%, based on the length of ICS-A). A database search for both ICS using BLASTN (Altschul et al. 1990) failed to identify similar sequences. Consensus sequences of the LSU-ISC-5S region of the rRNA-A unit and the complete rRNA-B unit were deposited at GenBank™ (AY305327 and AY305326, respectively).

To isolate and characterize the LSU, the 5S, and the ICS of *Perkinsus* sp. (*M. mercenaria*), amplicons were generated by PCR using the primers M5 and a primer based in a conserved region of the 5' end of the SSU (primer UPRA), as well as a primer in a conserved region of the 3' end of the SSU (primer UPRC) (Figure 4). The amplicons obtained were 6,881 bp and 5,073 bp long, respectively. Both amplicons contained a partial IGS sequence, the ITS1-5.8-ITS2 region, the complete LSU, and a region consisting of the ICS and the 5S that were contiguous with the LSU and the IGS, respectively. The amplicon generated by the primers UPRA and M5 contained, in addition, the complete SSU. The LSU sequence was 3,528 bp long, the 5S sequences was 121 bp, and the ICSs 109 bp.

A comparison of the rRNA genes and intergenic regions of the *Perkinsus* sp. (*M. mercenaria*) showed that all rRNA genes and intergenic regions were

almost identical to the rRNA-B genes and intergenic regions of *P. andrewsi*. The IGS showed 99.5% identity, the LSU 99.6% identity, the SSU 99.7 %, and the ITS1, ITS2, 5.8S, 5S, and ICS 100% identity to the respective sequences of the rRNA-B gene unit of *P. andrewsi*.

Sequence comparison between the *Perkinsus andrewsi* and *Perkinsus* sp. (*M. mercenaria*) rRNA genes and intergenic spacer with other *Perkinsus* species and isolates

Sequence comparisons of the rRNA genes and intergenic regions of the *P. andrewsi* rRNA-A and -B gene units, *Perkinsus* sp. (*M. mercenaria*), *P. marinus*, *P. olseni*, and *P. chesapeaki* are summarized in Table 3. Sequence differences were most pronounced in the IGS and the ICS. The sequences of the *P. andrewsi* rRNA-B and *Perkinsus* sp. (*M. mercenaria*) rRNA showed high

Table 3. Comparison of the rRNA genes and intergenic spacers of the *Perkinsus andrewsi* A and B rRNA units, *Perkinsus* sp. (*M. mercenaria*), *P. chesapeaki* (isolate G117), *P. marinus*, and *P. olseni*. ICS: intercluster spacer; IGS: intergenic spacer; ITS1, 2: internal transcribed spacer 1 and 2; LSU: large subunit rRNA gene; *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*); SSU: small subunit rRNA gene.

Percent identity of the 5S rRNA gene

	P. andrewsi rRNA-B	*Perkinsus* sp.	*P. marinus*	*P. olseni*
P. andrewsi rRNA-A	99.2	99.2	97.5	95.2
P. andrewsi rRNA-B		100.0	98.3	96.0
Perkinsus sp.			98.3	96.0
P. marinus				97.6

Table 3 continued

Percent identity of the IGS

	P. andrewsi rRNA-B	*Perkinsus* sp.	*P. marinus*	*P. olseni*
P. andrewsi rRNA-A	71.3	71.4	48.9	49.8
P. andrewsi rRNA-B		99.4	52.7	53.4
Perkinsus sp.			52.5	53.3
P. marinus				70.9

Percent identity of the SSU

	P. andrewsi rRNA-B	*Perkinsus* sp.	*P. chesapeaki*	*P. marinus*	*P. olseni*
P. andrewsi rRNA-A	99.8	99.7	99.3	98.5	99.1
P. andrewsi rRNA-B		99.7	99.4	98.7	99.3
Perkinsus sp.			99.2	98.5	99.1
P. chesapeaki				98.2	98.7
P. marinus					99.2

Percent identity of the ITS1

	P. andrewsi rRNA-B	*Perkinsus* sp.	*P. chesapeaki*	*P. marinus*	*P. olseni*
P. andrewsi rRNA-A	93.7	93.7	93.7	74.3	85.3
P. andrewsi rRNA-B		100.0	100.0	74.5	87.8
Perkinsus sp.			100.0	74.5	87.8
P. chesapeaki				74.5	87.8
P. marinus					77.5

Table 3 continued

Percent identity of the 5.8S rRNA gene

	P. andrewsi rRNA-B	*Perkinsus* sp.	*P. chesapeaki*	*P. marinus*	*P. olseni*
P. andrewsi rRNA-A	100.0	100.0	99.4	98.7	98.7
P. andrewsi rRNA-B		100.0	99.4	98.7	98.7
Perkinsus sp.			99.4	98.7	98.7
P. chesapeaki				98.1	98.1
P. marinus					100.0

Percent identity of the ITS2

	P. andrewsi rRNA-B	*Perkinsus* sp.	*P. chesapeaki*	*P. marinus*	*P. olseni*
P. andrewsi rRNA-A	91.3	91.3	91.3	77.6	77.3
P. andrewsi rRNA-B		100.0	99.2	77.4	75.9
Perkinsus sp.			99.2	77.4	75.9
P. chesapeaki				77.9	76.6
P. marinus					92.3

Percent identity of the LSU

	P. andrewsi rRNA-B	*Perkinsus* sp.	*P. marinus*	*P. olseni*
P. andrewsi rRNA-A	99.0	99.3	97.7	97.9
P. andrewsi rRNA-B		99.6	97.9	98.1
Perkinsus sp.			97.9	98.2
P. marinus				98.5

Table 3 continued

Percent identity of the ICS

	P. andrewsi rRNA-B	*Perkinsus* sp.	*P. marinus*	*P. olseni*
P. andrewsi rRNA-A	57.9	57.9	35.9	38.8
P. andrewsi rRNA-B		100.0	34.6	32.4
Perkinsus sp.			34.6	32.4
P. marinus				64.7

similarities to the available sequences of *P. chesapeaki* (isolate G117) (Table 3).

In *Perkinsus* species isolated from *M. arenaria* and *T. plebeius,* four distinct sequence types of the ITS1-5.8S-ITS2 region were reported (Dungan et al. 2002). One of the four reported variant types of ITS2-5.8S-ITS2 sequences (AF440465 and AF440469) of each *Perkinsus* species isolated from *M. arenaria* and *T. plebeius* showed 100% identity with the ITS1-5.8S-ITS2 region of *P. andrewsi* rRNA-A. The *P. andrewsi* unit B ITS1-5.8S-ITS2 sequence showed high sequence identity (99.6%) with the respective *P. chesapeaki* sequence and to two of the four variant sequence types (AF440464, AF440467, AF440468, and AF440471) of each *Perkinsus* species isolated from *M. arenaria* and *T. plebeius*, with 100%, 99.9%, 99.6% and 100% identity, respectively. One variant sequence form of the ITS1-5.8S-ITS2 sequences from the *Perkinsus* species from *T. plebeius* (AF440470) showed high sequence identity with the *P. andrewsi* unit A sequence at the 3' end and high sequence identities with *P. chesapeaki* and the *P. andrewsi* unit B sequence at the 5' end (Figure 6).

Assessment of representation of the rRNA-A and rRNA-B units

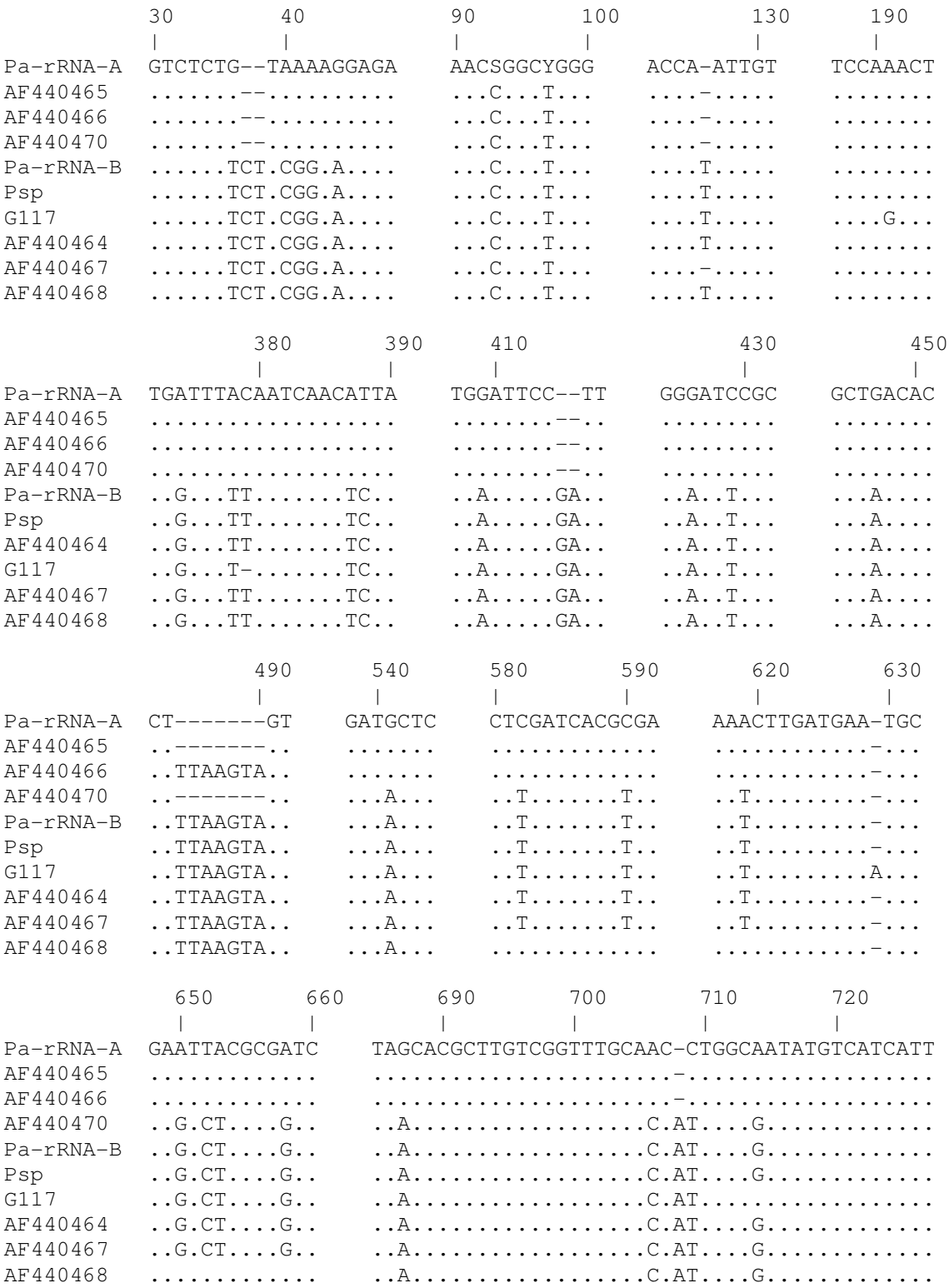
The relative amounts of *P. andrewsi* IGS-A and IGS-B amplicons varied with the amplification conditions selected during the PCR optimization (Figure 5, Figure 7). A semi-quantitative assessment carried out by titration of genomic DNA template showed that the detection limit for IGS-A and IGS-B was between 0.5 pg and 5 pg of genomic DNA (Figure 7). Analysis of the intensities of the amplicons obtained under the optimized PCR conditions showed that the intensity of the IGS-B amplicon was approximately 90% of the intensity of the IGS-A.

Single-cell IGS-specific PCR

The *P. andrewsi* clonal culture characterized in the present study had been subjected to three rounds of subcloning (Coss et al. 2001a). However, to rule out the possibility that rRNA-A and rRNA-B originated in different (non-clonal) *P. andrewsi* cells, PCR amplification of IGS-A and IGS-B was carried out from single *P. andrewsi* cells, using primers specific for IGS-A (Coss et al. 2001) and IGS-B (Pecher et al. 2004, present study). In each single cell, both primer

Figure 6 (next page). Sequence alignment of the ITS1-5.8S-ITS2 regions of *Perkinsus andrewsi* (ATCC 50807), *P. chesapeaki* (isolate G117), a *Perkinsus* species isolated from *Mya arenaria* (ATCC 50864), and a *Perkinsus* species isolated from *Tagelus plebeius* (ATCC 50866) using ClustalW. Only the regions where differences occur are depicted. Pa rRNA-A: rRNA gene unit A of *P. andrewsi* (AF102171); Pa rRNA-B: rRNA gene unit B of *P. andrewsi* (AY305326); G117: *P. chesapeaki* (isolate G117, AF091541); Psp: *Perkinsus* sp. (*M. mercenaria*) (deposited as *Perkinsus* sp. CCA2001; AF252288); AF440464, AF440465, AF440466, AF440467: ITS1-5.8S-ITS2 type a, b, c, and d sequences from the *Perkinsus* species isolated from *M. arenaria*; AF440468, AF440470: ITS1-5.8S-ITS2 type a and c sequences from the *Perkinsus* species isolated from *T. plebeius*. The type b and d sequences of the *T. plebeius* isolate (AF440469 and AF440471) that are identical to the type b and a sequences of the *M. arenaria* isolate (AF440465 and AF440464), respectively, are not depicted.

Figure 6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AF440465 .....--..... ...C...T... ....-..... .....
AF440466 .....--..... ...C...T... ....-..... .....
AF440470 .....--..... ...C...T... ....-..... .....
Pa-rRNA-B .....TCT.CGG.A.... ...C...T... ....T..... .....
Psp .....TCT.CGG.A.... ...C...T... ....T..... .....
G117 .....TCT.CGG.A.... ...C...T... ....T..... ....G...
AF440464 .....TCT.CGG.A.... ...C...T... ....T..... .....
AF440467 .....TCT.CGG.A.... ...C...T... ....-..... .....
AF440468 .....TCT.CGG.A.... ...C...T... ....T..... .....
380 390 410 430 450
Pa-rRNA-A TGATTTACAATCAACATTA TGGATTCC--TT GGGATCCGC GCTGACAC
AF440465 ....--.. ....
AF440466 ....--.. ....
AF440470 ....--.. ....
Pa-rRNA-B ..G...TT.....TC.. ..A.....GA.. ..A..T... ...A....
Psp ..G...TT.....TC.. ..A.....GA.. ..A..T... ...A....
AF440464 ..G...TT.....TC.. ..A.....GA.. ..A..T... ...A....
G117 ..G...T-.....TC.. ..A.....GA.. ..A..T... ...A....
AF440467 ..G...TT.....TC.. ..A.....GA.. ..A..T... ...A....
AF440468 ..G...TT.....TC.. ..A.....GA.. ..A..T... ...A....
490 540 580 590 620 630
Pa-rRNA-A CT-------GT GATGCTC CTCGATCACGCGA AAACTTGATGAA-TGC
AF440465 ..-------.. ....-...
AF440466 ..TTAAGTA.. ....-...
AF440470 ..-------.. ...A... ..T.....T.. ..T.....-...
Pa-rRNA-B ..TTAAGTA.. ...A... ..T.....T.. ..T.....-...
Psp ..TTAAGTA.. ...A... ..T.....T.. ..T.....-...
G117 ..TTAAGTA.. ...A... ..T.....T.. ..T.....A...
AF440464 ..TTAAGTA.. ...A... ..T.....T.. ..T.....-...
AF440467 ..TTAAGTA.. ...A... ..T.....T.. ..T.....-...
AF440468 ..TTAAGTA.. ...A... ....-...
650 660 690 700 710 720
Pa-rRNA-A GAATTACGCGATC TAGCACGCTTGTCGGTTTGCAAC-CTGGCAATATGTCATCATT
AF440465 ....-.....
AF440466 ....-.....
AF440470 ..G.CT....G.. ..A.....C.AT....G.....
Pa-rRNA-B ..G.CT....G.. ..A.....C.AT....G.....
Psp ..G.CT....G.. ..A.....C.AT....G.....
G117 ..G.CT....G.. ..A.....C.AT.....
AF440464 ..G.CT....G.. ..A.....C.AT....G.....
AF440467 ..G.CT....G.. ..A.....C.AT....G.....
AF440468 ....A.....C.AT....G.....

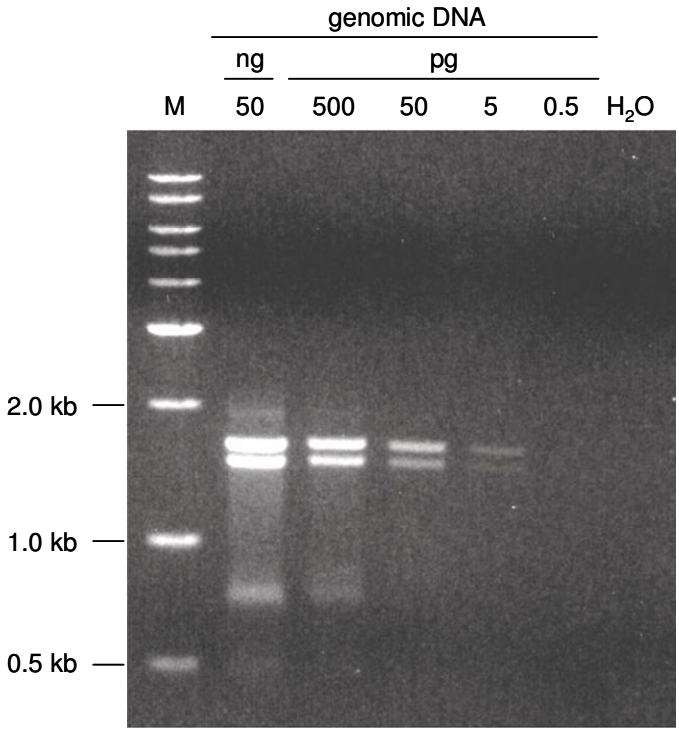


Figure 7. Agarose gel of PCR-amplified products with IGS primers using optimized conditions. Decreasing amounts of *Perkinsus andrewsi* genomic DNA (50 ng to 0.5 pg) were used to assess the relative representation of the two intergenic spacers. H_2O: negative control.

sets yielded amplicons of the expected sizes for IGS-A and IGS-B (Figure 8), confirming that each *P. andrewsi* cell contains both IGS sequences.

Intraclonal variability of IGS in *Perkinsus andrewsi*

A total of 12 clones containing IGS-A sequences and nine clones containing IGS-B sequences obtained from five independent PCR amplifications were sequenced. Within the clones that had the IGS-A sequence, variability occurred at three positions. Three clones had an insertion-deletion at position 573 that was preceded by a stretch of eight A's, whereas the remaining clones had an additional A at this position. Transitions from T to C occurred in three

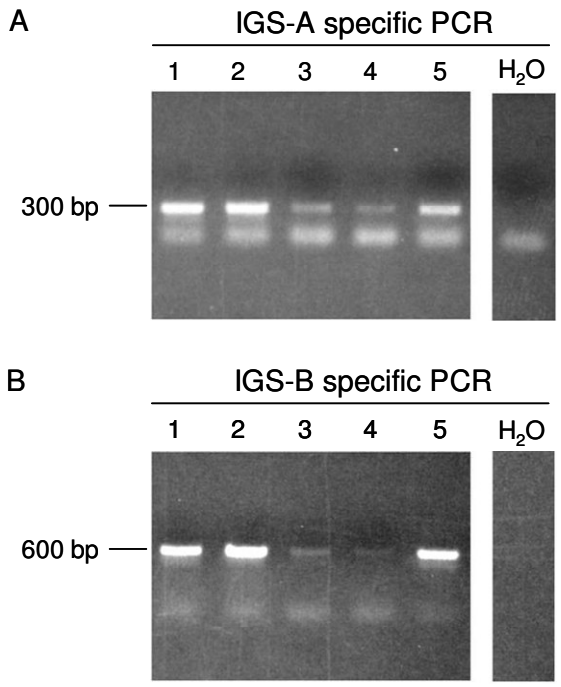


Figure 8. Agarose gels of amplified products of the single cell PCR experiment. **A.** Amplification using IGS-A specific primers. **B.** Amplification using IGS-B specific primers. 1-5: genomic DNA extracted from single cells; H_2O: negative control.

clones at position 1,038, and from C to T in two clones at position 1,047. A consensus IGS-A sequence of the 12 clones was deposited in GenBank™ (AY305328). All 12 clones, however, differed in nine positions (0.7%) from the previously described IGS-A (AF102171). Changes were transitions from A to G (four positions) and T to C (five positions). Sequence alignment of the IGS-A and IGS-B showed that the IGS-A was identical to IGS-B in these positions (data not shown). The IGS-B sequences of all nine clones analyzed were identical.

Southern analysis

To confirm the presence of the additional rRNA gene unit in the *P. andrewsi* genome, *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I restriction digests of *P.*

andrewsi genomic DNA, undigested *P. marinus* genomic DNA, and plasmids containing the IGS-A and IGS-B, respectively, were blotted and hybridized with probes specific for the IGS-A, IGS-B, and a probe designed to detect IGSs of all *Perkinsus* species (Figure 9).

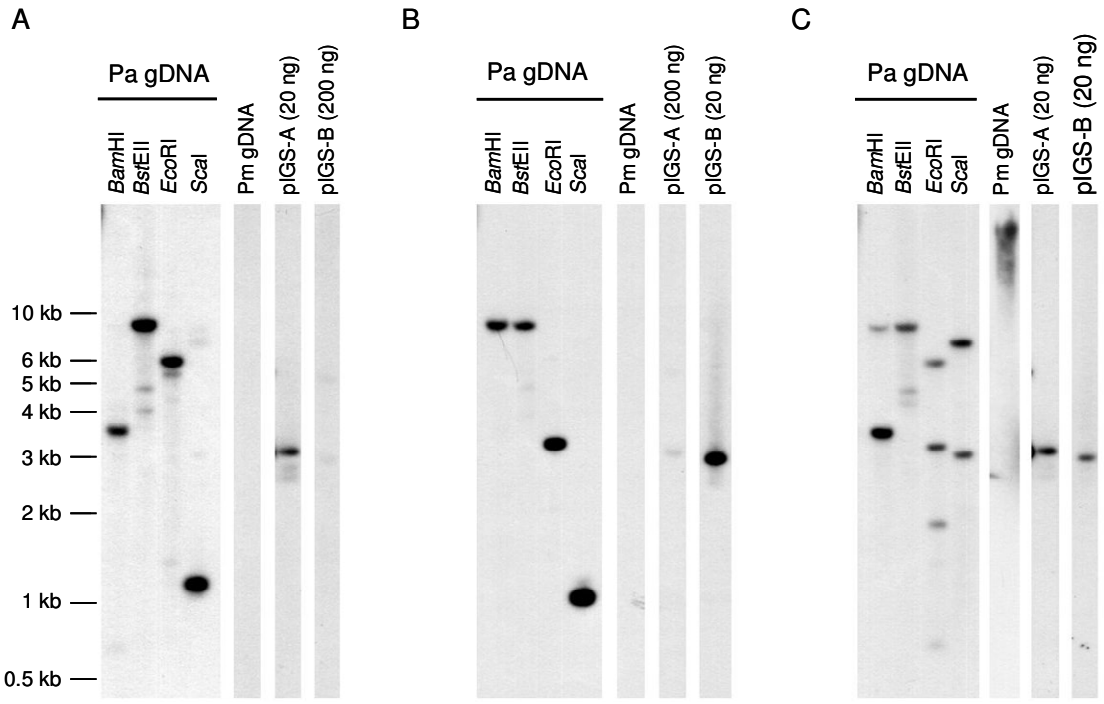


Figure 9. Southern analysis of *Perkinsus andrewsi* genomic DNA. *P. andrewsi* genomic DNA, digested with *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I was size separated on a 0.8% agarose gel, blotted onto a Hybond XL nylon membrane (Amersham Bioscience) and hybridized. **A.** Hybridization with an IGS-A specific probe. **B.** Hybridization with an IGS-B specific probe. **C.** Hybridization with a probe specific to *Perkinsus* species IGSs. Undigested *P. marinus* genomic DNA (4 µg) and 20 or 200 ng of plasmid DNA containing the IGS-A and IGS-B were used as controls. IGS: intergenic spacer; Pa gDNA: *P. andrewsi* genomic DNA; Pm gDNA: *P. marinus* genomic DNA, uncut; pIGS-A: plasmid containing the IGS-A; pIGS-B: plasmid containing the IGS-B.

The IGS-A specific probe hybridized strongly with fragments of about 3.3 kb, 8.0 kb, 5.5 kb, and 1.1 kb in the *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I digested *P. andrewsi* genomic DNA, respectively (Figure 9 A). Additional weak hybridization

was observed with fragments of 4.5 kb and 3.8 kb, as well as with 4.9 kb and 4.0 kb fragments in the *Bst*EII and *Eco*RI digested *P. andrewsi* DNA, respectively (Figure 9 A). The IGS-A-specific probe hybridized very weakly with plasmid containing the IGS-B (200 ng), but did not hybridize with *P. marinus* DNA, (4 µg; no signal was detected after an 18 h exposure at -80 °C) (Figure 9 A).

The IGS-B-specific probe strongly hybridized with fragments of approximately 8.0 kb, 8.0 kb, 3.0 kb, and 0.9 kb in the restriction-digested *P. andrewsi* DNA, but only hybridized very weakly with 200 ng DNA from a plasmid containing the IGS-A and did not hybridize with *P. marinus* DNA (4 µg) (Figure 9 B). These results revealed the specificity of the probes to the IGS-A and IGS-B.

The probe specific for *Perkinsus* species IGSs (probe PER) hybridized with two fragments of approximately 8.0 kb and 3.3 kb in the *Bam*HI digested *P. andrewsi* DNA, with one fragment of 8.0 kb in the *Bst*EII digested *P. andrewsi* DNA, three fragments of approximately 5.5 kb, 3.0 kb, and 1.8 kb in the *Eco*RI digested *P. andrewsi* DNA, and with two fragments of 6.5 kb and 2.9 kb in the *Sca*I digested *P. andrewsi* DNA (Figure 9 C). Probe PER hybridized strongly with both plasmids and somewhat weaker with *P. marinus* genomic DNA. A stronger signal with *P. marinus* genomic DNA was obtained by a longer exposure (18 h) of the autoradiograhic film (Figure 9 C). The banding patterns observed with the three probes were consistent with fragments predicted from the rRNA-A and rRNA-B sequences (Figure 10), with the exception of additional weak hybridizations of probe A and PER with the *Bst*EII and *Eco*RI digests of *P. andrewsi* DNA (Figure 9 A, C).

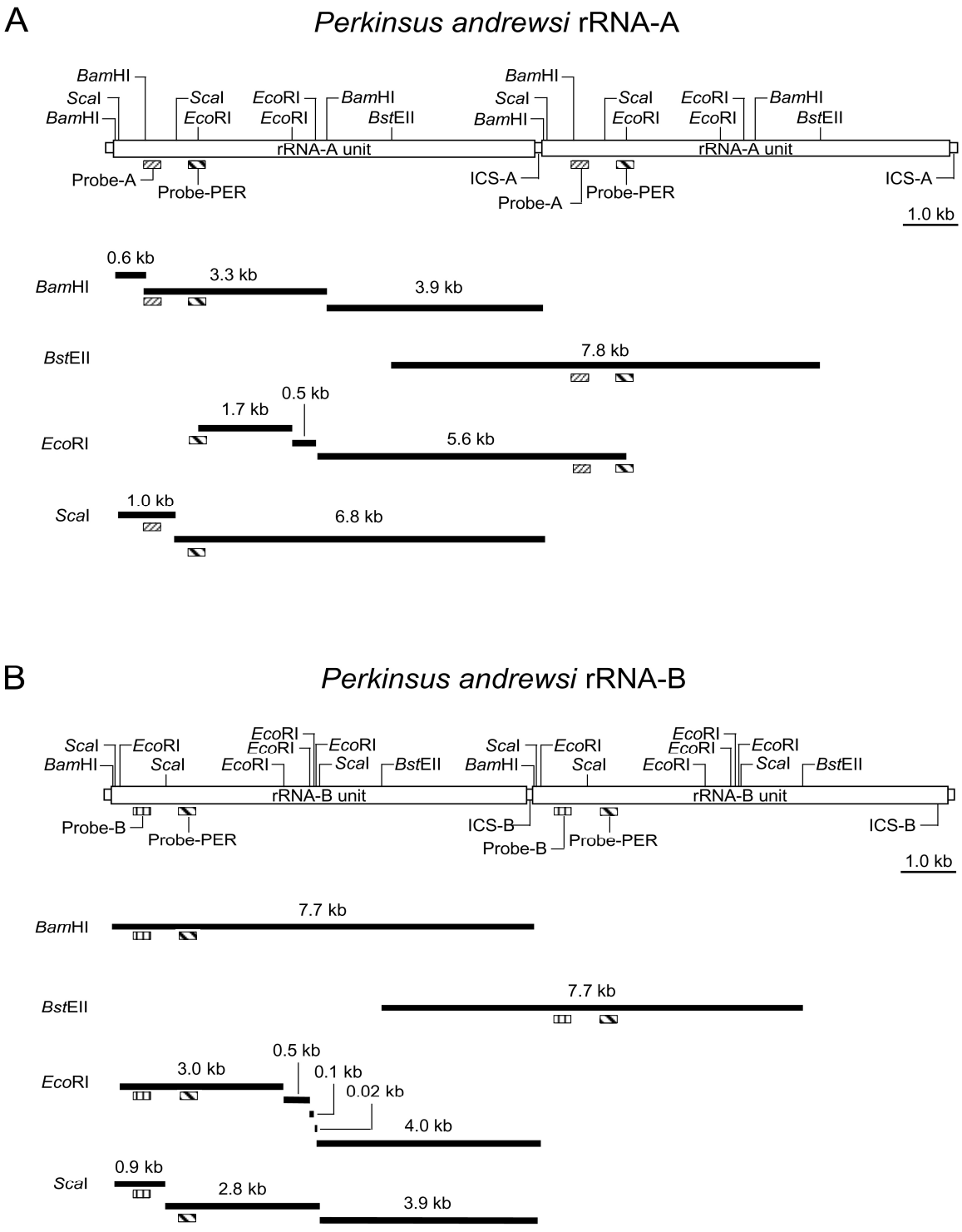
To investigate whether the *Perkinsus* sp. (*M. mercenaria*) genome contained the other rRNA gene unit of the *P. andrewsi* genome (rRNA-A), *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I restriction digests of *Perkinsus* sp. (*M. mercenaria*) and *P. andrewsi* genomic DNA, and plasmids containing the IGS-A and IGS-B, respectively, were blotted and hybridized with probes specific for the IGS-A and IGS-B (Figure 11).

The IGS-A specific probe hybridized strongly with fragments of about 3.3 kb, 8.0 kb, 5.5 kb, and 1.1 kb in the *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I digested *P. andrewsi* genomic DNA, respectively (Figure 11), but no hybridization was observed in the *Perkinsus* sp. (*M. mercenaria*). The IGS-A-specific probe did not hybridize with a plasmid containing the IGS-B (15 ng).

The IGS-B-specific probe strongly hybridized with fragments of approximately 8.0 kb, 8.0 kb, 3.0 kb, and 0.9 kb in the restriction-digested *Perkinsus* sp. (*M. mercenaria*) and *P. andrewsi* DNA. In addition, weaker hybridization with a 4.5 kb fragment was observed in the *Perkinsus* sp. (*M. mercenaria*) DNA digested with *Bst*EII. The IGS-B specific probe did not hybridize with a plasmid containing the IGS-A (15 ng).

Figure 10 (next page). Predicted restriction map of two *Perkinsus andrewsi* rRNA gene units digested with *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I and the fragments created by these endonucleases. A. *P. andrewsi* rRNA-A. B. *P. andrewsi* rRNA-B. The hybridization sites of probe A (▨), probe B (▥), and probe PER (◪) are indicated. ISC-A: intercluster spacer of the rRNA-A gene unit; ICS-B: intercluster spacer of the rRNA-A gene unit.

Figure 10


A
Perkinsus andrewsi rRNA-A
BamHI
ScaI
EcoRI
BstEII
rRNA-A unit
Probe-A
Probe-PER
ICS-A
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0.6 kb
3.3 kb
3.9 kb
7.8 kb
0.5 kb
1.7 kb
5.6 kb
1.0 kb
6.8 kb
B
Perkinsus andrewsi rRNA-B
rRNA-B unit
Probe-B
Probe-PER
ICS-B
1.0 kb
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0.5 kb
3.0 kb
0.1 kb
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4.0 kb
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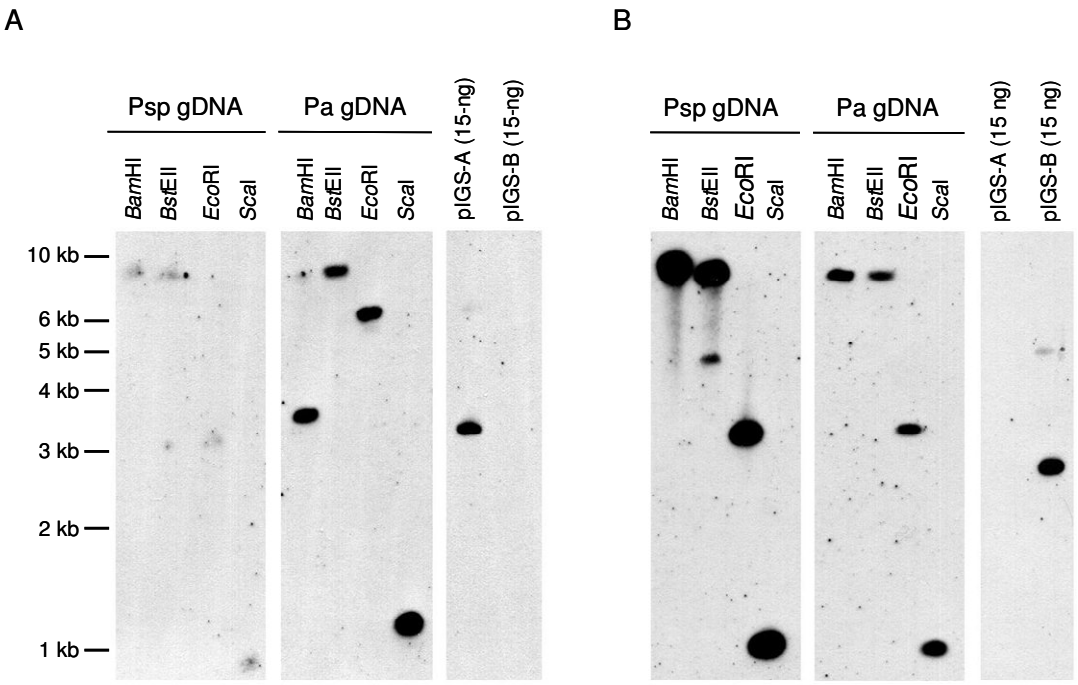


Figure 11. Southern analysis of *Perkinsus* sp. (*M. mercenaria*) and *P. andrewsi* genomic DNA. *Perkinsus* sp. (*M. mercenaria*) and *P. andrewsi* genomic DNA, digested with *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I were size separated on a 0.8% agarose gel, blotted onto a Hybond XL nylon membrane (Amersham Bioscience) and hybridized. A. Hybridization with an IGS-A specific probe. B. Hybridization with an IGS-B specific probe. 15 ng plasmid DNA containing the IGS-A and IGS-B were used as controls. IGS: intergenic spacer; Pa gDNA: *P. andrewsi* genomic DNA; Psp gDNA: *Perkinsus* sp. (*M. mercenaria*) genomic DNA; pIGS-A: plasmid containing the IGS-A; pIGS-B: plasmid containing the IGS-B.

DISCUSSION

Further characterization of the rRNA gene loci of *Perkinsus andrewsi* and *Perkinsus* sp. (*M. mercenaria*)

PCR amplification of *P. andrewsi* genomic DNA using primers that anneal to conserved regions of the 5S and the SSU resulted in two distinct amplicons representing IGS sequences (IGS-A and IGS-B). These were confirmed by the presence of the 5S rRNA and the SSU gene flanking sequences. The sequence

of the IGS-A was identical to the previously described *P. andrewsi* IGS (Coss et al. 2001b), except in nine positions. Because the IGS-A sequence described in the present study represents a consensus sequence of 12 clones obtained from five independent PCR reactions, these differences most likely represent intraclonal IGS sequence variability (see below). The IGS-B, however, differed considerably from the IGS-A.

Using primers specific to the IGS-B sequence and IGS-A specific primers from Coss et al. (2001b), the presence of both IGS sequences in a single *P. andrewsi* cell was confirmed. Single cells were isolated by the "limiting dilution" method, routinely used for sub-cloning of *Perkinsus* cultures. Although for the single cell PCR assay, serial dilutions were carried out in distilled water to facilitate concentration of the DNA at the following step, I have experimentally demonstrated by 4',6'-diamidino-2-phenylindole (DAPI) staining that during the time taken to complete the procedure, the nucleus and nuclear DNA are retained (data not shown). Therefore, since the IGS-A and -B specific primers target sequences in the non-transcribed spacer region of the IGS, potential cross contamination is highly unlikely. Variability of amplicon intensities may have been due to differences in DNA yields by the crude DNA extraction procedure used. Alternatively, the different cells could be in different stages of the cell cycle and thus might contain more than one genome equivalent.

No intraclonal sequence variability within the IGS-B was observed and only low sequence variability within the IGS-A, strongly suggesting that *P. andrewsi* possesses two distinct IGS sequences.

There are at least two possible organizations of the two IGS sequences within the *P. andrewsi* rRNA gene cluster(s). In one model, both IGS-A and IGS-B could be randomly dispersed and associated with identical rRNA genes, resulting in rRNA gene units that only differ in their IGSs. Alternatively, *P. andrewsi* could possess two distinct rRNA gene units, containing either IGS-A or IGS-B associated with different rRNA genes and intergenic regions. To address this question, amplicons obtained by PCR using an IGS-B-specific primer and conserved primers located in the 3' end of the SSU or in the 5' end of the LSU were cloned and sequenced. In all clones, the IGS-B sequences were associated with rRNA genes and intergenic regions. The rRNA genes and intergenic regions associated with the IGS-B differed in their sequences from the rRNA genes and intergenic regions associated with the IGS-A. Results from Southern analysis also corroborated the presence of two distinct rRNA units in the *P. andrewsi* genome. Hybridization patterns of restriction digests of *P. andrewsi* genomic DNA were consistent with restriction length fragment polymorphisms predicted on the basis of two distinct rRNA gene units being present in a single cell. Weak hybridizations observed in some digests tested with the IGS-A-specific probe, and with the probe specific to *Perkinsus* species IGS, could represent intraclonal variability either in the rRNA-A gene unit, especially in the IGS-A, or alternatively, in the 5' or 3' end sequences of the rRNA gene clusters. Lack of sufficient sequence information upstream or downstream of the rRNA gene clusters prevents me from confirmation of either alternative.

Taken together, these observations provide strong evidence that *P. andrewsi* contains two distinct rRNA gene units, which were designated as rRNA-A and rRNA-B. Further, Southern analysis and PCR suggest that each rRNA gene unit is arranged as a "head to tail" tandem repeat. All three probes hybridized with only one fragment in the genomic DNA samples that were digested with *Bst*EII, an endonuclease that cuts once in each rRNA gene unit (based on restriction maps on the complete rRNA sequences). The observed fragments are slightly smaller than 8.0 kb, which corresponds approximately to the length of each rRNA gene unit (7.8 kb for the rRNA-A gene unit, and 7.7 kb for the rRNA-B gene unit).

The presence of multiple distinct rRNA gene units has been described in vertebrates (Cortadas and Pavon 1982), in the kinetoplastid flagellate *Trypanosoma cruzi* (Souto and Zingales 1993), the dinoflagellate *Alexandrium* (Scholin et al. 1993, Scholin et al. 1994, Yeung et al. 1996), and the apicomplexans *Theileria* (Bishop et al. 2000), *Babesia* (Dalrymple et al. 1996, Reddy et al. 1991), *Cryptosporidium* (Le Blancq et al. 1997), and *Plasmodium* (reviewed by McCutchan et al. 1995). In *Plasmodium* species, there are four rRNA gene units (A, B, C, and D). The exception is *Pl. falciparum* that carries seven rRNA gene units (Carlton et al. 2002, Dame and McCutchan 1984, Gardner et al. 2002, Waters 1994, Waters et al. 1997, Wellems et al. 1987, Qari et al. 1994).

The gene units transcribe at least two distinct ribosomal types (A and S) that are stage-specifically expressed (Dame and McCutchan 1984, Waters 1994,

Waters et al. 1997, Wellems et al. 1987). Sequence comparison showed that high percent identities are found between rRNA gene units that transcribe for the same rRNA types (McCutchan et al. 1995). There are lower percent identities between rRNA gene units that transcribe for the different ribosomal types. In *Pl. berghei* for example, sequence analysis of the SSU-A and SSU-C showed that the two genes are 96.5% identical (Gunderson et al. 1986, Gunderson et al. 1987).

Based on the structural differences postulated for the different life stage-specific expressed ribosome types in *Plasmodium* species, it has been proposed that these ribosome types are also functionally different (Gunderson et al. 1987, Rogers et al. 1996, Thompson et al. 1999), and that the parasite relies on functionally distinct ribosomes to complete its life cycle in the mammalian and insect hosts (Gunderson et al. 1987). However, a recent study showed that in *Pl. berghei*, the rRNA genes encoded by the respective gene units do not differ in the sequence of important core regions and lack the structurally differences observed in other *Plasmodium* species. Furthermore, both ribosome types, A and S, are expressed in the life stages in the insect vector van Spaendonk et al. (2001). The observations in *Pl. berghei* question the presence of functional different ribosomal types and their proposed requirement for completion of the life cycle. Instead, based on the atypical organization of the rRNA gene, van Spaendonk et al. (2001) proposed that each unit is controlled by different promoters, an arrangement that would enable the parasite to adjust the production of ribosomes depending on the need.

In *P. andrewsi,* genes of the rRNA-A and -B units show a high degree of identity: SSU-A and -B show 99.8% identity, LSU-A and -B 99.0% identity, the 5S-A and -B 99.2% identity, and the 5.8S 100.0% identity, suggesting that the two units do not transcribe structurally and functionally distinct ribosomal types. However, the IGSs of rRNA-A and -B units show only 71.3% identity. The IGS carries all elements required for transcription and termination of the RNA genes, and is critical for the regulation of the rDNA metabolism (De Lucchini et al. 1997, Reeder 1990, Sollner-Webb and Mougey 1991). In contrast to *P. olseni* and *P. marinus*, which possess a single rRNA gene unit type (Robledo et al. 2002; Robledo and Vasta, unpublished), *P. andrewsi* has two distinct rRNA gene units. Whether these units are selectively regulated, as proposed for *Pl. berghei* (van Spaendonk et al. 2001), remains to be demonstrated.

The rRNA gene unit of *Perkinsus* sp. (*M. mercenaria*) shares high sequence identity with the rRNA-B gene unit of the *P. andrewsi* hapantotype, which explains the cross-reactivity of *P. andrewsi* with the PCR assay originally designed for *Perkinsus* sp. (*M. mercenaria*). Southern analysis on genomic DNA of *Perkinsus* sp. (*M. mercenaria*) suggests that this *Perkinsus* isolate lacks the rRNA-A gene unit of *P. andrewsi*. Similar observations have been reported for *T. cruzi*, where isolates have been identified that possess either two distinct rRNA gene units (rRNA unit 1 and 2), or one of the two rRNA gene units (Souto et al. 1996, Stolf et al. 2003, Zingales et al. 1999). Thus, like in *T. cruzi*, *Perkinsus* sp. (*M. mercenaria*) may be a *P. andrewsi* strain with only one of the two rRNA gene units.

Known life stages of *Perkinsus* species include trophozoites, tomonts, prezoosporangia, zoosprangia, and motile biflagellated zoospores. Within the host, trophozoites develop into tomonts. Tomonts undergo palintonomy and, through successive karyokineses and cytokineses, generate up to 64 immature torphozoites that are released by rupture of the tomont cell wall (Perkins 1996). Once released into the water column, trophozoites enlarge and become prezoosporangia that develop into zoosporangia. Within zoosporangia numerous zoospores are formed that are released through a discharge tube (Perkins 1996) (Figure 1). In *Perkinsus* species, sexual stages have not been identified, but their presence cannot be ruled out. Interestingly, prezoosporangia have been observed that developed outgrowths. These outgrowths appeared to fuse, however, whether these stages truly fused and cytoplasmic mixing occurred has not been determined (Perkins 1996). Nonetheless, these events are reminiscent of the formation of dikaryons in fungi and would suggest that *Perkinsus* species sexually reproduce. If this suggestion proves correct, the presence of two distinct rRNA genes could be attributed genetic recombination and the presence of different alleles. Although the ploidy status of *Perkinsus* species has not yet been resolved, it has been suggested that *P. marinus* trophozoites are diploid (Reece et al. 1997, Reece et al. 2001). Assuming that this assertion is correct, and that it can be extended to *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*), then the *P. andrewsi* clonal culture would be heterozygous for the rRNA units and *Perkinsus* sp. (*M. mercenaria*) homozygous.

Alternatively, both rRNA unit types in *P. andrewsi* could be contiguously located on the same chromosome or on different chromosomes. Evidence suggests that *Perkinsus* species have multiple copies of rRNA gene units (de la Herrán et al. 2000). Like other repetitive sequences, rRNA gene units are homogenized by processes such as unequal crossing over, gene conversion, and transposition, regardless of their chromosomal distribution (Dover 1982,Ohta and Dover 1984). Homogenization results in multiple copies of the rRNA gene units of similar or identical sequences. Nevertheless, it is possible that the additional rRNA gene unit in *P. andrewsi* was a product of partial homogenization of the rRNA gene unit. Partial homogenization or homogenization that does not equally affect the entire length of rRNA genes and intergenic spacers have been proposed to have caused sequence polymorphisms between the rRNA gene operons within *E. coli* strains (Cilia et al. 1996, Martinéz-Murcia et al. 1999). However, until further investigations are conducted to address the linkage of the two rRNA units in *P. andrewsi*, their localization and organization within the rRNA array(s), and which biological processes may have led to two distinct rRNA gene units remain open questions.

In *T. cruzi*, the presence of different rRNA gene units correlates with other traits that led to divide *T. cruzi* strains into group I and II. Whether *Perkinsus* sp. (*M. mercenaria*) differs from *P. andrewsi* not only in the number of rRNA gene units but also in other traits such as its carbohydrate surface composition is currently being investigated (Saito and Vasta, unpublished data).

Implication of multiple distinct rRNA gene units on taxonomic analysis and species designations of *Perkinsus* species

The presence of multiple distinct rRNA gene units in a single *Perkinsus* species adds new complexity to the use of rRNA sequences for taxonomic analysis and species designations based on molecular characters. It also underscores the need of rigorous characterization of the complete rRNA gene locus sequences to determine whether polymorphisms observed in intergenic regions represent inter- or intraclonal variability or, as in the case for *P. andrewsi*, represent distinct rRNA gene units. The present study shows low sequence variability within the IGS of each rRNA gene unit of *P. andrewsi*. Only 0.7% variability was observed in the IGS-A sequence, and none for the IGS-B. In *P. marinus*, two distinct sequence types (type I and II) differing in only seven nucleotide positions out of 307 bp within the non-transcribed spacer of the IGS were identified (Robledo et al. 1999), suggesting that in *P. marinus* the IGS also has a low degree of sequence variability.

In a recent study, (Dungan et al. 2002) reported intraclonal variability of ITS sequences in clonal cultures of *Perkinsus* species isolated from *M. arenaria* and *T. plebeius*. Four variant ITS1-5.8S-ITS2 types ("a" to "d") were described. Sequence comparison of the eight sequences with *P. andrewsi* A and B unit ITS-5.8S-ITS2 sequences show that the type "a" of the *M. arenaria* isolate and type "d" of the *T. plebeius* isolate are identical to the ITS1-5.8S-ITS2-B sequences of *P. andrewsi*. Similarly, the type "b" of both *Perkinsus* isolates are identical to the rRNA-A ITS1-5.8S-ITS2 sequence of *P. andrewsi*. The remaining two types of

each isolate appear to be intermediate, sharing sequences with the *P. andrewsi* A and B unit, respectively. Dungan et al. (2002) obtained the ITS1-5.8S-ITS2 sequences by PCR amplification using conserved primers, which has the potential to produce sequence artifacts by *in vitro* recombination, and thus, may mislead genetic analysis (Judo et al. 1998, Meyerhans et al. 1990, Tanabe et al. 2002). Tanabe et al. (2002) analyzed PCR amplifications of a low copy gene (*Msp1*) from different *Pl. falciparum* strains, and observed up to 11.5% *in vitro* recombination when conserved primers were used. *In vitro* recombination sequence artifacts can be avoided by either sequencing the genes of interest directly from digested genomic DNA, or by the use of at least one allele-specific primer (Tanabe et al. 2002). In the present study, the second approach was chosen and either rRNA-A- or rRNA-B-specific primers were used in combination with a conserved primer to amplify partial rRNA sequences that contain the ITS1-5.8S-ITS2 region. No *in vitro* recombination was observed with this system, but if primers based on conserved regions were used to co-amplify partial A and B type rRNA sequences, sequence “chimeras” were identified (data not shown).

Sequence comparison of the two rRNA units of *P. andrewsi*, the rRNA gene unit of *Perkinsus* sp. (*M. mercenaria*) and the available sequences that are attributed to *P. chesapeaki* (the sequences were first deposited as *Perkinsus* sp. G117), revealed that the SSU-B gene and the ITS1-5.8-ITS2-B sequence of *P. andrewsi* and the respective regions of *Perkinsus* sp. (*M. mercenaria*) have high percent identity to the respective sequences of *P. chesapeaki* strain G117. These observations suggest that strain G117, *P. andrewsi* and *Perkinsus* sp. (*M.*

mercenaria) are closely related. Based on the sequence information available the findings presented here suggest that the strain G117 is closely related to *Perkinsus* sp. (*M. mercenaria*). However, only the SSU and ITS1-5.8S-ITS2 region have been described (Kotob et al. 1999a). The IGS that is more informative on the species and strain level has not been described. Unfortunately, the clonal culture of the strain G117 has never been deposited in a recognized depository and apparently has been lost (Burreson et al. 2005). Therefore, further molecular characterization cannot be pursued to clarify the relationship of the G117-*Perkinsus* isolate to *P. andrewsi* or *Perkinsus* sp. (*M. mercenaria*). For the *Perkinsus* isolates from *M. arenaria* and *T. plebeius*, only partial rRNA sequences have been characterized. Characterization of the complete rRNA gene loci from these isolates would reveal whether the observed sequence polymorphisms represent either the presence of distinct rRNA gene units or variability within a single unit. Furthermore, it would confirm the presence of the intermediate forms described, and contribute to resolving the relationship between *P. andrewsi, P. chesapeaki*, *Perkinsus* sp. (*M. mercenaria*), and the *Perkinsus* isolates from *M. arenaria* and *T. plebeius*.

Recently, Burreson et al. (2005) reported the characterization of a *Perkinsus* isolate from *M. arenaria* from which a clonal culture has been established. This isolate has been designated to be the “neotype” of *P. chesapeaki* based on its host, and sampling location in Chesapeake Bay (Burreson et al. 2005). In addition to morphological data, Burreson et al. (2005) compare the ITS1-5.8S-ITS2 region of the rRNA gene locus submitted for the

Perkinsus isolate G117 (later designated as *P. chesapeaki* ; AF091541) with the respective region of the rRNA-B gene unit of *P. andrewsi* (AY305326), *Perkinsus* sp. (*M. mercenaria*) (deposited as *Perkinsus* sp. CCA2001; AF252288), *Perkinsus* sp. ATCC 50864 (AF440464–AF440467), *Perkinsus* sp. ATCC 50866 (AF440468–AF440471), and the new *Perkinsus* isolate. Interestingly, sequence information available on the rRNA-A gene unit of *P. andrewsi* has not been included in their analysis.

Analysis of actin and partial LSU sequences from several *Perkinsus* species of which clonal cultures are available, support the close relationship of *P. andrewsi* to the new *Perkinsus* isolate (Burreson et al. 2005). Based on these findings, Burreson et al. (2005) suggest to synonymize *P. andrewsi* and *P. chesapeaki* with *P. andrewsi* being the junior synonym.

However, I consider *P. andrewsi* as a valid and distinct *Perkinsus* species because of the following reason. The species designation of *P. chesapeaki* was based on morphology of the trophozoite and zoospore stage (McLaughlin et al. 2000) and was supported by rRNA sequence data obtained from a clonal *Perkinsus* species culture derived from a different *Perkinsus* species isolate from *M. arenaria* (Kotob et al. 1999a, Kotob et al. 1999b, McLaughlin et al. 2000). In contrast, the species designation of *P. andrewsi* is based on rRNA gene and intergenic sequences obtained from a clonal *Perkinsus* species culture (*P. andrewsi*, PAND-A8-4a, ATCC 50807) that differed from other *Perkinsus* species (including the putative *P. chesapeaki* culture, G117) (Table 3 in the present study; Coss et al. 2001b). Morphological characteristics of this isolate were

considered insufficient to support species designation (Coss et al. 2001a, Coss et al. 2001b). Unfortunately, the only reference material available that possibly represents *P. chesapeaki* is a slide containing histological sections of gill tissue of infected *M. arenaria* that does not contain all life stages of the species described. Neither the clonal culture of *Perkinsus* species G117 nor any material of the non-clonal primary culture on which the *P. chesapeaki* species designation is based have been fixed and deposited in a recognized public depository. Thus, it is difficult to address the relationship of *P. chesapeaki* to *P. andrewsi*, *Perkinsus* sp. (*M. mercenaria*) and other *Perkinsus* isolates, including isolates from *M. arenaria*. Despite that, all presumptive *P. chesapeaki* isolates and cultures, including the culture that was used to support the synonymizing of *P. andrewsi* and *P. chesapeaki* were designated as such solely based on their isolation from *M. arenaria*.

Regardless, Burreson et al. (2005) corroborated the findings presented in the present study that suggest that the G117 isolate, the *Perkinsus* isolates from *M. arenaria*, and *T. plebeius*, *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) are closely related isolates (Coss 2000, Pecher et al. 2004, present study).

A preliminary search in *P. marinus* and *P. olseni* by PCR, the two other *Perkinsus* species from which the complete rRNA locus has been characterized, failed to reveal the presence of additional rRNA gene units (Robledo et al. 2002; Robledo and Vasta, unpublished). In this regard, the *P. andrewsi* hapantotype appears to be unique among the *Perkinsus* species characterized at present time.

CHAPTER 3

ASSESSMENT OF THE NORTHERN DISTRIBUTION OF SELECTED *PERKINSUS* SPECIES IN EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*) AND HARD CLAMS (*MERCENARIA MERCENARIA*) USING PCR-BASED DETECTION ASSAYS

SUMMARY

Perkinsus species are protistan parasites of mollusks. In Chesapeake Bay, *P. marinus*, *P. chesapeaki*, and *P. andrewsi* are sympatric, infecting oysters and clams. *P. marinus* was found in a variety of bivalves such as *C. virginica Macoma balthica, M. mitcheli, M. mercenaria*, *Geukensia demissa*, *Ischadium recurvum*, and *Mya arenaria*. *P. andrewsi* infections were observed not only in *M. balthica* but also in *C. virginica*, *M. mitcheli*, and *M. mercenaria*, suggesting a broad host range for these two *Perkinsus* species. However, *in vitro* studies on the proliferation rate of *P. marinus* revealed that plasma of the clams *M. mercenaria* and *Anadara ovalis*, and of the mussel *Mytilus edulis* reduced the proliferation rate of *P. marinus* compared to plasma of *C. virginica*, suggesting a possible host preference of *P. marinus* for its type host, *C. virginica*. Although *P. marinus* is a well characterized pathogen for the eastern oyster, *C. virginica*, it

remains unknown whether *P. andrewsi* and *P. chesapeaki* are equally pathogenic. Infections with *Perkinsus* species were reported in *C. virginica* as far north as Maine, sometimes with high prevalence and high infection densities, but low mortalities. Thus, it was hypothesized that, in addition to *P. marinus*, *Perkinsus* species and/or strains with little or no pathogenicity for *C. virginica* may be present. Accordingly, I investigated the distribution of the genus *Perkinsus*, and the individual species *P. marinus*, *P. andrewsi*, and a *Perkinsus* species isolated from *M. mercenaria* [referred to as *Perkinsus* sp. (*M. mercenaria*)] in *C. virginica* and the hard clam, *M. mercenaria* by applying available PCR-based assays. The distribution of *P. chesapeaki* was not assessed since a specific diagnostic assay was not available. *C. virginica* and *M. mercenaria* specimens were collected monthly from June to September 2002 from eight sites from Maine to Virginia. Eighteen to 60 specimens from each sampling site and collection date were dissected. From each individual, gut, gill, and mantle tissue was collected and pooled, and DNA was extracted using a commercially available kit. DNA samples of *M. mercenaria* possessed potent PCR inhibitory activity, which was overcome by the addition of 1mg/ml BSA and 5% (v/v) dimethyl sulfoxide (DMSO) to the PCR reaction mixture. Infections with *P. marinus, P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) were observed in *C. virginica* and *M. mercenaria* populations as far north as Maine. The prevalence of *P. marinus* in *C. virginica* (58.4%) was significantly higher than in *M. mercenaria* (4.4%; Fisher's exact test, p-value < 0.001). In contrast, the prevalence of *Perkinsus* sp. (*M. mercenaria*) was lower in *C. virginica* (2.2%)

than in *M. mercenaria* (7.6%; Fisher's exact test, p-value < 0.001). The prevalence of *P. andrewsi* in *C. virginica* (1.3%) and in *M. mercenaria* did not differ. These findings suggest that *M. mercenaria* is not an optimal host for *P. marinus*.

INTRODUCTION

Perkinsus species (Perkinsozoa, Alveolata) are the causative agent of perkinsosis in a variety of mollusk species. For some host species, such as the eastern oyster *C. virginica*, *Perkinsus* species infections caused widespread mortalities in both natural and farmed populations, resulting in severe economic loss for the shellfishery, and detrimental effects on the environment (Andrews 1988, Ford 1996, Villalba et al. 2004). Currently, three *Perkinsus* species are recognized along the eastern USA coast: *P. marinus,* isolated from the eastern oyster, *C. virginica* (Mackin et al. 1950), *P. chesapeaki* from the soft shell clam, *M. arenaria* (McLaughlin et al. 2000), and *P. andrewsi* from the Baltic clam, *M. balthica* (Coss et al. 2001a). In addition, various *Perkinsus* isolates have been reported, including an isolate from the hard clam *M. mercenaria* [*Perkinsus* sp. (*M. mercenaria*)] that appears to be closely related to *P. andrewsi* (Andrews 1955, Coss 2000, Perkins 1988, Chapter 2).

The standard diagnostic method for *Perkinsus* infections has been the fluid thioglycollate medium (FTM) assay (Ray 1952, 1966) that is considered to be more sensitive than histological diagnosis (McLaughlin and Faisal 1999). However, neither diagnostic method is able to discriminate among *Perkinsus*

species (reviewed in Villalba et al. 2004). Additional diagnostic assays based on anti-*Perkinsus* antibodies (Choi et al. 1991, Dungan and Robertson 1993, Montes et al. 2002, Ottinger et al. 2001) have not been rigorously validated, and may exhibit cross-reactivity with dinoflagellates (Bushek et al. 2002, Dungan and Robertson 1993, Villalba et al. 2004).

The development of culture methods for *Perkinsus* species (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, La Peyre et al. 1993) greatly facilitated the development of specific PCR-based diagnostic assays. The first PCR-based assay, developed for *P. marinus*, was species-specific and more sensitive than the FTM assay (Marsh et al. 1995, Robledo et al. 1998). Subsequently, PCR-based assays specific for *P. olseni* (de la Herrán et al. 2000, Robledo et al. 2000), *P. andrewsi* (Coss et al. 2001b) and for the genus *Perkinsus* (Robledo et al. 2002) were developed. Quantitative PCR assays for *P. marinus* (Gauthier et al. 2006, Yarnall et al. 2000), a multiplex PCR assay detecting *P. marinus* and *Haplosporidium* species (Penna et al. 2001), and modified PCR-based assays have been developed that can distinguish between *P. marinus*, *P. olseni*, *P. mediterraneus,* and *P. andrewsi*/*P. chesapeaki* or *P. marinus* and *P. olseni* (Abollo et al. 2006, Elandalloussi et al. 2004).

Prior to 2000, all surveys for *Perkinsus* species were conducted using histology or FTM-based assays, and all *Perkinsus* infections observed in *C. virginica* were attributed to *P. marinus*, the only *Perkinsus* species described in the Americas at that time. By 2001, two new species, *P. andrewsi* and *P. chesapeaki*, were described from clams (*M. balthica* and *M. arenaria*,

respectively) in Chesapeake Bay, USA (Coss 2000, Coss et al. 2001b, McLaughlin et al. 2000). However, in addition to its type host, *P. andrewsi* was also found in *C. virginica* (the type host of *P. marinus*) and in the clams *M. mercenaria*, and *M. mitchelli* (Coss 2000, Coss et al. 2001b). Conversely, *P. marinus* was detected in *M. arenaria*, *M. balthica*, and *M. mitchelli* (Coss 2000, Coss et al. 2001b, Kotob et al. 1999a, McLaughlin et al. 2000), suggesting a broad host range of these *Perkinsus* species.

Infections by *Perkinsus* species have been observed in oysters from Tabasco, Mexico, to Maine, USA (reviewed in Burreson and Ragone Calvo 1996, Ford 1996, Soniat 1996). In some areas of the northeastern USA, mortalities in oyster populations were low to moderate, despite high prevalence and infection densities of *Perkinsus* species (Ford 1996, Karolus et al. 2000). This observation led to the hypothesis that, in addition to *P. marinus*, other *Perkinsus* species are present in the Northeastern regions that may be less virulent towards *C. virginica*. I therefore surveyed *C. virginica* and *M. mercenaria* obtained from selected sites from Maine to Virginia for the presence of *Perkinsus* species, *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) using specific PCR-based assays.

MATERIALS AND METHODS

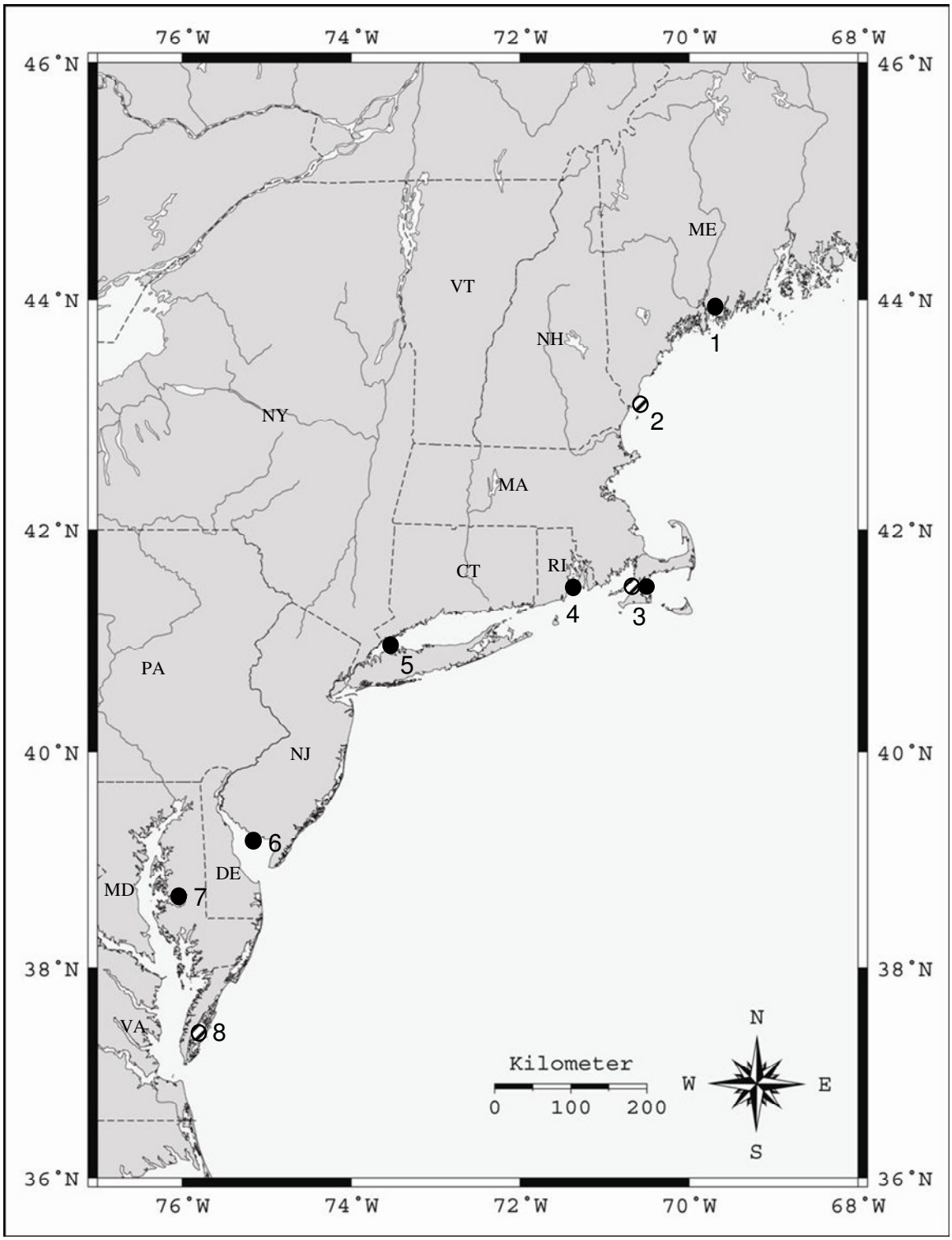
Collection of tissue specimens and DNA extraction

C. virginica and *M. mercenaria* specimens, collected monthly from June to September 2002, were obtained from shellfish farmers and academic institutions from eight different sites along the Atlantic coast of the USA as follows: *C. virginica* were obtained from six sites - Walpole, ME, Martha's Vineyard, MA, Narragansett Bay, RI, Oyster Bay, NY, Delaware Bay, NJ, and Sandy Point, MD; *M. mercenaria* were obtained from three sites - Eliot, ME, Martha's Vineyard, MA, and Cheriton, VA (Figure 12). Upon arrival the shellfish were stored at 4 °C until further processing.

Eighteen to 60 specimens from each sampling site and collection date were individually dissected. From each individual, gut, gill, and mantle tissue was collected and pooled (50-100 mg wet weight of total tissue/pool) and DNA was extracted using a commercially available kit (DNeasy, 96 well-format, QIAGEN, Valencia, CA, USA). The remaining soft bodies were stored at -20 °C. DNA concentration and purity was estimated by spectrophotometry at wavelengths of 260 nm and 280 nm. The DNA samples were stored at -20 °C until tested.

Figure 12 (next page). Sample site locations for *Crassostrea virginica* (●) and *Mercenaria mercenaria* (⊘). Specimens were collected each month from June to September 2002. 1: Walpole, ME; 2: Eliot, ME; 3: Martha's Vineyard, MA; 4: Narragansett Bay, RI; 5: Oyster Bay, NY; 6: Delaware Bay, NJ; 7: Sandy Point, MD; 8: Cheriton, VA. The map was generated with the Generic Mapping Tools, v.4.1.4 (Smith and Wessel 1990, Wessel and Smith 1998).

Figure 12


76°W
74°W
72°W
70°W
68°W
46°N
44°N
42°N
40°N
38°N
36°N
ME
VT
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NY
MA
CT
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1
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PCR assays

PCR-based assays specific for the genus *Perkinsus*, *P. marinus*, *P. andrewsi*, and *P. olseni* were used according to Coss et al. (2001b), Marsh et al. (1995), and Robledo et al. (2002).

Development of a PCR-based diagnostic assay specific for the *Perkinsus* species isolated from *Mercenaria mercenaria*

Primers designated M6L (sense 5' - GCG GCG CAA ATT CAT CAC TTG AG - 3') and M5 (antisense 5' - AAC CAT CCC GAC TAC CAT CTG G - 3') were designed based on the intergenic spacer of the rRNA gene cluster of *Perkinsus* sp. (*M. mercenaria*) using Oligo Calculator v3.07 (available at http://www.basic.northwestern.edu/biotools/oligocalc.html). Thermocycler conditions were 94 ℃ for 4 min, 35 cycles of 94 ℃ for 1 min, 65 ℃ for 30 sec with an extension of 1 sec per cycle, 72 ℃ for 1 min, with a final extension at 72 ℃ for 7 min.

PCR reaction mixtures

Three different PCR reaction mixtures (A to C) were used. PCR reaction mixture A consisted of 1x QIAGEN PCR Master mix (contains *Taq* DNA Polymerase (250 mU/µl), KCl, Tris-Cl, $(NH_4)_2SO_4$, 1.5 mM MgCl, and 200µM of each dNTP) (QIAGEN), and 40nM of each primer. To obtain PCR reaction mixture B, heat treated BSA (New England Biolabs, Ipswich, MA, USA) and DMSO (Sigma-Aldrich) were added to a final concentration of 1 mg/ml BSA and 5% (v/v) DMSO to PCR reaction mixture A. PCR reaction mixture C consisted of

TaKaRa *Ex Taq*™ DNA Polymerase (250 mU/µl) (TaKaRa Bio Inc.), 1x of the proprietary Ex Taq reaction buffer (contains 2 mM $MgCl_2$), 200µM of each dNTP (TaKaRa Bio Inc.), 1 mg/ml BSA, 5% (v/v) DMSO, and 40 nM of each primer.

Assessment of the specificity and sensitivity of the PCR assays

To assess the specificity of each PCR assay, 50 ng of DNA from *P. marinus* (ATCC 50489), *P. andrewsi* (ATCC 50807), and *Perkinsus* sp. (*M. mercenaria*) were used as templates in the PCR reactions. Sensitivities of the species-specific assays were assessed using decreasing amounts of genomic DNA (100 pg to 1 fg) from the respective *Perkinsus* species. For the genus-specific assay, the sensitivity was assessed using *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) genomic DNA, and for the *Perkinsus* sp. (*M. mercenaria*)-specific assay using *Perkinsus* sp. (*M. mercenaria*) and *P. andrewsi* genomic DNA. Assay sensitivities were assessed in the presence or absence of 500 ng of host (*C. virginica* or *M. mercenaria*) genomic DNA. Sensitivity of the PCR assays in the presence of *C. virginica* genomic DNA was assessed using PCR reaction mixture A. For assessment of the sensitivity in the presence of *M. mercenaria* genomic DNA, PCR reaction mixtures A and C were used. Negative PCR controls contained similar PCR reaction mixtures except that the template was replaced by sterile double distilled H_2O.

PCR-based detection of selected *Perkinsus* species in oyster and clam samples

Five hundred nanograms of DNA extracted from *C. virginica* and *M. mercenaria* were tested for *Perkinsus* species, *P. marinus, P. andrewsi,* and *Perkinsus* sp. (*M. mercenaria*). For *C. virginica* DNA samples, PCR reaction mixture A was used, and for *M. mercenaria* DNA samples, PCR reaction mixture C. Positive controls consisted of similar PCR reaction mixtures except that 1 ng of genomic DNA extracted from cultured *Perkinsus* species was used as a template. In negative PCR controls, the DNA template was substituted by sterile double distilled H_2O.

To minimize false-negatives, the SSU was amplified from all *M. mercenaria* samples using the universal primers UPRA and UPRB from Medlin et al. (1988). For *C. virginica*, 45 out of 176 samples that tested negative for *Perkinsus* species infections were tested for the amplifiability of the SSU. PCR reaction mixtures were identical to those used to detect *Perkinsus* infections in *C. virginica* and *M. mercenaria*. Positive PCR amplification controls consisted of similar PCR reaction mixtures except that 500 ng of genomic DNA extracted from either *C. virginica* or *M. mercenaria* was used that was known to be amplifiable. Cycling conditions were 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, with a final extension at 72 °C for 7 min.

Attenuation of inhibitory effects on the PCR amplification

To establish and optimize PCR conditions that would attenuate the observed inhibition of PCR amplification (see Results section), spike-recovery

experiments were conducted by spiking *C. virginica* and *M. mercenaria* genomic DNA (500 ng) with 10 pg and 1 pg *P. olseni* genomic DNA. These mixtures were tested for *P. olseni* as described elsewhere (Robledo et al. 2000) using the PCR mixtures A, B, and C. PCR amplification controls consisted of similar PCR reaction mixtures except that the addition of *C. virginica* and *M. mercenaria* genomic DNA was omitted.

Sequencing

Forty amplicons generated by the *Perkinsus* genus-specific PCR assay of samples that tested negative with all of the *Perkinsus* species-specific assays were cored from agarose gels and reamplified using the genus-specific assay as described above. The amplified products were again separated on 1.5% agarose gels, purified from the gels (QIAquick, QIAGEN), and sequenced from both directions with the primers PER1 and PER2 (Robledo et al. 2002). Fragment assembly was performed using the Staden Package v1.6.0 on a Mac OS X (Apple Computer, Inc., Cupertino, CA, USA) or Linux Fedora™ Core 5 (Red Hat, Inc., Raleigh, NC, USA) based computer.

Ribosomal RNA sequences for assay design and sequence comparisons

Sequences of rRNA genes and intergenic spacers of the rRNA sequences of *P. andrewsi* (accession number AF102171 and AY305326), *P. marinus* (AF497479), *P. olseni* (syn. *P. atlanticus*, AF140295) and *Perkinsus* sp. (*M. mercenaria*) (deposited as *Perkinsus* sp. CCA2001, AF252288) were obtained

from GenBank™. Sequence alignments were performed using the Needleman-Wunsch global alignment algorithm within EMBOSS (Rice et al. 2000).

Statistical analysis

The spatial distributions of the genus *Perkinsus*, and the species *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) were analyzed by comparing monthly prevalences between each site using the Fisher's exact test. Within each site, monthly prevalence changes were analyzed also using the Fisher's exact test. Prevalence differences between *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) were analyzed by comparing monthly prevalences of each *Perkinsus* species within each site using McNemar's χ^2 test with continuity correction. All statistical tests were performed on R (R Development Core Team 2006).

RESULTS

Specificity of the diagnostic assays

To assess the specificity of the four PCR diagnostic assays used in the present study, 50 ng genomic DNA from clonal cultures of *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) were tested. The genus-specific assay amplified a fragment of approximately 300 bp from each DNA preparation, whereas the assays designed to be specific for *P. marinus* and *P. andrewsi*

amplified fragments of expected size (approximately 300 bp) only from genomic DNA preparations of the respective *Perkinsus* species (Figure 13). The assay

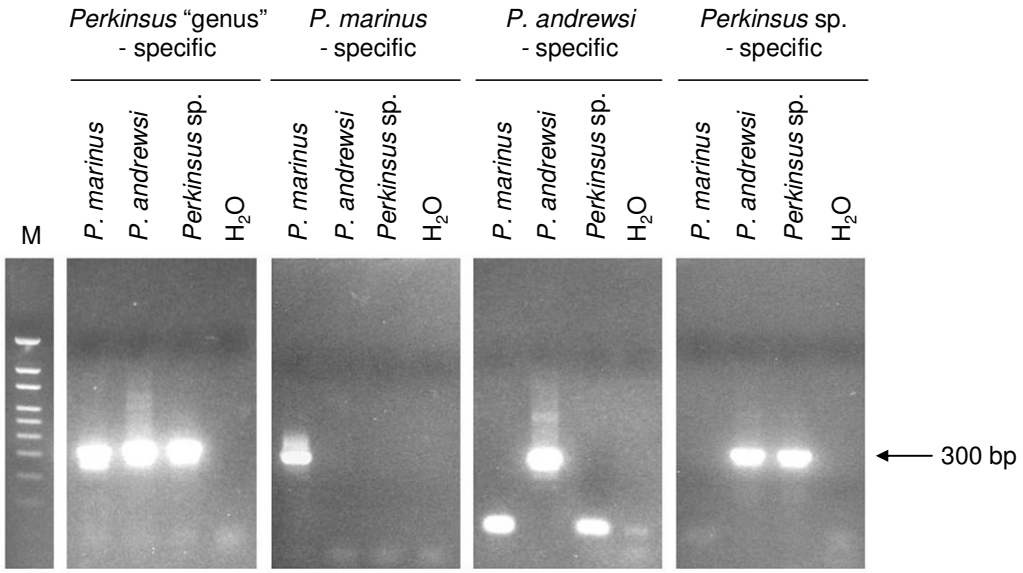


Figure 13. Specificity of the PCR-based assays. Fifty nanograms of genomic DNA of *Perkinsus marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) were tested with PCR-based assays specific for the *Perkinsus* genus, *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) using the PCR reaction mixture A. *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*); H_2O: negative PCR control.

designed for *P. andrewsi* showed intense bands of small size (approximately 100 bp) when *P. marinus* and *Perkinsus* sp. (*M. mercenaria*) were used as templates. Since this band also appeared with lesser intensity in the negative PCR control, they likely represent primer dimers. The PCR assay designed for *Perkinsus* sp. (*M. mercenaria*) amplified a fragment of expected size (approximately 300 bp) from *Perkinsus* sp. (*M. mercenaria*) and from *P. andrewsi* (Figure 13).

Sensitivity of the diagnostic assays

The sensitivity of the *Perkinsus* genus-, and species-specific assays was assessed by performing the respective assays on serial diluted genomic DNA (100 pg to 1 fg) using the standard PCR reaction mixture A. The *Perkinsus* genus-specific assay amplified 100 fg of *P. marinus* and 1 pg of *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) genomic DNA (Figure 14 A). The *P. marinus*- and the *P. andrewsi*-specific assays amplified 1 pg of *P. marinus* and *P. andrewsi* genomic DNA, respectively (Figure 14 B, C). The assay designed for the *Perkinsus* sp. (*M. mercenaria*) amplified 100 fg *Perkinsus* sp. (*M. mercenaria*) and 1 pg of *P. andrewsi* genomic DNA (Figure 14 D). In the genus-specific assay, the addition of 500 ng *C. virginica* DNA had no effect on the detection limit of *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*), but reduced the sensitivity by about 10-fold for *P. marinus*. No effects of 500 ng *C. virginica* DNA were observed on the *P. marinus*-, and *P. andrewsi*-specific assays (Figure 14 B, C). In the *Perkinsus* sp. (*M. mercenaria*)-specific assay, while the 500 ng of *C. virginica* DNA had no effect on the detection limit of *P. andrewsi*, it reduced the sensitivity by about 10-fold for *Perkinsus* sp. (*M. mercenaria*) (Figure 14 D). The addition of 500 ng of *M. mercenaria* genomic DNA to the PCR reactions reduced the sensitivity by at least 1000-fold in all four assays for all *Perkinsus* species tested (Figure 14 A-D), suggesting that the *M. mercenaria* genomic DNA preparations possessed potent PCR inhibitory activity.

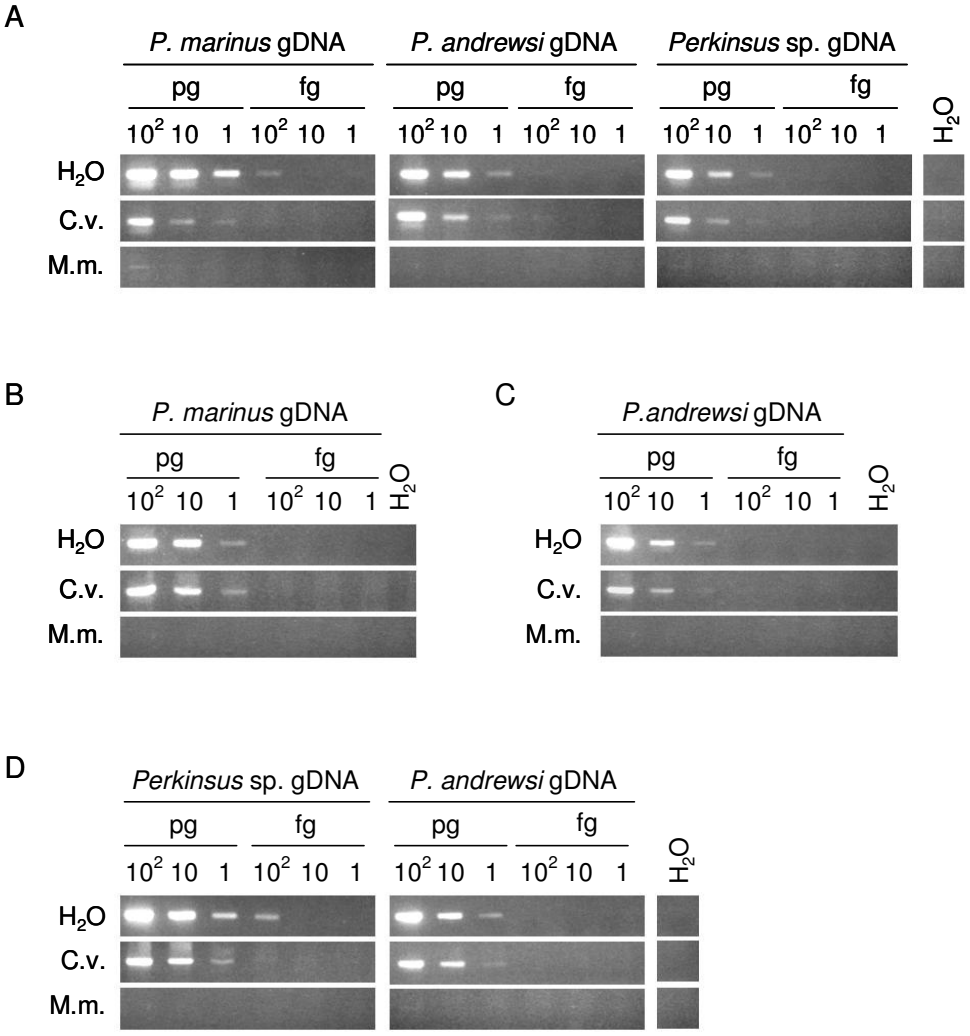


Figure 14. Sensitivity of the PCR-based assays using standard PCR reaction conditions. Decreasing amounts of genomic DNA (100 pg to 1 fg) from *Perkinsus marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) were tested with the respective PCR-based assays using the PCR reaction mixture A. Assays were performed in the absence (H_2O) or presence of 500 ng *Crassostrea virginica* (C.v.) and *Mercenaria mercenaria* (M.m.) genomic DNA. **A.** Sensitivity of the genus-specific assay. **B.** Sensitivity of the *P. marinus*-specific assay. **C.** Sensitivity of the *P. andrewsi*-specific assay. **D.** Sensitivity of the *Perkinsus* sp. (*M. mercenaria*)-specific assay. C.v.: *C. virginica*; M.m.: *M. mercenaria*; *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*); H_2O: negative PCR control.

Attenuation of PCR amplification inhibition

To establish PCR conditions that would attenuate the inhibition of the PCR amplification, 500 ng of *C. virginica* and *M. mercenaria* DNA were spiked with 10

pg and 1 pg *P. olseni* genomic DNA. *P. olseni* DNA was used because infections with this species have not been reported in the USA. These mixtures were tested for *P. olseni* using PCR mixture A, B, and C. PCR reaction mixture A did not amplify *P. olseni* in the presence of *M. mercenaria* genomic DNA, confirming PCR-amplification inhibition by DNA extractions from *M. mercenaria* (Figure 15).

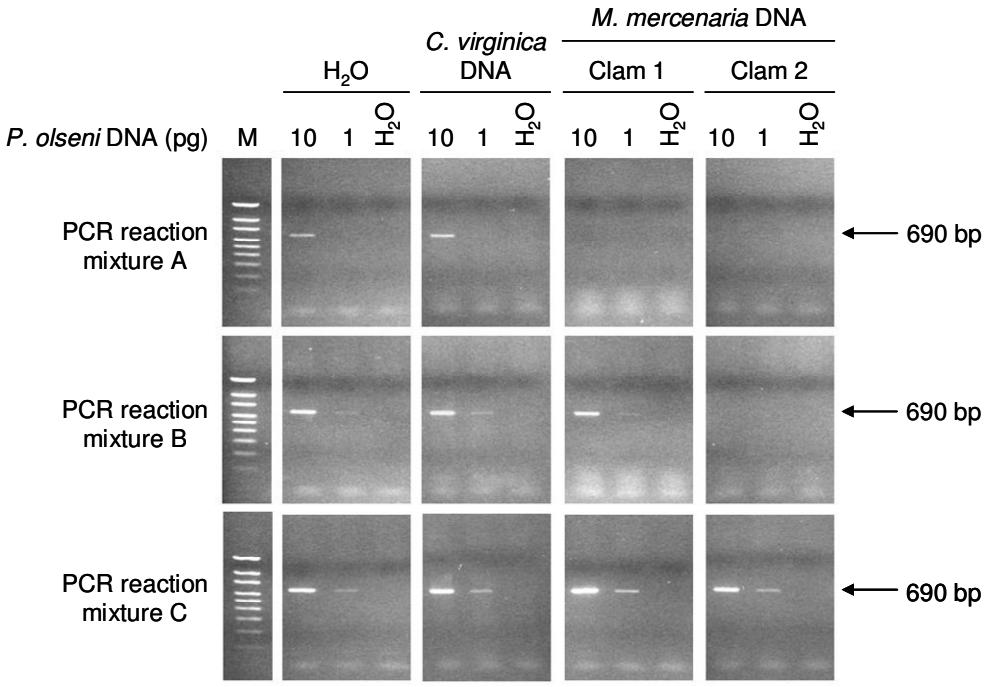


Figure 15. Attenuation of PCR amplification inhibition. Five hundred nanograms genomic DNA of one individual of *Crassostrea virginica* and two *Mercenaria mercenaria* specimens were spiked with 10 pg and 1 pg *Perkinsus olseni* genomic DNA. Samples were tested with a *P. olseni*-specific PCR-based assay using the PCR reaction mixture A, B, and C. In the positive PCR control (H_2O), host DNA was omitted. H_2O: negative PCR control.

The use of PCR reaction mixture B, which contains BSA (1 mg/ml) and DMSO (5% v/v), alleviated PCR inhibitory effects in most clam DNA preparations (Figure 15). However, DNA extracted from some individual clams still inhibited PCR amplification in mixture B. Using PCR reaction mixture C (containing TaKaRa *Ex*

Taq™, 1x of the proprietary Ex Taq reaction buffer, 1 mg/ml BSA, and 5% (v/v) DMSO), PCR amplification inhibition was removed from all DNA extractions tested (Figure 15). Under these conditions, the detection limits of the genus- and the species-specific assays were 1 pg to 10 fg (Figure 16).

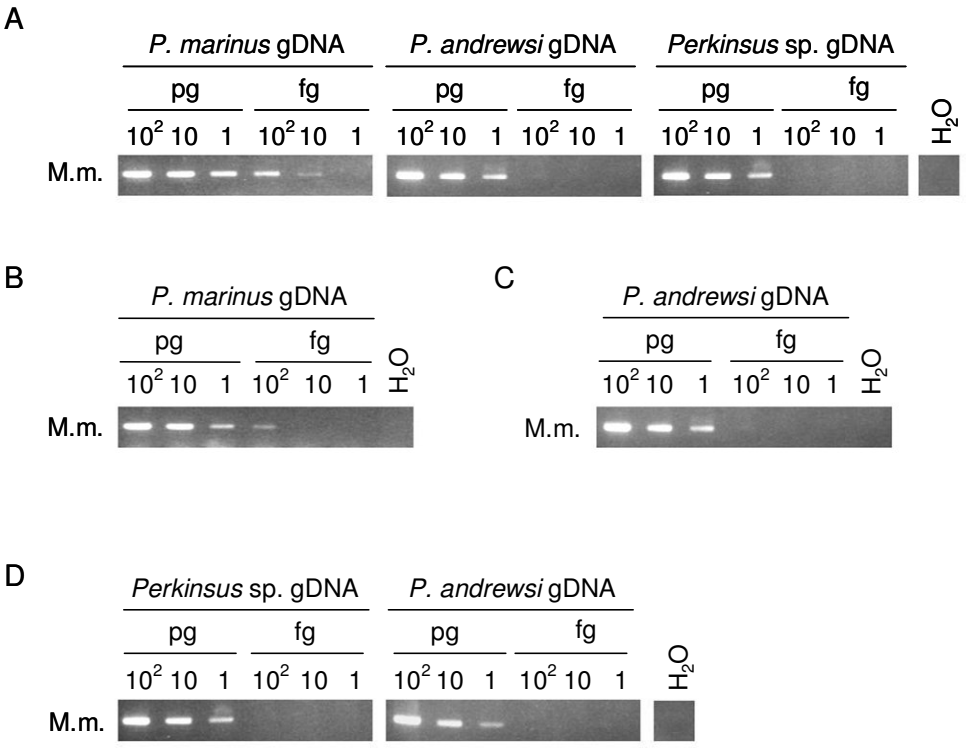


Figure 16. Sensitivity of the PCR-based assays using optimized PCR conditions. Decreasing amounts of genomic DNA (100 pg to 1 fg) from *Perkinsus marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) were tested with the respective PCR-based assays using the PCR reaction mixture C. Assays were performed in the presence of 500 ng *Mercenaria mercenaria* (M.m.) genomic DNA. **A.** Sensitivity of the genus-specific assay. **B.** Sensitivity of the *P. marinus*-specific assay. **C.** Sensitivity of the *P. andrewsi*-specific assay. **D.** Sensitivity of the *Perkinsus* sp. (*M. mercenaria*)-specific assay. gDNA: genomic DNA; M.m.: *M. mercenaria*; *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*); H_2O: negative PCR control.

Validation of PCR negative results

To exclude false negative PCR results from the analysis, the SSU of DNA samples was amplified using primers that anneal in conserved regions of the

SSU (Medlin et al. 1988). Forty five of 176 *C. virginica* that tested negative with the diagnostic PCR assays were tested. The SSU was amplified in all samples tested (data not shown). Using PCR reaction mixture C, the SSU of 225 out of 244 *M. mercenaria* samples could be amplified (data not shown). The 19 *M. mercenaria* samples (7.8%) for which no amplification was observed were excluded from further analysis.

Distribution and prevalences of *Perkinsus* species in *Crassostrea virginica* from Walpole, ME to Sandy Point, MD

To assess the distribution and prevalences of *Perkinsus* species in *C. virginica* along the Northeast Atlantic coast of the USA, a total of 625 *C. virginica* were collected monthly from June to September 2002 from Walpole, ME, Martha's Vineyard, MA, Narragansett Bay, RI, Oyster Bay, NY, Delaware Bay, NJ, and Sandy Point, MD (Figure 12). The oysters were tested for the presence of the genus *Perkinsus* and the individual species *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) with specific PCR-based assays as described in materials and methods.

***Comparison of the prevalences of the different* Perkinsus species *in* Crassostrea virginica.** A total of 449 (66.5%) *C. virginica* tested positive for a *Perkinsus* species with the genus-specific assay, 394 (58.4%) oysters were positive for *P. marinus*, nine (1.3%) for *P. andrewsi*, and 15 (2.3%) for *Perkinsus* sp. (*M. mercenaria*) (Table 4). Coinfection with *P. marinus* and *P. andrewsi* (0.9%) or *P. marinus* and *Perkinsus* sp. (*M. mercenaria*) (2.2%) were also

observed (Table 4). However, none of the oysters that tested positive for *P. andrewsi* tested positive for *Perkinsus* sp. (*M. mercenaria*).

Fifty two oysters that tested positive for a infection with a *Perkinsus* species with the genus-specific assay were negative for *P. marinus*, *P. andrewsi*, or *Perkinsus* sp. (*M. mercenaria*) (Table 4). Sequence analysis of amplicons obtained by the genus-specific PCR assay from 22 of the 52 oysters suggests that 13 oysters carried *P. marinus*, three *P. andrewsi*, and two *Perkinsus* sp. (*M. mercenaria*). Four samples showed extensive sequence ambiguities, possibly due to infections with more than one *Perkinsus* species.

To compare the prevalences between *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*), infection frequencies were analyzed using the McNemar's χ^2 test with continuity correction. The prevalence of *P. marinus* was significantly higher than the prevalence of *P. andrewsi* (McNemar's χ^2 test, p-value <0.001) and the prevalence of *Perkinsus* sp. (*M. mercenaria*) (McNemar's χ^2 test, p-value < 0.001). In contrast, the prevalences of *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) did not differ.

This trend held true for all sampling sites with the exception of Walpole. Here prevalences of *P. marinus* (0.9%), *P. andrewsi* (2.6%), and *Perkinsus* sp. (*M. mercenaria*) (0.0%) did not differ from each other.

Table 4. Prevalence of *Perkinsus* species infections in *Crassostrea virginica* collected from June to September 2002. 1: Walpole, ME; 3: Martha's Vineyard, MA; 4: Narragansett Bay, RI; 5: Oyster Bay, NY; 6: Delaware Bay, NJ; 7: Sandy Point, MD; N: number of individuals examined; n: number of infected individuals; (%): prevalence in percent; *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*).

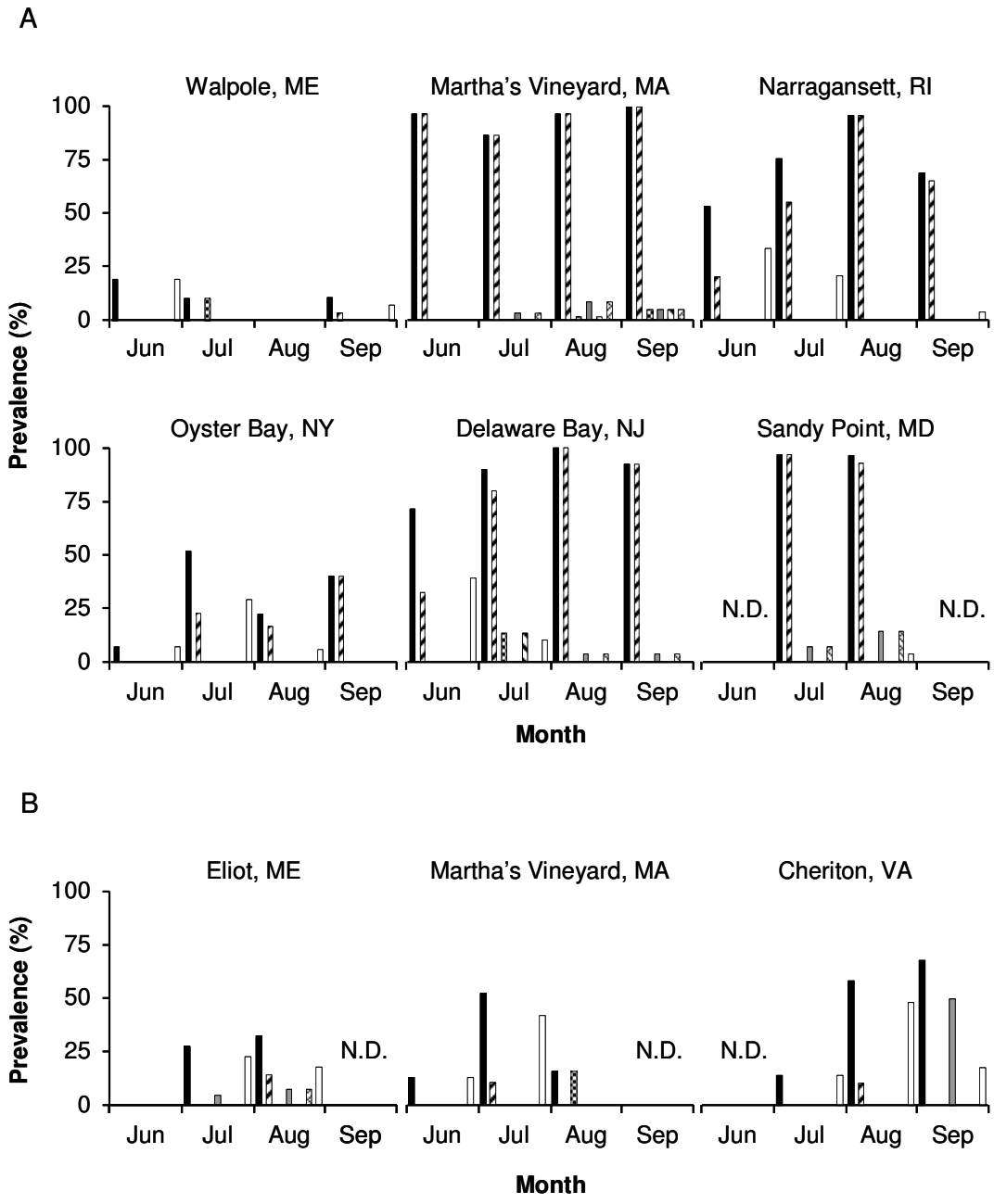
		Perkinsus species infections in *Crassostrea virginica*													
		Genus *Perkinsus* [a]		*P. marinus*		*P. andrewsi*		*Perkinsus* sp.		*P. marinus* & *P. andrewsi* [b]		*P. marinus* & *Perkinsus* sp. [b]		Genus *Perkinsus* only [c]	
Site	N	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
1	116	12	10.3	1	0.9	3	2.6	0	0.0	0	0.0	0	0.0	8	6.9
3	180	173	96.1	173	96.1	2	1.1	7	3.9	2	1.1	7	3.9	0	0.0
4	112	81	72.3	64	57.1	0	0.0	0	0.0	0	0.0	0	0.0	17	57.1
5	99	30	30.3	18	18.2	0	0.0	0	0.0	0	0.0	0	0.0	12	12.1
6	111	98	88.3	84	75.7	4	3.6	2	1.8	4	3.6	2	1.8	14	12.6
7	57	55	96.5	54	94.7	0	0.0	6	10.5	0	0.0	6	10.5	1	1.8
All	675	449	66.5	394	58.4	9	1.3	15	2.2	6	0.9	15	2.2	52	7.7

[a] All *Perkinsus* infections detected with the generic PCR-based assay.
[b] Note that coinfections with *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) were not observed.
[c] Infections that were detected with the generic PCR-based assay, but not with any of the species-specific assays.

***Spatial distribution of* Perkinsus species *in* Crassostrea virginica.** To assess the spatial distribution of *Perkinsus* infections in *C. virginica*, monthly prevalences between the different sites were compared pair-wise with the Fisher's exact test. In all four months a significant increase in prevalence was observed from Walpole (19.4%, 10.3%, 0%, and 10.7%) in the north to Martha's Vineyard (96.7%, 86.7%, 96.6%, and 100.0%; Fisher's exact test, p-values < 0.001), the next sampling site to the south, and from Walpole to Narragansett Bay (53.3%, 75.9%, 95.8%, and 69.0%; Fisher's exact test, p-values < 0.009), a sampling site south of Martha's Vineyard (Figure 17 A). In contrast, in June and September, the prevalence decreased significantly from Martha's Vineyard (96.7% and 100.0%) to Narragansett Bay (53.3% and 69.0%; Fisher's exact test, p-values < 0.001) and did not differ in July and August, with prevalences of 86.7% and 96.6% in Martha's Vineyard, and 75.9% and 95.8% in Narragansett Bay (Figure 17 A). Comparison between the sites Narragansett Bay, Oyster Bay, and Delaware Bay revealed that prevalences decreased in June and August from Narragansett Bay (53.3% and 95.8%) to Oyster Bay (6.7% and 22.2%; Fisher's exact test, p-values < 0.001), which is located south of Narragansett Bay and north of Delaware Bay, but did not differ between Narragansett Bay and Delaware Bay (71.4 % and 100.0%). However, prevalences increased from

Figure 17 (next page). Monthly *Perkinsus* infection prevalence in *Crassostrea virginica* and *Mercenaria mercenaria.* Percent prevalences of *Perkinsus* species (detected by the genus-specific assay) (■), *P. marinus* (◪), *P. andrewsi* (▩), *Perkinsus* sp. (*M. mercenaria*) (▣), dual infections with *P. marinus* and *P. andrewsi* (▧), as well as *P. marinus* and *Perkinsus* sp. (*M. mercenaria*) (▨), and infections with *Perkinsus* species only (□) are shown for all sampling sites. **A.** Prevalence in *C. virginica*. **B.** Prevalence in *M. mercenaria*. Jun: June; Jul: July; Aug: August; Sep: September.

Figure 17


A
Walpole, ME
Martha's Vineyard, MA
Narragansett, RI
Oyster Bay, NY
Delaware Bay, NJ
Sandy Point, MD
N.D.
N.D.
Prevalence (%)
100
75
50
25
0
Jun
Jul
Aug
Sep
Month
B
Eliot, ME
Martha's Vineyard, MA
Cheriton, VA
N.D.
N.D.
N.D.
Prevalence (%)
Month

Oyster Bay to Delaware Bay (Fisher's exact test, p-value < 0.001). In July, prevalences did not differ between Narragansett Bay (75.9%) and Oyster Bay (51.3%), and Narragansett Bay and Delaware Bay (90.0%), but increased from Oyster Bay to Delaware Bay (Fisher's exact test, p-value = 0.002). In September, while the prevalence did not differ between Narragansett Bay (69.0%) and Oyster Bay (40.0%), it decreased significantly from Narragansett Bay to Delaware Bay (92.3%; Fisher's exact test, p-value = 0.044), and increased significantly from Oyster Bay to Delaware Bay (Fisher's exact test, p-value < 0.001) (Figure 17 A). In Sandy Point, prevalence data was not available for June and September. The prevalences observed in Sandy Point in July (96.6%) and August (96.4%) did not differ from prevalences found in Delaware Bay (90.0% and 100.0%, respectively) (Figure 17 A).

***Spatial distribution of* Perkinsus marinus *in* Crassostrea virginica.**

The presence of *P. marinus* was observed at all sampling sites with the *P. marinus*-specific assay. The spatial distribution of *P. marinus* was similar to the spatial distribution observed for the genus *Perkinsus* with one exception. In July, where the prevalences of the genus *Perkinsus* did not differ between Narragansett Bay (75.9%) and Oyster Bay (51.6%), the prevalence of *P. marinus* decreased significantly from Narragansett Bay (55.2%) to Oyster Bay (22.6%; Fisher's exact test, p-value = 0.016). Like with the genus *Perkinsus*, the prevalence of *P. marinus* did not differ between Narragansett Bay (55.2%) and Delaware Bay (80.0%) (Figure 17).

***Spatial distribution of* Perkinsus andrewsi *in* Crassostrea virginica.** With the *P. andrewsi*-specific assay, *P. andrewsi* infections were detected at low prevalences in Walpole in July (10.3%), in Martha's Vineyard in August (1.7%) and September (1.6%), and in Delaware Bay in July (13.3%). Statistical analysis showed that the prevalences did not differ between all sites in the four months.

***Spatial distribution of* Perkinsus *sp. (*M. mercenaria*) in* Crassostrea virginica.** *Perkinsus* sp. (*M. mercenaria*) was detected by the *Perkinsus* sp. (*M. mercenaria*)-specific assay at low prevalences in Martha's Vineyard in July (3.3%), in August (8.4%), and in September (3.3%), in Delaware Bay in August (3.7%) and in September (3.8%), and in Sandy Point in July (6.9%) and in August (14.3%). Like with *P. andrewsi*, prevalences did not differ between all sites in the four months.

***Temporal distribution of* Perkinsus *species in* Crassostrea virginica.** Changes of *Perkinsus* infection prevalences at each site did not show a general temporal pattern over the four months. In Walpole, Martha's Vineyard, and Delaware Bay prevalences did not change significantly from month to month. Furthermore, no significant difference was observed between early summer (June) and late summer (September). In contrast, in Oyster Bay, prevalences increased significantly from June (6.7%) to July (51.6%; Fisher's exact test, p-value < 0.001), and did not change significantly between July (51.6%) and August (22.2%), and between August and September (40.0%). In Narragansett Bay, prevalences did not differ between June (53.3%) and July (75.9%), and

between July and August (95.8%), however decreased significantly from August to September (69.0%; Fisher's exact test, p-value 0.015) (Figure 17 A).

***Temporal distribution of* Perkinsus marinus *in* Crassostrea virginica.** The temporal distribution of *P. marinus* was similar to the temporal distribution of the genus *Perkinsus* with exception of Narragansett Bay and Delaware Bay. In both sites, in contrast to the genus *Perkinsus*, where no differences in prevalence were observed between June and July, and between July and August, the prevalence of *P. marinus* increased significantly from June (20.0% in Narragansett Bay, and 32.1% in Delaware Bay) to July (55.2% in Narragansett Bay; Fisher's exact test, p-value = 0.007, and 80.0% in Delaware Bay; Fisher's exact test, p-value < 0.001), and from July to August (95.8% in Narragansett Bay; Fisher's exact test, p-value = 0.001, and 100.0% in Delaware Bay; Fisher's exact test, p-value = 0.02).

***Temporal distribution of* Perkinsus andrewsi *and* Perkinsus *sp. (*M. mercenaria*) in* Crassostrea virginica.** Prevalences of *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) did not differ from month to month in all sites.

Distribution and prevalences of *Perkinsus* species in *Mercenaria mercenaria* from Eliot, ME to Cheriton, VA

To assess the prevalence of *Perkinsus* species infections in the hard clam *M. mercenaria*, 225 specimens were tested with the PCR-based diagnostic assays as described in materials and methods. The specimens tested were collected monthly from June to August 2002 from Eliot, ME, and Martha's Vineyard, MA, and from July to September 2002 from Cheriton, VA (Figure 12).

Comparison of the prevalences of the different* Perkinsus *species in Mercenaria mercenaria. Overall, a total of 72 (32.0%) *M. mercenaria* tested positive for a *Perkinsus* species with the genus-specific assay. Ten (4.4%) clams tested positive for *P. marinus*, three (1.3%) for *P. andrewsi*, and 17 (7.6%) for *Perkinsus* sp. (*M. mercenaria*). Coinfection with *P. marinus* and *Perkinsus* sp. (*M. mercenaria*) was observed in two (0.9%) clams (Table 5). None of the clams that tested positive for *P. andrewsi* tested positive for *Perkinsus* sp. (*M. mercenaria*).

The prevalences of *P. marinus* and *P. andrewsi*, and *P. marinus* and *Perkinsus* sp. (*M. mercenaria*) did not differ significantly. In contrast, overall, significantly more clams were infected with *Perkinsus* sp. (*M. mercenaria*) than with *P. andrewsi* (McNemar's χ^2 test; p-value = 0.004).

However, when the prevalences of the three *Perkinsus* species were compared at each sampling site, only in Cheriton significant differences between *P. andrewsi* (0.0%) and *Perkinsus* sp. (*M. mercenaria*) (7.6%) were observed (McNemar's χ^2 test, p-value < 0.001). No significant differences were detected between the prevalences of *P. marinus* and *P. andrewsi* as well as *P. marinus* and *Perkinsus* sp. (*M. mercenaria*) in Cheriton, and between the three *Perkinsus* species in Eliot and Martha's Vineyard.

Forty four specimens tested positive for a *Perkinsus* infection but negative for any of the *Perkinsus* species or isolate tested. Eighteen of the 44 amplicons generated by the *Perkinsus* genus-specific PCR were sequenced. Fourteen of the obtained sequences were highly similar or identical to the sequence of *P.*

Table 5. Prevalence of *Perkinsus* species infections in *Mercenaria mercenaria* collected from June 2002 to September 2002. 2: Eliot, ME; 3: Martha's Vineyard, MA; 8: Cheriton, VA; N: number of individuals examined; n: number of infected individuals; (%): prevalence in percent; *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*).

		Perkinsus species infections in *Mercenaria mercenaria*													
		Genus *Perkinsus* [a]		*P. marinus*		*P. andrewsi*		*Perkinsus* sp.		*P. marinus* & *P. andrewsi* [b]		*P. marinus* & *Perkinsus* sp.[b]		Genus *Perkinsus* only [c]	
Site	N	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
2	79	15	19.0	4	3.8	0	0.0	3	3.8	0	0.0	2	2.5	10	12.7
3	61	16	26.2	2	3.3	3	4.9	0	0.0	0	0.0	0	0.0	11	18.0
8	85	41	48.2	4	4.7	0	0.0	14	16.5	0	0.0	0	0.0	23	27.1
All	225	72	32.0	10	4.4	3	1.3	17	7.6	0	0.0	2	0.9	44	19.6

[a] All *Perkinsus* infections detected with the generic PCR-based assay.
[b] Note that coinfections with *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) were not observed.
[c] Infections that were detected with the generic PCR-based assay, but not with any of the species-specific assays.

marinus and three sequences to *P. andrewsi*. One sequence showed extensive ambiguities, suggesting an infection with more than one *Perkinsus* species.

***Spatial distribution of* Perkinsus *species in* Mercenaria mercenaria.**

To assess the spatial distribution of *Perkinsus* infections in *M. mercenaria*, monthly prevalences between the different sites were compared. In Eliot, with the genus-specific assay, 0%, 27.3%, and 32.1% clams were found to be infected with a *Perkinsus* species in June, July, and August, respectively. In Martha's Vineyard, 13,0%, 52.6%, and 15.8% of the clams were infected. In Cheriton, 14.3%, 58.6%, and 67.9% of the clams were found to carry a *Perkinsus* species in July, August, and September (Figure 17 B). Statistical analysis using the Fisher's exact test revealed that the prevalences between Eliot and Martha's Vineyard did not differ significantly in all three months. Likewise, the prevalences observed in Eliot in July and August did not differed from those observed in Cheriton. However, the prevalences differed significantly between Martha's Vineyard Cheriton in July and August (Fisher's exact test, p-values < 0.009). (Figure 17 B).

***Spatial distribution of* Perkinsus marinus *in* Mercenaria mercenaria.**

Using the *P. marinus*-specific assay, *P. marinus* infections were observed in Eliot only in August in 14.3% of the clams. *P. marinus* infections were not observed in Martha's Vineyard. In Cheriton, *P. marinus* was detected in 10.3% of the clams in July, and in 3.6% of the clams in September (Figure 17 B). Monthly prevalences did not differ between the sampling sites.

***Spatial distribution of* Perkinsus andrewsi *in* Mercenaria mercenaria.** With the *P. andrewsi*-specific assay, only 15.8% of the clams collected from Martha's Vineyard in August tested positive for *P. andrewsi*. In Eliot and in Cheriton *P. andrewsi* infections were not observed over the study period. (Figure 17 B). Like with *P. marinus*, monthly prevalences did not differ between the sampling sites.

***Spatial distribution of* Perkinsus *sp. (*M. mercenaria*) in* Mercenaria mercenaria.** Infections with *Perkinsus* sp. (*M. mercenaria*) were observed with the specific assay in June (4.5%) and August (7.1%) in Eliot, and in September (50.0%) in Cheriton. Prevalences between Eliot and Martha's Vineyard did not differ in June, July, and August. They also did not differ between Eliot and Cheriton, and Martha's Vineyard and Cheriton in July and August.

***Temporal distribution of* Perkinsus *species in* Mercenaria mercenaria.** In Eliot, the *Perkinsus* prevalence increased significantly from June (0%) to July (27.3%; Fisher's exact test, p-value = 0.004), but did not differ between July and August (32.1%). In Martha's Vineyard, the *Perkinsus* prevalence also increased from June (13.0%) to July (52.6%; Fisher's exact test, p-value = 0.008), however decreased from July to August (15.8%, Fisher's exact test, p-value = 0.03). In Cheriton, the prevalence increased from July (14.3%) to August (58.6%; Fisher's exact test, p-value) and did not differ between August and September (67.9%) (Figure 17 B).

***Temporal distribution of* Perkinsus marinus*, Perkinsus andrewsi, and* Perkinsus *sp. (*M. mercenaria*) in* Mercenaria mercenaria.** At all sites, prevalences of *P. marinus* and *P. andrewsi* did not differ from month to month. Similarly, the prevalence of *Perkinsus* sp. (*M. mercenaria*) did not differ from month to month in Eliot and Martha's Vineyard. However, in Cheriton, it increased significantly between August (0.0%) and September (50.0%; Fisher's exact test, p-value <0.001).

Comparison of *Perkinsus* species prevalences in *Crassostrea virginica* and *Mercenaria mercenaria*

Overall, the prevalence of *Perkinsus* species and *P. marinus* in *M. mercenaria* (32.0% and 4.4%, respectively) was significantly lower than in *C. virginica* (66.5% and 58.6%, respectively; Fisher's exact test $p < 0.001$). The *P. andrewsi* prevalence in *M. mercenaria* (1.3%) was similar to the prevalence in *C. virginica* (1.3%). In contrast, the prevalence of *Perkinsus* sp. (*M. mercenaria*) was significantly higher in *M. mercenaria* (7.6%) than in *C. virginica* (2.2%; Fisher's exact test, $p < 0.001$).

A side by side comparison was most informative at the sampling sites in Martha's Vineyard, were oysters and clams were collected from proximal locations. Here 96.1% of the *C. virginica* were infected with a *Perkinsus* species, compared to only 26.2% of the *M. mercenaria* (Figure 18). All but three infected *C. virginica* specimens carried *P. marinus*. Infections with *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) were 1.1% and 3.9%, respectively. Nine (5.0%)

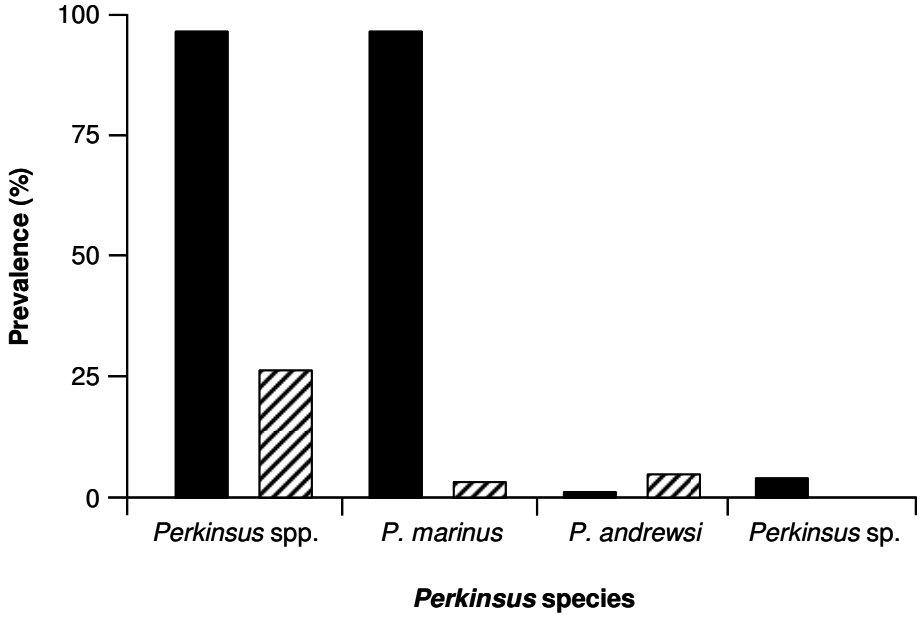


Figure 18. Comparison of the *Perkinsus* infection prevalence in *Crassostrea virginica* and *Mercenaria mercenaria.* Percent prevalences of *Perkinsus* species, *P. marinus, P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) in *C. virginica* (■) and *M. mercenaria* (▨) collected from June 2002 to September 2002 in Martha's Vineyard, MA are shown. *Perkinsus* sp.: *Perkinsus* sp. (*M. mercenaria*).

C. virginica were additionally infected with either *P. andrewsi* (1.1%) or *Perkinsus* sp. (*M. mercenaria*) (3.9%). In *M. mercenaria*, *P. marinus*, and *P. andrewsi*, were present at low prevalences (3.3%, and 4.9% respectively). *Perkinsus* sp. (*M. mercenaria*) was not detected (Figure 18, Table 4, 5).

Differences in prevalence between *C. virginica* and *M. mercenaria* were statistically significant for infections with the genus *Perkinsus* and *P. marinus* (Fisher's exact test, $p < 0.001$), but not for *P. andrewsi* or *Perkinsus* sp. (*M. mercenaria*).

DISCUSSION

The aim of the present study was to assess the presence of *P. marinus, P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) in two economically important bivalves, *C. virginica*, and *M. mercenaria*, using PCR-based assays. Along the Gulf of Mexico and Atlantic coast of the USA three *Perkinsus* species have been described, *P. marinus* (Mackin et al. 1950, type host *C. virginica*), *P. chesapeaki* (McLaughlin et al. 2000, type host *M. arenaria*), and *P. andrewsi* (Coss et al. 2001b, type host *M. balthica*). Although the synonymization of *P. andrewsi* and *P. chesapeaki* has been suggested (Burreson et al. 2005), at this point, I consider *P. andrewsi* as a valid and distinct *Perkinsus* species for the reasons outlined in Chapter 2.

Diagnostic assays for *Perkinsus* species

The diagnostic assays for *Perkinsus* species that are based on the FTM method do not distinguish between the sympatric *Perkinsus* species found along the Atlantic coast (Coss 2000, Coss et al. 2001b, McLaughlin et al. 2000, Ray 1952). However, several *Perkinsus* species-specific assays are available (Coss et al. 2001b, Marsh et al. 1995, Robledo et al. 2002, Yarnall et al. 2000). No PCR-based assay has been developed for *P. chesapeaki*, mainly due to the lack of a *bona fide* type culture that would allow design and validation of such an assay.

For the purpose of the present study, available PCR-based assays specific for the genus *Perkinsus*, and for the species *P. marinus*, and *P. andrewsi*

(Coss et al. 2001b, Marsh et al. 1995, Robledo et al. 2002) were used, and a new PCR-based assay for a *Perkinsus* species isolated from *M. mercenaria* [*Perkinsus* sp. (*M. mercenaria*)] was developed. The species-specific assays are based on sequence differences within the IGS of the rRNA gene locus of *Perkinsus* species that links the 5S and small subunit rRNA genes, whereas the genus-specific assay is based on a conserved region at the 3' end of the IGS (Coss et al. 2001b, Marsh et al. 1995, Robledo et al. 2002). As expected, the genus-specific assay detected all *Perkinsus* species tested in the present study, including *Perkinsus* sp. (*M. mercenaria*). *Perkinsus* sp. (*M. mercenaria*) was not detected by the *P. marinus*-specific or the *P. andrewsi*-specific assays, demonstrating the capacity of the genus-specific *Perkinsus* assay to detect new *Perkinsus* species or strains.

The assay designed for *Perkinsus* sp. (*M. mercenaria*) also amplified *P. andrewsi* genomic DNA extracted from the *P. andrewsi* type culture. This is to be expected for it was shown previously that *P. andrewsi* has two distinct rRNA gene units (type A and B) (Pecher et al. 2004, Chapter 2). Sequence analysis of the rRNA gene unit of the *Perkinsus* sp. (*M. mercenaria*) revealed that, while this isolate has only one rRNA gene unit, this unit is very similar in sequence to the rRNA-B gene unit of the *P. andrewsi* type culture (Chapter 2). In particular, the IGS of *Perkinsus* sp. (*M. mercenaria*) is 99.4% identical to the IGS of the *P. andrewsi* rRNA-B gene unit (Chapter 2), explaining the cross amplification. The *P. andrewsi*-specific assay does not detect *Perkinsus* sp. (*M. mercenaria*), since

it has been developed based on the IGS of the rRNA-A gene unit that is only 71.4% identical to the IGS of *Perkinsus* sp. (*M. mercenaria*).

The sensitivity of each species-specific assay observed in the present study (0.1 to 1 pg genomic DNA of the respective *Perkinsus* species) is similar to the sensitivities of the diagnostic assays for the *P. marinus* and *P. andrewsi* reported by Marsh et al. (1995) and Coss et al. (2001b). The data collected in the present study suggest that the sensitivity of the genus-specific assay (10 fg to 1 pg genomic *Perkinsus* species DNA) is equal to, or greater than the respective species-specific assays, allowing the detection of low density *Perkinsus* infections.

Inhibition of PCR amplification

Inhibition of PCR amplification is frequently observed in environmental and biological samples (reviewed by Wilson 1997). Inhibitory substances include organic and phenolic compounds, humic acids, heavy metals, fats, and polysaccharides. PCR inhibition has been reported in mollusks, with glycogen being the responsible component (Andersen and Omiecinski 1992, Atmar et al. 1993, Hill et al. 1991). Modified DNA extraction protocols, such as extraction with phenol chloroform and precipitation in the presence of cetyltrimethylammonium bromide (CTAB), have been used to overcome PCR inhibition in oysters (Atmar et al. 1993). In addition, commercially available kits have been developed that can be used to extract DNA from plants, animals, and fungi from complex sources such as the soil and other environmental samples. Alternatively, additives such as BSA, DMSO, Tween 20, polyethylen glycol, and

glycerol are commonly included in the PCR reaction mixture at various concentrations and combination to attenuate the effects of the interfering substances. The nature and combination of the additives and the concentrations used are determined empirically and vary based on the sample source and DNA extraction method (reviewed in Wilson 1997).

PCR inhibition in *C. virginica* DNA extracts was not observed. In contrast, marked inhibition of PCR amplification was observed with DNA from clams (*M. mercenaria*) and scallops (*Argopecten irradians*) extracted with a commercial kit (Pecher and Vasta, unpublished data). To obtain PCR amplification conditions that overcome inhibitory effects, the addition of BSA and DMSO at various concentrations to the PCR reaction mixture were tested by spike recovery experiments. Only the addition of 1 mg/ml BSA in combination with 5% (v/v) DMSO to the PCR reaction mixture, however, successfully attenuated the PCR amplification inhibition in *M. mercenaria*. Using the modified PCR reaction mixture with a standard *Taq* DNA polymerase and a standard PCR reaction buffer, the SSU gene of 70% of the *M. mercenaria* could be amplified (data not shown). The use of a specialty *Taq* DNA polymerase with its optimized buffer system that is designed to amplify large DNA fragments further facilitated PCR amplification, and enabled the amplification of the SSU gene of 91% of the clams. However, similar reaction conditions failed to amplify scallop DNA samples (Pecher and Vasta, unpublished data). These observations underline the importance of validation of PCR conditions for each sample type used, including the assessment and validation of potential false negatives, vis-à-vis

inhibition of PCR amplification. Once DNA is extracted from samples and PCR conditions are optimized, the PCR-based assays enable detection of any *Perkinsus* species and different *Perkinsus* species in the same sample. Thus, the application of the genus- and species-specific assays presents a valuable alternative to the FTM assay.

Distribution of *Perkinsus* infections in *Crassostrea virginica* and *Mercenaria mercenaria*

Studies based on the FTM assay documented the distribution of *Perkinsus* species in *C. virginica* from the Yucatán peninsula to Maine (Burreson et al. 1994, Ford 1996, Soniat 1996). These infections have been attributed to *P. marinus*. However, the discovery of additional *Perkinsus* species and development of specific PCR assays for them has provided tools to test this assumption. In the present study, commercially-harvested *C. virginica* and *M. mercenaria* populations were tested for the presence of *Perkinsus* species, *P. marinus*, *P. andrewsi*, and a *Perkinsus* sp. isolated from *M. mercenaria* using specific PCR-based assays.

In accordance with the studies mentioned above, by using the genus-specific assay, *Perkinsus* species infections were observed in both host species as far north as Maine. In *C. virginica*, *P. marinus* was the most prevalent *Perkinsus* species. *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*) were present, but at significantly lower prevalences. Therefore, discrepancies between high infection density, prevalence, and low mortality observed in other studies in *C. virginica* (Ford 1996, Karolus et al. 2000) cannot be attributed to the

presence of a different *Perkinsus* species with less pathogenicity towards the oyster.

Alternative hypotheses that will require further studies are (a) the presence of *P. marinus* strains with different pathogenicity, rather than different *Perkinsus* species, and (b) differences in environmental factors such as cooler summer peak temperatures and colder winter temperatures that influence the outcome of an infection (Burreson and Ragone Calvo 1996, Ford 1996). Of course, the presence of host populations with different genetic background could be another component leading to lower host mortality.

The effect of temperature and salinity on the distribution, abundance, and severity of *Perkinsus* infections is well documented (Andrews 1988, Andrews and Hewatt 1957, Andrews and Ray 1988, Chu and La Peyre 1993a, Chu et al. 1993, Chu 1996, Hewatt and Andrews 1956) and it has been shown in field studies that *Perkinsus* infection prevalences and densities of infections decreased with decreasing temperatures and salinities (Chu and La Peyre 1993a, Chu et al. 1993). Furthermore, it was reported that in laboratory experiments the mortality of infected oysters kept at 15 °C was lower than the mortality of infected oysters kept at 26 °C (Hewatt and Andrews 1956). If temperature indeed is shown to be a major factor contributing to the currently observed discrepancies between high prevalence, infection density, and low mortality, then it is conceivable that, due to the projected world wide warming trends, not only a further distribution range extension of *Perkinsus* species is possible (Harvell et al. 1999), but also more sever *Perkinsus* infections outbreaks associated with mass mortalities in *C.*

virginica populations will occur in the northeastern Atlantic coast of the United States.

In contrast to *C. virginica*, even though *P. marinus* appears to be abundant in the study area, in *M. mercenaria*, *Perkinsus* sp. (*M. mercenaria*), and not *P. marinus*, was the most prevalent *Perkinsus* species. This observation indicates that, as discussed below, *M. mercenaria* might not be an optimal host for *P. marinus*, and conversely, *C. virginica* might not be an optimal host for *Perkinsus* sp. (*M. mercenaria*).

Due to sequence similarities of the second rRNA gene unit (rRNA-B) of the *P. andrewsi* hapantotype to the *Perkinsus* sp. isolated from *M. mercenaria* (Chapter 2), the *P. andrewsi* hapantotype is detected by the *Perkinsus* sp. (*M. mercenaria*)-specific assay with a 10-fold lower sensitivity. In the present study however, none of the clams and oysters that tested positive for *P. andrewsi* tested positive for *Perkinsus* sp. (*M. mercenaria*). Certainly, the lower sensitivity of the *Perkinsus* sp. (*M. mercenaria*) towards *P. andrewsi* may partially explain this observation, but the presence of *P. andrewsi* isolates that contain only the rRNA-A gene unit, and thus are not detected by the *Perkinsus* sp. (*M. mercenaria*) assay, cannot be ruled out.

Application of the genus-specific PCR-based assay to both *C. virginica* and *M. mercenaria* specimens resulted in the detection of *Perkinsus* infections that could not be attributed to *P. marinus*, *P. andrewsi*, or *Perkinsus* sp. (*M. mercenaria*) by the species-specific PCR assays. However, sequence analysis of selected amplicons generated by the genus-specific PCR failed to reveal novel

sequences that could suggest the presence of yet undescribed *Perkinsus* species or strains. It rather suggested that the density of *Perkinsus* infections in these specimens was below the detection limit of the species-specific assays, but high enough to be detected by the genus-specific assay.

In *C. virginica*, prevalences of *Perkinsus* infections (as assessed by the genus-specific assay) and of *P. marinus* varied from site to site. Prevalences increased from north to south in June and September 2002, with the exception of Martha's Vineyard and Oyster Bay. Martha's Vineyard had surprisingly high prevalences compared to those in Narragansett Bay, whereas prevalences in Oyster Bay were significantly lower than in Narragansett Bay.

The high prevalences of *P. marinus* in Martha's Vineyard are also in contrast to observations by Russell et al. (2004). These authors did not observe *P. marinus* infections in *C. virginica* specimens that were collected in September 2000 and tested by a multiplex PCR-based assay, suggesting that Martha's Vineyard may have experienced a localized *P. marinus* epizootic. This assertion is also supported by high oyster mortalities observed at the sampling site by oyster growers (R. Karney, personal communication).

Why the prevalence in Oyster Bay was lower than in Narragansett Bay is unclear. Possible explanations include lower salinities at the sampling site in Oyster Bay and different genetic background of the oysters and the parasite, or both.

Interestingly, the observed prevalences in Oyster Bay in 2002 were higher than those reported by Russell et al. (2004). These authors observed *P. marinus*

prevalences of 0% and 3% in *C. virginica* collected in June and August 2000 from a site in Oyster Bay. In the present study, also no *P. marinus* infections were observed in Oyster Bay in June 2002. However, in August 2002, 17% of *C. virginica* were infected with *P. marinus*. These differences may be due to higher temperatures and drier conditions in 2002 as compared to 2000, as judged by monthly mean air surface temperatures and precipitation recorded by a nearby weather station (Mineola, NY, COOP ID 305377). Mean monthly air temperatures in 2000 were 16.9 °C, 20.4 °C, 21.2 °C, and 21.9 °C in May, June, July, and August. Monthly precipitation was 108.2 mm, 111.3 mm, 137.7 mm, and 61.2 mm over the same time period. In 2002, mean monthly temperatures were 15.2 °C, 21.0 °C, 25.9 °C, and 24.8 °C from May to August. Monthly precipitation was 93.0 mm, 95.8 mm, 22.9 mm, and 183.9 mm. Higher temperatures and drier conditions may result in higher water temperatures and higher salinity, both conditions favorable to *P. marinus* infections (Andrews 1988, Burreson and Ragone Calvo 1996). However, even though sensitivity differences between the PCR assay used in the two studies are minor, they also may have contributed to the observed differences in prevalences. Based on the following rough calculation, the PCR-based assay used in the present study is about 2.5 fold more sensitive than the assay used by Russell et al. (2004). The assay used in the present study has a detection limit of 1 pg *P. marinus* genomic DNA. Based on available sequences, *P. marinus* has an estimated genome size between 70 and 80 Mb (El-Sayed, Robledo and Vasta, unpublished data; http://www.tigr.org/tdb/e2k1/pmg/), and a single rRNA gene unit is approximately

7 kb long (Robledo and Vasta, unpublished data). If a genome size of 80 Mb is assumed, then 1 pg genomic DNA represent 13 genome equivalent. If it is furthermore assumed, that, similar to *P. atlanticus* (de la Herrán et al. 2000), *P. marinus* rRNA genes are encoded by 5% of its genome, then about 570 copies of a single rRNA gene unit are present per genome equivalent. Thus, the PCR assay used in the present study would detect 7,500 copies of the rRNA gene unit, or, since each gene unit contains one IGS, 7,500 copies of the IGS. The multiplex PCR assay used by Russell et al. (2004), detects 100 fg of plasmid DNA containing the *P. marinus* IGS (Russell et al. 2004). Because the plasmid with the IGS sequence is approximately 4.5 kb, 100 fg plasmid DNA represent about 20,000 copies of the IGS.

Epizootiological studies based on the FTM assay on *Perkinsus* species in *C. virginica* in Chesapeake Bay and Delaware Bay suggest that *Perkinsus* species prevalences in *C. virginica* are generally lower in early summer than in late summer in these locations (reviewed in Andrews 1988, Burreson and Ragone Calvo 1996, Ford 1996). In the present study, a similar trend is observed for *Perkinsus* infections (as judged by the genus-specific assay) only in Oyster Bay. In the other sites, including Delaware Bay, prevalences did not differ between the months and between June and September. A similar trend was observed for *P. marinus*, with the exception for Delaware Bay and Narragansett Bay. In both sites, in contrast to infections with the genus *Perkinsus*, prevalences of *P. marinus* increased from June to August. However, since differences in prevalence between the genus *Perkinsus* and *P. marinus* cannot

be explained by the presence of other *Perkinsus* species, they may possibly be due to sensitivity differences between the genus-specific assay and the assay for *P. marinus*, as discussed above, and thus be an artifact.

Interestingly, in the present study *P. andrewsi* infections were not observed in farmed oysters in Chesapeake Bay. This finding is in contrast to reports of Coss et al. (2001b), according to which 65% of 125 *C. virginica* collected from natural populations throughout Chesapeake Bay were infected with *P. andrewsi*, 64% with *P. marinus*, and 34% had dual infections. In the present study, 94.7% of oysters were infected with *P. marinus* and 7.0% with *Perkinsus* sp. (*M. mercenaria*). A possible explanation is that the conditions at the particular location were favorable for a *P. marinus* infection. Alternatively, and not mutually exclusive, it is conceivable that, once a *P. marinus* infection has been established in an oyster population, it may outgrow other *Perkinsus* infections. But since the present study does not provide any evidence to support either hypothesis, and knowledge on the infection dynamics of the different *Perkinsus* species is limited at best, both assertions remain speculation. However, with the ability to discriminate between infections of *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*), it is now feasible to test the above mentioned hypotheses and to address general questions on the infection dynamics of the three *Perkinsus* species, such as whether co-infections with the three *Perkinsus* species have an influence on the outcome and/or virulence of either *Perkinsus* species infection.

In contrast to *C. virginica*, *Perkinsus* infection prevalences in *M. mercenaria* did not differ between Eliot, the northern most sampling site, and Martha's Vineyard. In contrast, they differed between Cheriton and Martha's Vineyard, but did not show a consistent decrease from north to south: in July, prevalences were significantly higher in Martha's Vineyard than in Cheriton, but in August, prevalences were significantly lower in Martha's Vineyard than in Cheriton. Since prevalences between Cheriton and Eliot did not differ over the two months, it is possible that the prevalence observed in July in Martha's Vineyard may have been exceptionally high. However at this point, this remains speculation.

Prevalences of the genus *Perkinsus* increased at all three sites from June to July or, in Cheriton, from July to August. In contrast, prevalences of the individual species did not differ from month to month. Like in *C. virginica*, the presence of a new *Perkinsus* species could not be confirmed in the present study. Therefore, the discrepancy between the prevalences of infections with the genus *Perkinsus* and with the individual species is most likely a result of lower sensitivities of the species-specific assays.

Host preference of *Perkinsus* species

While *Perkinsus* species appear to lack strict host-specificity, they may have adapted best to the hostile environment of one particular host species. Studies on the effects of plasma of different mollusk species on the *in vitro* proliferation of *P. marinus* show that its growth rate is reduced by plasma or sera from bivalve mollusks (*Andara ovalis, Geukensia demissa*, *M. mercenaria*, and

M. edulis) that are naturally exposed to the parasite compared to plasma or sera from *C. virginica* (Anderson 2001, Gauthier and Vasta 2002). These observations suggest indeed a preference of *P. marinus* for its type host, *C. virginica*. Results from the present study on the distribution of *P. marinus* provide further evidence for its host preference: even though *P. marinus* was detected in both *M. mercenaria* and *C. virginica*, prevalences in *M. mercenaria* were significantly lower. The next two chapters of the this work describe studies aimed at elucidating the basic molecular mechanisms responsible for the above mentioned host preference.

CHAPTER 4

CHARACTERIZATION OF THE ANTIOXIDANT PATHWAY OF *PERKINSUS MARINUS*: SEARCH FOR PEROXIDASES [2]

SUMMARY

Perkinsus marinus is a protistan parasite of the eastern oyster *C. virginica*. Infections are initiated by phagocytosis of *P. marinus* trophozoites by oyster hemocytes. Upon phagocytosis, *P. marinus* actively suppresses the oxidative burst normally observed during phagocytosis of yeast or heat-killed *P. marinus*, possibly by scavenging produced ROS such as $^{\bullet}O_2^-$ and H_2O_2 and/or by interfering with their production through parasite-derived enzymes. Enzymes that scavenge ROS include SOD that dismutases $^{\bullet}O_2^-$ to H_2O_2. In most aerobic eukaryotes, H_2O_2 in turn is degraded to H_2O by CAT. In addition, H_2O_2 can be reduced by peroxidases such as glutathione peroxidases (GPXs). Studies on the antioxidant pathway suggest that *P. marinus* possesses at least two SODs, but neither CAT nor other peroxidases have been identified yet. In the present

[2] The experimental work to assess the ability of *Perkinsus marinus* and *Vibrio splendidus* to remove H_2O_2 was published in Schott et al. (2003a) and is presented in amended form with kind permission from Elsevier.

study, I demonstrate that, while *P. marinus* actively removes H_2O_2, it appears to lack CAT. Instead, my findings suggest the presence of an APX commonly found in plants and algae.

INTRODUCTION

Perkinsus marinus is a protistan parasite of marine bivalves along the Gulf of Mexico and Atlantic coast of the USA. Despite its apparent broad host range, *in vitro* experiments on the proliferation rate of *P. marinus* (Anderson 2001, Gauthier and Vasta 2002) and distribution studies of *Perkinsus* species in eastern oyster (*C. virginica*) and hard clam (*M. mercenaria*) populations from the Northeastern Atlantic coast of the USA suggest that *C. virginica* is its preferred host (Chapter 3).

Factors that contribute to successful transmission, entry, survival, and proliferation of *P. marinus* may also influence its host preference. *P. marinus* infections likely occur after ingestion of trophozoites or zoospores by filter feeding, followed by their phagocytosis by host hemocytes, located in the gut epithelium, or mantle and gills, thereby mediating the distribution of *P. marinus* to the host tissues and body fluids (Allam and Ford 2002, Mackin 1951, Perkins 1976). Like vertebrate neutrophils, oyster hemocytes produce ROSs aimed at the destruction of invading microorganisms. These ROSs include $^{\bullet}O_2^-$ that is generated by a NADPH oxidase (Takahashi and Mori 2000). $^{\bullet}O_2^-$ is converted to H_2O_2 by SODs, and in the presence of chloride ions, MPO catalyzes the conversion of H_2O_2 into HOCl (Anderson et al. 1992, Greger et al. 1995).

However, upon phagocytosis *P. marinus* actively suppresses the oxidative burst normally observed during phagocytosis of yeast or heat-killed *P. marinus*, possibly through the production of antioxidant enzymes such as SODs and peroxidases and/or by interfering with the production of ROSs through parasite-derived enzymes (Anderson 1999, Volety and Chu 1995).

P. marinus is resistant to relatively high levels of exogenous $^{\bullet}O_2^-$ and H_2O_2, which may be attributed to the parasite's ability to scavenge ROSs. In contrast, it is highly susceptible to HOCl (Schott et al. 2003a). The high tolerance to $^{\bullet}O_2^-$ and H_2O_2 raises the question of how and at which rates *P. marinus* is able to remove the respective ROSs. As mentioned above, $^{\bullet}O_2^-$ is enzymatically removed by SODs that produce H_2O_2 in that process. Most eukaryotes detoxify H_2O_2 by CAT that degrades H_2O_2 to H_2O and O_2. No enzymatic mechanism is known to degrade HOCl. Studies on the antioxidant pathway in *P. marinus* showed that the parasite possesses ample SOD activities and two distinct iron type SODs have been identified and characterized (Ahmed et al. 2003, Gauthier 1998, Schott et al. 2003b, Wright et al. 2002). The antioxidant enzymes that may contribute to the removal of H_2O_2 in *P. marinus*, however, have not yet been identified. This chapter describes experiments undertaken to investigate the ability of *P. marinus* to remove H_2O_2 and the attempts to identify CAT activity and CAT genes of *P. marinus*.

MATERIALS AND METHODS

Cell cultures

If not otherwise noted, *P. marinus* strain Texas (*P. marinus* TxSc; ATCC 50849) was grown at 28 °C in DMEM:HAM's F12 (1:2) medium with 5% (v/v) FBS and 15 ppt ASW following Gauthier and Vasta (1995). *Escherichia coli* K12 was grown at 37 °C in Luria-Bertani-Miller (LB) medium under constant agitation, *Vibrio splendidus* (ATCC 33125) on LB agar (Difco, Detroit MI, USA) at 22 °C, and *Saccharomyces cerevisiae* EG103 at 30 °C in YPD medium under constant agitation.

Isolation of DNA

For PCR, DNA was extracted from log-phase *Perkinsus* cultures using the DNeasy Tissue kit from QIAGEN, following the manufacturer's instructions. DNA from *S. cerevisiae* was extracted from cultures grown to stationary phase according to Hoffman (1997). Briefly, *S. cerevisiae* cells were lysed mechanically by vortexing cells in equal volume breaking buffer (2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris Cl, 1 mM EDTA, pH8.0), phenol:chloroform:isoamyl alcohol (25:24:1), and glass beads. DNA was ethanol precipitated and resuspended in TE buffer, treated with RNAse A (1 mg/ml) and again ethanol precipitated. DNA concentration and purity were estimated by spectrophotometry at wavelengths of 260 nm and 280 nm.

Hydrogen peroxide removal assay (FOX assay)

To test the ability of live *P. marinus* and *V. splendidus* cells to remove H_2O_2 from solution, cells were freshly harvested and resuspended in PBS500 (500 mM NaCl, 20 mM phosphate buffer, pH 7.2), at an OD_{600} of 2.0 and 0.025, respectively. Cells were challenged with 75 µM H_2O_2 in triplicate reactions in microtiter wells (200 µl final volume) at 22 °C. Samples were withdrawn at 25, 40, and 60 min, and assayed for remaining H_2O_2 with a SpectraMax 340 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at a wavelength of 560 nm using a microtiter adaptation of the FOX assay of Jiang et al. (1992). Standard curves were constructed using the same reagents as used for experiments. Viability of *P. marinus* and *V. splendidus* cells after 1 h exposure to peroxides, was assessed by neutral red staining and dilution plating on LB, respectively.

For analysis of H_2O_2 destruction by cytosolic cell extracts, *P. marinus* and *V. splendidus* were lysed in low osmotic buffer (25 mM phosphate buffer, pH 7.2) containing 0.5% Triton X-100. Cell debris was removed by centrifugation (10,000 x g), and supernatants dialyzed overnight at 4 °C against 0.5 M NaCl buffered with 25 mM phosphate buffer (pH 7.2) and supplemented with 1 mM ascorbic acid (ASA). *P. marinus* and *V. splendidus* extracts were processed in parallel, and maintained no more than 24 h at 0 °C until assayed. Ten microliters of extract were incubated in the presence of 75 µM H_2O_2. After 20 min, 40 min, and 60 min 10 µl aliquots were withdrawn and tested for H_2O_2 removal with the FOX assay at 22 °C in a final volume of 200 µl.

Detection of catalase activity

To investigate possible CAT activity in *P. marinus*, *P. marinus* trophozoites were either exposed to an excess of iron, or iron starved by addition of the iron chelator desferroxamine (DFO) in the culture medium. Briefly, *P. marinus* was grown to stationary phase (10 days) in DMEM:HAM's F12 (1:2) supplemented with 1.7 mg/ml or 3 mg/ml fetuin alone (controls), 1.7 mg/ml fetuin and 100 µM $FeCl_2$, or 3 mg/ml fetuin and 20 µg/ml DFO. As control, *E. coli* (K12) grown in LB for 16 h at 37 °C was used. Cells were lysed by sonication in extraction buffer (50 mM Tris Cl, 150 mM NaCl, 1 mM phenylmethylsulphonylfluoride (PMSF), pH 7.4). A total of 20 µg protein per cell extract was separated on non-denaturing 9% polyacrylamide gels under non-reducing conditions. Gels were assayed for catalytic hydroperoxidase activity by ferricyanide negative staining following Wayne and Diaz (1986). Briefly, gels were washed in double distilled H_2O and incubated in 18 mM H_2O_2 for 5 min at 22 °C. After three washes in double distilled H_2O gels were incubated in a solution containing 2% (w/v) $K_3Fe(CN)_6$ and 2% (w/v) $FeCl_3$ at 22 °C until a blue-green color developed and subsequently washed in double distilled H_2O. In this assay, areas where H_2O_2 is removed by catalytic hydroperoxidase activity appear as clear bands on a dark blue-green background.

Catalase and catalase peroxidase sequences

CAT protein sequences from *Homo sapiens* (Accession number P04040), *T. gondii* (AAD45528), *Dictyostelium discoideum* (O77229) and *S. cerevisiae* (NP_010542), and *V. vulnificus* CMCP6 (NP_763353) and catalase-peroxidase

(CPX) sequences from *S. cerevisiae* (AAS56092) and *V. vulnificus* CMCP6 (AAO11099) were obtained from GenBank™.

Identification of putative catalase genes through PCR-based approaches

The identification of putative *P. marinus* CAT genes was attempted by PCR amplification using degenerate oligonucleotide primers under various stringency conditions or using a "touchdown" approach. Degenerate oligonucleotides were designed based on sequence alignments of CAT protein sequences of *T. gondii*, *D. discoideum* and *S. cerevisiae* and are listed in Table 6.

Cycling conditions for the amplifications at constant annealing temperatures were 94 ℃ for 3 min followed by 30 cycles of 93 ℃ for 30 sec, 45 sec at different annealing temperatures (38.3 ℃, 41.1 ℃, 45.5 ℃, 49.6 ℃, and 52.2 ℃, respectively), 72 ℃ for 50 sec with a final extension of 72 ℃ for 7 min. Cycling conditions for the "touchdown" approach were as follows: after an initial denaturation step at 94 ℃ for 3 min, 17 cycles of 92 ℃ for 25 sec, 55 ℃ for 35 sec with a decrease of 1 ℃ per cycle, and 72 ℃ for 45 sec were followed by 25 cycles of 92 ℃ for 30 sec, 37 ℃ for 45 sec with a time increment of 1 sec per cycle, and 72 ℃ for 45 sec. The final extension was performed at 72 ℃ for 4 min.

PCR products were size-separated on 1.5% agarose gels prestained with 0.5 μg/ml EtBr. Selected amplicons from the amplification of *P. marinus* and *S. cerevisiae* genomic DNA were cored and reamplified with the respective primers used for the primary amplification. The amplified products were again separated

on 1.5% agarose gels, purified from the gels (QIAquick, QIAGEN) and sequenced with the respective primers. Similarity searches with the obtained sequences were performed with the program "translated query vs. protein database BLAST" (BLASTX).

Table 6. Selected PCR primers used for the identification of CAT genes in *Perkinsus marinus*. K: G or T; I: inosin; M: A or C; N: A, G, T, or C; R: A or G; S: C or G; W: A or T; Y: T or C.

Primer	Sequence (5'- 3')
cat1F	TGA TCG ATA AGC TGG CCC AYT TYR AYM G
cat2F	CCG ATA CCG AGC GCG AYC CNM GNG G
cat3F	AGT TCC CCG ACT TCA TCC AYA CNC ARA A
cat4R	TGG GGG TGG CGC TTY TGN GTR TG
cat5R	GGC CTG CTC CAC CTC CTC CKS RAA RWA RTT
cat6R	GCC CAG GCG GTG GCK RTG NGY RTC
cat11F	GCA CTA CGT GCA GTG GCA YWT HAA RAC
cat12R	AGC ACA AGA ACA ACT AGG TGA AYW TNC AYA C
cat13F	ACT GCC AGC AGG ACC TGT TYR ANG CNA T
cat14R	GCA ACT TGT CCA GGA CGA CCS KYA TYA GNC C
cat15F	TGT TCG ACG TGA CCA AGG TNT GGC CNC A
cat16R	CCC CCA TGA CCC GGC ACN CCG GTN TG
cat21F	GCI CAI TTC IAR KGI GAA IIR ATR CC
cat22F	GGI GCC III GGI TAT TTR GAY GTI AC
cat29R	GGI AGI AGI CAI ATR TTY AAC RII CG
cat30R	CAA ACI CCI AII GII AAY ACY CAM AC

Similarity search

CAT protein sequences from *H. sapiens*, *T. gondii*, *S. cerevisiae*, and *V. vulnificus* CMCP6 as well as CPX sequences from *S. cerevisiae* and *V. vulnificus* CMCP6 were queried against a local *P. marinus* expressed sequence tag (EST) database (PmEST_TIGR_COMB) with the program "protein query vs. translated nucleotide database BLAST" (TBLASTN) using default parameters. BLAST searches were performed using NCBI-BLAST v.2.2.11 on a Mac OS X (Apple Computer, Inc.) or Linux Fedora^{TM} Core 5 (Red Hat, Inc.) based computer. The EST database contained 31,419 sequences and was generated within an ongoing functional genome project and the genome sequence project in collaboration of our laboratory with J. Kissinger at the University of Georgia and with TIGR, respectively.

Sequence hits with an expect value (E-value) equal or below $1.0 \times e^{-6}$ were considered significant. These EST sequences were submitted to the BLAST server at the National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) and queried against NCBI default databases using BLASTX with default parameters.

RESULTS

Removal of hydrogen peroxide by live *Perkinsus marinus* cells

To comparatively investigate the ability of live *P. marinus* trophozoites and *V. splendidus* to degrade H_2O_2, approximately 3.3×10^7 *P. marinus* and 2.5×10^7

V. splendidus cells were suspended in PBS500 and exposed to 75 µM H_2O_2 over a 1 h period. The rate of H_2O_2 consumption per 10^8 cells was calculated for three time intervals. *P. marinus* removed 19.9, 21.9, and 21.3 nmol of H_2O_2 per 10^8 cells/h at incubation times of 25, 40, and 60 min (Figure 19). The rate of H_2O_2 removal by *P. marinus* remained constant over the 1 h assay period. In comparison, *V. splendidus* removed 35, 30, and 24 nmol per 10^8 cells/h at these three time points, and the H_2O_2 removal rate by *V. splendidus* decreased by more than 30% (Figure 19).

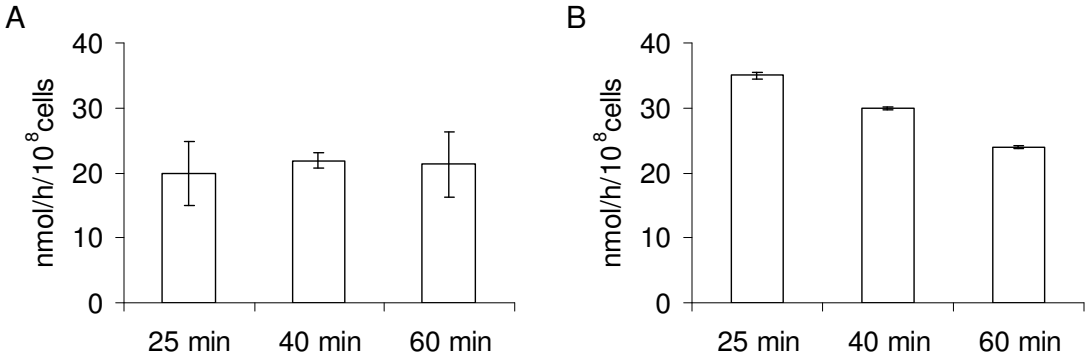


Figure 19. Hydrogen peroxide removal by live cells. The removal rate of H_2O_2 is expressed in nmol H_2O_2 removed/h/10^8 cells. Each time point is an average of triplicate samples. Error bars represent the standard deviation. A. H_2O_2 removal of *Perkinsus marinus* cells. B. H_2O_2 removal of *Vibrio splendidus* cells.[3]

After the 1 h exposure to 75 µM H_2O_2, 96% of *P. marinus* trophozoites remained viable as judged by neutral red staining. In contrast, only 1% of *V. splendidus* survived the exposure (data not shown).

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[3] Figure 19 is reprinted in modified form from Experimental Parasitology, 105, Schott, E. J., Pecher, W. T., Okafor, F., Vasta, G. R.: The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species, pp. 232-240, Copyright (2003) Elsevier Inc., with kind permission from Elsevier.

Identification of catalase genes using PCR-based approaches

The previous experiment showed that *P. marinus* has the ability to actively remove H_2O_2, however, the mechanism of removal remains elusive. In most eukaryotes, H_2O_2 is degraded by CAT. It was therefore attempted to identify putative CAT genes in the *P. marinus* genome by degenerate oligonucleotide-based amplifications from genomic *P. marinus* DNA. Several combinations of degenerate primers, designed based on multiple alignments of CAT protein sequences from *T. gondii*, *S. cerevisiae*, and *D. discoideum*, were used to amplify putative CAT genes in *P. marinus* genomic DNA under different stringency conditions, or using a "touchdown" approach. PCR amplifications of genomic *P. marinus* DNA with annealing temperatures of 38.3 ℃, 41.4 ℃, 45.5 ℃, and 49.6 ℃ yielded multiple amplicons with all primers used. Representative results are shown for the primers cat21F and cat30R, and cat21F and cat29R. Single amplicons of expected size of 300 bp and 400 bp were obtained from *S. cerevisiae* genomic DNA with the primers cat21F and cat29R and cat21 and cat30R at an annealing temperature of 51.2 ℃ (Figure 20 A, B). The "touchdown" approach using the primers cat3F and cat5R yielded one amplicon of approximately 1,500 bp from *P. marinus* genomic DNA and one amplicon of the expected size (600 bp) from *S. cerevisiae* genomic DNA (Figure 20 C). Amplicons of approximately 400 bp, 500 bp, and 1,000 bp obtained with the primers cat21F and cat30R, the prominent amplicon of about 850 bp obtained with the primers cat21F and cat29R, and the 1,500 bp amplicon obtained with the primers cat3F and cat5R, all from PCR on *P. marinus* genomic DNA, were

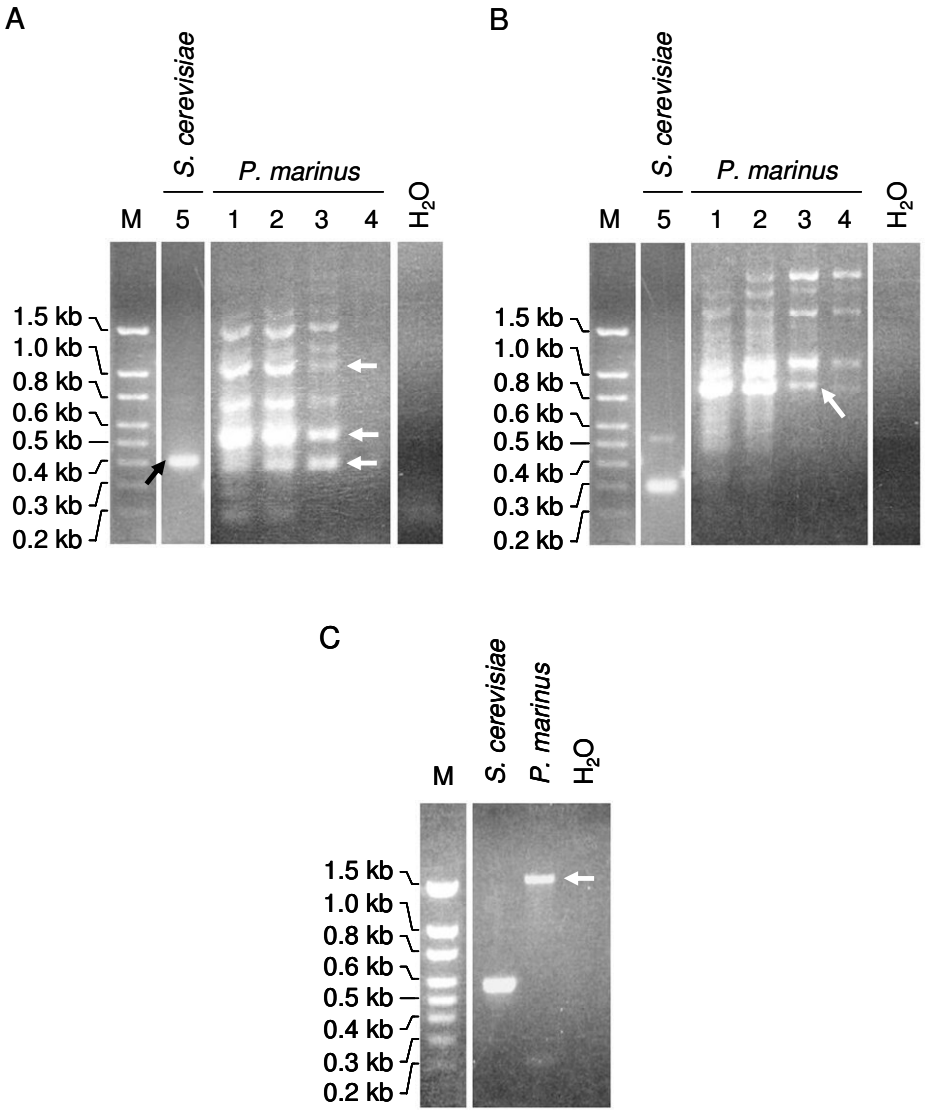


Figure 20. Agarose gel electrophoresis of PCR-amplified products from *Perkinsus marinus* and *Saccharomyces cerevisiae* genomic DNA using CAT primers. **A, B**. PCR reactions were conducted with the primers cat21F and cat30R (A) and cat21F and cat29R (B) using different stringency conditions. **C.** PCR reactions were conducted with the primers cat3F and cat5R using a touchdown approach. 1 - 5: annealing temperatures as follows: 1 = 38.3 °C, 2 = 41.4 °C, 3 = 45.5 °C, 4 = 49.6 °C, 5 = 51.2 °C; M: DNA size marker; H_2O: negative PCR control. Arrows indicate products that have been cored, reamplified, and sequenced.

reamplified and sequenced. Similarity searches using BLASTX with sequences obtained from the 500 bp, 850 bp, and 1,500 bp amplicons revealed no similarities to CAT genes. Instead, highest similarities were observed to a gene

product of *Drosophila melanogaster*, aspartate aminotransferases, and a human ATP-dependent RNA helicase, respectively. The E-values, however, were above 0.05, suggesting that the similarities observed were not significant. Sequences obtained from the 400 bp and 1,000 bp amplicons showed extensive ambiguities possibly due to the presence of more than one sequence. In addition, the 400 bp amplicon obtained by the PCR of *S. cerevisiae* genomic DNA with the primers cat21F and cat30R was sequenced. The sequence was identical to the respective *S. cerevisiae* catalase sequence stretch, confirming the validity of the PCR approach (data not shown).

Similarity search for putative catalase sequences of *Perkinsus marinus*

In addition to the PCR-based approaches described above, a *P. marinus* EST database with 31,419 sequences was searched for putative CAT genes using TBLASTN with CAT amino acid (aa) sequences from *H. sapiens*, *T. gondii*, *S. cerevisiae*, and *V. vulnificus*, and with CPX aa sequences from *S. cerevisiae*, and *V. vulnificus*. No significant hits with an E-value equal or below 1 x e^{-6} were obtained with CAT sequences and with the CPX sequence from *S. cerevisiae*. In contrast, the *V. vulnificus* CPX sequence had significant hits to the following *P. marinus* EST sequences: PMDBO52TR (E-value = 8.0 x e^{-14}), PMDAH94TR (E-value = 1.0 x e^{-12}), PMEA443TR (E-value = 9.0 x e^{-10}), PMDAL76TR (9.0 x e^{-10}), PMDCN60TR (E-value = 9.0 x e^{-10}), PMDAH94TO (E-value = 1.0 x e^{-7}), and PMDBO52TO (E-value = 1.0 x e^{-6}). To confirm their identity, these EST sequences were submitted to the NCBI BLAST server and queried using

BLASTX. All seven sequences showed high similarities to APXs from plants and algae, but not to CATs or CPXs (Table 7).

Table 7. Results of the similarity search using BLASTX of Perkinsus marinus ESTs. Seven *P. marinus* EST sequences that showed high similarities to the *Vibrio vulnificus* CAT gene were queried against the NCBI BLAST server databases using BLASTX. Only the first hit is shown.

EST	Hit	Score	E-value
PMDBO52TR	Chloroplast thylakoid-bound APX of *Vigna unguiculata* (AAS55852)	265	8.0 e^{-70}
PMDAH94TR	APX of *Oryza sativa* (CAH67301)	255	2.0 e^{-66}
PMEA443TR	Chloroplast APX of *Ostreococcus tauri* (CAL50268)	196	1.0 e^{-48}
PMDAL76TR	Chloroplast APX of *O. tauri* (CAL50268)	197	4.0 e^{-49}
PMDCN60TR	Chloroplast APX of *O. tauri* (CAL50268)	197	4.0 e^{-49}
PMDAH94TO	Chloroplast thylakoid-bound APX of *V. unguiculata* (AAS55852)	199	1.0 e^{-49}
PMDBO52TO	Chloroplast thylakoid-bound APX of *V. unguiculata* (AAS55852)	177	5.0 e^{-43}

Peroxidase activity of *Perkinsus marinus*, *Escherichia coli*, and *Vibrio splendidus* extracts

To investigate whether *P. marinus* cell extracts contain CAT activity "in-gel" assays were performed. Whereas *E. coli* extracts that were used as positive controls show ample CAT activity, CAT activity was not detected in *P. marinus* cell extracts (Figure 21).

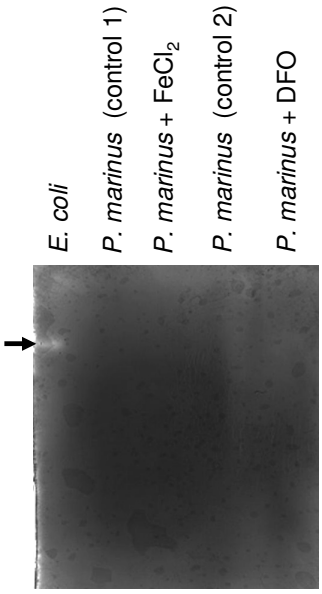


Figure 21. Detection of catalytic hydroperoxidase activity in polyacrylamide gels. The clear band observed in the lane of the *Escherichia coli* extract (arrow) indicates removal of H_2O_2. *Perkinsus marinus* was grown in DMEM:HAM's F12 (1:2) supplemented with fetuin [1.7 mg/ml (control 1), or 3 mg/ml (control 2)], 1.7 mg/ml fetuin and 100 µM $FeCl_2$, or 3 mg/ml fetuin and 20 µg/ml DFO.

The inability to detect CAT activity in *P. marinus* extracts by multiple approaches motivated a search for alternative peroxidase activities. A preliminary search of 31,419 *P. marinus* EST sequences suggested the possible presence of APXs. This peroxidase activity requires the constant presence of ascorbate (ASC) in cell extracts. Thus, soluble cell extracts (1 mg/ml) of *P. marinus* and *V. splendidus* were dialyzed in buffer containing 1 mM ASA and assayed the extracts with the FOX assay as described above. Again, the rate of H_2O_2 removal was measured at 20, 40, and 60 min. The *P. marinus* extract removed H_2O_2 at a rate of 3.0, 2.9, and 3.5 nmol H_2O_2/h/mg protein, while *V. splendidus* extract removed 16.4, 12.1, and 8.3 nmol H_2O_2/h/mg protein (Figure 22). Similar to live cells, the rate of H_2O_2 removal of the dialyzed extract of *P.*

marinus remained at a constant level, while it decreased by nearly half over the course of the experiment in the *V. splendidus* extract (Figure 22).

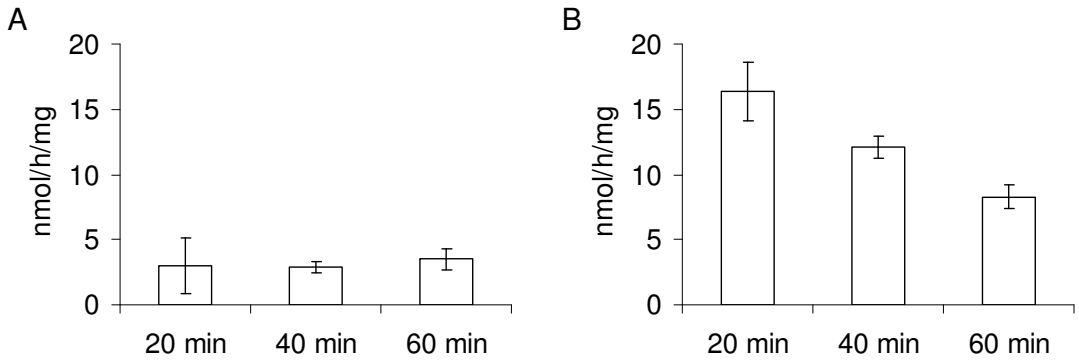


Figure 22. Hydrogen peroxide removal by cell extracts in the presence of 1 mM ascorbate. The H_2O_2 removal rate is expressed as nmol H_2O_2 removed/h/mg protein. Each time point is an average of triplicate samples. Error bars represent the standard deviation. **A**. H_2O_2 removal by *Perkinsus marinus* cell extracts. **B**. H_2O_2 removal by *Vibrio splendidus* cell extracts.[4]

DISCUSSION

Perkinsus marinus is a protistan endoparasite of a variety of mollusks and causes “Dermo” disease in the eastern oyster, *C. virginica* (Andrews and Hewatt 1957, Mackin 1962). Infections are believed to be initiated by ingestion of *P. marinus* trophozoites or zoospores that have been released by moribund oysters into the water column (Andrews 1996) (Figure 1). The *P. marinus* cells are subsequently phagocytosed by hemocytes located in the gut epithelium (Mackin

[4] Figure 22 is reprinted in modified form from Experimental Parasitology, 105, Schott, E. J., Pecher, W. T., Okafor, F., Vasta, G. R.: The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species, pp. 232-240, Copyright (2003) Elsevier Inc., with kind permission from Elsevier.

1951, Perkins 1976), or mantle and gills (Allam and Ford 2002). Within the hemocytes, *P. marinus* resides in a phagosome like structure where it proliferates, counteracting possible cytocidal activities such as the production of ROSs that are mounted by the oyster hemocyte against invading microorganisms. Indeed, once live *P. marinus* are engulfed, no measurable accumulation of ROSs is observed. In contrast, killed *P. marinus* elicit a strong oxidative burst (Anderson 1999, Volety and Chu 1995). Two hypotheses, that are not mutually exclusive, can be proposed to explain this phenomenon. (1) *P. marinus* inhibits the production of ROSs through parasite-derived enzymes such as phosphatases and (2) it detoxifies produced ROSs through its own antioxidant enzymes. Schott et al. (2003a) demonstrated that *P. marinus* trophozoites are over a 100-fold less sensitive to $^{\bullet}O_2^-$ and H_2O, compared to *V. splendidus*, a bacterium associated with disease in the Pacific oyster, *C. gigas.* However, both are sensitive to HOCl (Schott et al. 2003a). These findings are congruent with the notion that *P. marinus* is removing ROSs. Antioxidant enzymes that degrade $^{\bullet}O_2^-$ and H_2O_2 are SODs and peroxidases. In *P. marinus*, ample SOD activity has been reported and two iron type SODs, PmSOD1 and PmSOD2, have been identified and characterized. PmSOD1 localizes to the mitochondria and appears to be upregulated upon exposure of *P. marinus* trophozoites to *C. virginica* serum. PmSOD2 is found in a yet unidentified punctuate structure at the cortex of the cell membrane and is constitutively expressed (Ahmed et al. 2003, Gauthier 1998, Schott et al. 2003b, Wright et al. 2002). However, peroxidases have not been reported. Therefore, in the present study the

capability and efficiency of *P. marinus* to remove H_2O_2 was investigated, and it was attempted to identify possible peroxidases in *P. marinus*.

Removal of H_2O_2 by *Perkinsus marinus* and *Vibrio splendidus*

In comparison to *V. splendidus* that has, like other *Vibrio* species, ample CAT activity (Schott et al. 2003a), *P. marinus* removes H_2O_2 at a slightly lower rate (21 nmol/10^8 cells/h vs. 30 nmol/10^8 cells/h, respectively), which appears incongruent with their susceptibilities to it. As mentioned above, *V. splendidus* is over a 100-fold more sensitive to H_2O_2. Indeed, during the assays to measure H_2O_2 removal, conducted at 75 µM H_2O_2, the viability of *V. splendidus* decreased by 99%, while that of *P. marinus* dropped only 4%. A comparison of the removal rate per cell basis may not be ideal for that the removal rate of *V. splendidus* may have been underestimated. In *Vibrio* species, CATs are localized in the cytosol and possibly in the periplasm (Yumoto et al. 2000). In *P. marinus*, neither the identity nor the cellular localization of any H_2O_2 degrading enzyme is known. However, if it is assumed that, similar to *Vibrio* species, peroxidase activity is cellular, then exogenous H_2O_2 must diffuse into the cells through the cell wall, plasma membrane, and, if the peroxidase activity is compartmentalized, through organelle membranes. Thus, factors that will contribute to the ability of cells to degrade H_2O_2 include cell wall/membrane permeability to H_2O_2, the cell volume, and the cell surface to volume ratio. Under the culture conditions used, size heterogenity of *P. marinus* TxSc trophozoites is minimal, and the diameter of the majority of the *P. marinus* TxSc trophozoites is 4 µm (Gauthier and Vasta 1995). Therefore, I estimate that *P. marinus* trophozoites are approximately 34 μm^3

spheres with a cell surface to volume ration of 1.5, while *Vibrio* species are approximately 1.0 μm^3 rods with a cell surface to volume ratio of 6.3 (Jiang and Chai 1996). If the cell volume is taken into consideration, the rate of H_2O_2 removal per cell by *V. splendidus* would be approximately 50-fold higher compared to the ration of the much larger *P. marinus* cells. Conversely, however, slower diffusion of H_2O_2 into the much larger *P. marinus* cells could also result in an underestimation of their internal peroxidatic potential. To what extent the permeability of the cell wall may contribute to the relative efficacy of both organisms to remove exogenous H_2O_2 is difficult to assess, since although both organisms are surrounded by substantial cell walls in addition to plasma membranes (Montes et al. 2002, Perkins and Menzel 1967), the relative permeability of *P. marinus* to H_2O_2, as compared to *V. splendidus*, is unknown.

Identification of peroxidases in *Perkinsus marinus*

Most eukaroytes remove H_2O_2 by CATs. CATs belong to catalytic hydrogen peroxidases (CHPs). In contrast to other peroxidases, CHPs use H_2O_2 as electron donor and acceptor and do not require any additional reductant to degrade H_2O_2 (reviewed in Klotz and Loewen 2003, Noctor and Foyer 1998). CHPs can be divided into three major groups: (1) heme-containing monofunctional CATs that are considered the “classical” CATs, (2) heme-containing bifunctional catalase-peroxidases (CPXs), and (3) non-heme containing CAT. Monofunctional CATs are found both in eukaryotes and prokaroytes. CPXs exhibit, in addition to their catalytic activity, a peroxidatic activity. The majority of CPXs are described from prokaryotes, however, their

presence has also been reported in fungi, but not in other eukaryotes. No non-heme CATs have been reported in eukaroytes (reviewed in Klotz and Loewen 2003). In contrast to *E. coli*, CAT activity in *P. marinus* cell extracts was not detected by "in-gel" assays. CAT gene sequences in *P. marinus* were also not identified by degenerate oligonucleotide-based amplifications from genomic *P. marinus* DNA or by similarity searches of CAT sequences from *H. sapiens*, *T. gondii*, *S. cerevisiae*, and *V. vulnificus* against a *P. marinus* EST database that contains more than 31,000 sequences. However, seven *P. marinus* EST sequences showed significant similarities to a *V. vulnificus* CPX sequence. Interestingly, BLAST similarity searches of the seven *P. marinus* EST sequences revealed high percent similarity to APX sequences of plants and algae, but not to CPXs. APXs belong to class I peroxidases that include CPXs, APXs, and cytochrome C peroxidases. This finding is consistent with the apparent lack of catalytic hydroperoxidase activity of *P. marinus* cell extracts, which would be observed if CATs or CPXs were present, and suggests the presence of APX activities in *P. marinus*.

APX requires the constant presence of ASC as electron acceptor for the reduction of H_2O_2 to H_2O. Therefore, *P. marinus* and *V. splendidus* cell extracts that have been dialyzed in the presence of ASC were tested for their ability to remove H_2O_2 using the FOX assay. Indeed, under these conditions, *P. marinus* cell extracts removed H_2O_2 at a constant rate. *Vibrio splendidus* cell extracts also degraded H_2O_2. When compared to *P. marinus* cell extracts, however, their removal rate was somewhat higher, but the H_2O_2 degrading activity decreased

over time. This observation is consistent with reports of the inactivation of some CAT activities due to high concentrations of H_2O_2 (e.g., DeLuca et al. 1995). To my knowledge, APX activity is not inhibited by H_2O_2. However, whether this phenomenon is relevant to the observations reported here will require further research.

The findings of the present study suggest that *P. marinus* lacks CAT. Even though CAT is used to detoxify H_2O_2 by most eukaryotes there are several other protistan parasites that are also CAT negative, including *Trypanosoma* species and *Leishmania* species (reviewed by Mehlotra 1996). *P. marinus* may possess an APX activity instead. APX activities, even though uncommon in non-autotrophic organisms, have been reported in the protozoan parasites *T. cruzi* and *L. major* (Adak and Datta 2005, Boveris et al. 1980, Wilkinson et al. 2002). APX catalyzes the oxidation of ASC. Using a standard APX assay that follows the oxidation of ASC, significant APX activity was observed in *P. marinus* extracts (Schott et al. 2003a), and two APXs (PmAPX1 and PmAPX2) have been identified in *P. marinus* that are currently further characterized. Immunolocalization studies suggest that APX1 is localized in the cytosol whereas, APX2 has a perivacuolar localization (Schott and Vasta, unpublished data).

As an early sister-group of the dinoflagellate lineage, the Perkinsozoa belong to the Alveolata. Most members of the Alveolata contain secondary plastids that are chloroplast-like organelles. These plastids are thought to be of red or green algae origin that have been acquired by secondary endosymbiontic

events (Stoebe and Maier 2002). Within the Alveolata, genes of plastids have been relocated into the host nucleus through progressive horizontal gene transfer that converted the endosymbionts to semiautonomous organelles under tight control of the host genome (Stoebe and Maier 2002). In *P. marinus*, nucleus-encoded plant-like ferredoxin (ptFd) and ferredoxin reductase (ptFnr) were identified that are molecular markers for plastids and therefore suggest the presence of secondary plastids (Stelter et al. 2007). Thus, it is plausible, that *P. marinus* also obtained the plant-like APX genes through gene transfer from the plastid genome and lost subsequently its own CAT genes, due to the redundant functions of APX and CAT.

In the process of reducing H_2O_2 to H_2O, APXs oxidize ASC to monodehydroascorbate (MDHA), a short-lived radical. MDHA may be rapidly converted into ASC and dehydroascorbate (DHA), or may be reduced by monoascorbate reductases (MDHAR) to ASC using NAD(P)H as electron donor. DHA is reduced to ASC by DHA reductases (DHARs) that oxidize reduced glutathione (GSH) to glutathione disulfide (GSSG) in that process. GSSG in turn is reduced by a glutathione reductase (GR) using NAD(P)H as electron donor (reviewed by Noctor and Foyer 1998).

In *P. marinus*, neither MDHA reductases nor DHA reductases have been identified. However, GR activity has been observed (Schott and Vasta, unpublished data), allowing us to propose an antioxidant pathway for *P. marinus* that is similar to the one described above. SODs dismutase exogenous or endogenous $^{\bullet}O_2^-$ to H_2O_2. Equally, APXs reduce exogenous or endogenous

produced H_2O_2 to H_2O while oxidizing ASC to MDHA. MDHA is reduced by a MDHA to DHA using NAD(P)H as an electron donor and/or converted into DHA and ASC. DHA is reduced to ASC by a yet to be identified DHAR activity oxidizing GSH to GSSG. GSSG is recycled to GSH by GR under the expense of NAD(P)H (Figure 23).

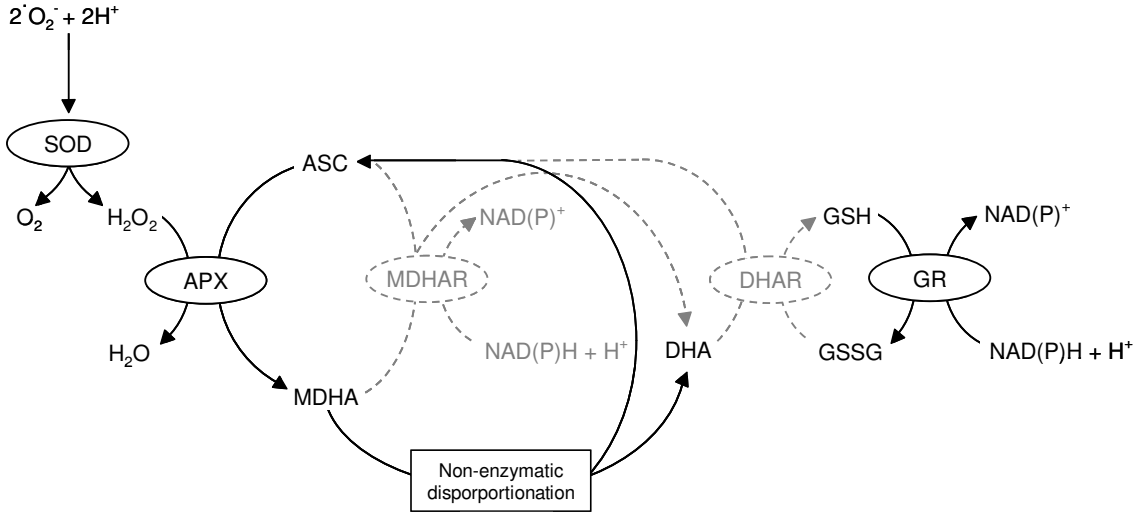


Figure 23. Proposed antioxidant pathway of *Perkinsus marinus*. Dashed lines indicate pathway components that have not yet been identified in *P. marinus*. For a detailed description see text. Modified from Noctor and Foyer (1998).

Other peroxidases such as GPX that in addition to APXs may contribute to the removal of H_2O_2 have not yet been identified in *P. marinus* (Schott and Vasta, unpublished data). However, their presence in *P. marinus* cannot be excluded by the present study. Further analysis of the *P. marinus* genome will aid in the identification of such peroxidases, if present.

To what extent the antioxidant pathway alone protects *P. marinus* against the ROS assault by oyster hemocytes remains unknown, and it is likely that other factors - possibly by modulating the ROS production - contribute to the *P. marinus* defense against ROSs, as proposed by others (Anderson 1999).

CHAPTER 5

IDENTIFICATION AND CHARACTERIZATION OF PHOSPHATASES WITH POTENTIAL ROLE IN INTRACELLULAR SURVIVAL

SUMMARY

Perkinsus marinus is a protistan parasite of a variety of mollusks including the eastern oyster *C. virginica* that appears to be its preferred host. However, the molecular mechanisms that determine its virulence and host preference remain elusive. Infections are initiated by phagocytosis of *P. marinus* trophozoites. In *C. virginica, P. marinus* actively suppresses the oxidative burst normally observed during phagocytosis of yeast or heat-killed *P. marinus*, possibly by interfering with the production of ROSs through parasite-derived enzymes, such as phosphatases. Phosphatases are a heterogeneous group of enzymes involved in a variety of biological processes, including the modulation of host/pathogen interactions. In the present study, the partial biochemical characterization of extracellular ACP activity of *P. marinus* is reported that is comparable to that of other virulent protistan parasites. In addition to the ACP activity, several putative phosphatase sequences were identified from *P. marinus*

ESTs obtained from an ongoing functional genomics project. Among those were a PP2B- and two PP2C-like genes that were designated as *PmPP2B*, *PmPP2C1*, and *PmPP2C2*, respectively. Computational analysis of obtained coding sequences (CDSs) of the three genes suggested that PmPP2C2 possesses a 19 aa long signal peptide. Western blot analysis using crude antiserum against recombinant PmPP2C2 (rPmPP2C2) showed that the crude antiserum recognized several proteins of *P. marinus* cell extracts that were separated by SDS-PAGE. Immunoaffinity-purified anti-rPmPP2C2 IgGs recognized only one protein band in *P. marinus* cell extracts separated under non-denaturing PAGE. However, no protein was recognized in culture supernatants, indicating that PmPP2C2 may not be secreted under the conditions tested. Preliminary analysis of the expression pattern of the *PmPP2C2*-like gene by Western blot analysis of *P. marinus* trophozoites exposed to ASW and serum of *C. virginica* and *M. mercenaria* failed to reveal clear evidence of a direct involvement of the PmPP2C2 in host preference or virulence of *P. marinus*.

INTRODUCTION

Perkinsus marinus is a protistan parasite first described in the eastern oyster *C. virginica* in the Gulf of Mexico (Mackin et al. 1950). It is recognized as a highly pathogenic organism for *C. virginica* that led to mass mortalities in farmed and natural *C. virginica* populations. Its current distribution range extends from the Yucatán Peninsula (Mexico) in the south as far north as Maine (USA) (Burreson et al. 1994, Ford 1996, Chapter 3). Recent studies showed that

P. marinus can also be found in other bivalve species such as *M. arenaria*, *M. balthica*, and *M. mercenaria* (Coss 2000, Coss et al. 2001b, Kotob et al. 1999a, Chapter 3). However, the proliferation rate of *P. marinus* is reduced *in vitro* upon exposure to host plasma of several bivalves [*M. mercenaria*, *Anadara ovalis*, *M. edulis*, *C. gigas* and *C. ariakensis* (syn. *C. rivularis*)] (Gauthier and Vasta 2002). Together with results from the distribution study presented in Chapter 3, these observations suggest a host preference of *P. marinus* for its type host *C. virginica*.

Infections are initiated by phagocytosis of *P. marinus* by host hemocytes. To establish an infection, *P. marinus* must be able to overcome or evade host defense responses, acquire nutrients, and proliferate. Host defense reactions include the production of ROSs by host hemocytes. However, the oxidative burst that is normally observed in oyster hemocytes, does not take place upon phagocytosis of live *P. marinus* (Anderson 1999, Volety and Chu 1995), suggesting that *Perkinsus* trophozoites remove ROS through antioxidant enzymes (e. g. SODs, APXs) and/or express enzymes that inhibit the production of ROS.

Phosphatases have been identified in other parasites as factors that may interfere with ROS production. Phosphatases play crucial roles in intracellular survival of a variety of protistan parasites and microbial pathogens. Not only have phosphatase been proposed to modulate host cell responses such as the inhibition of ROS production, but also to facilitate entry into a host cell, acquisition of nutrients, and to regulate expression of virulence factors

(Archambaud et al. 2005, Lovelace et al. 1986, Reilly et al. 1996, Remaley et al. 1985b, Saha et al. 1985, Vannier-Santos et al. 1995, Xia et al. 2001).

Phosphatases are a heterogeneous group of enzymes that have been classified based on physiological criteria (e.g. ACPs and alkaline phosphatases), substrate specificity (e.g. non-specific phosphatases, PPs, PTPs, and DSPs) or sequence similarities (e.g. histidine ACPs, PPP and PPM families of PPs, and PTPs). Phosphatases suggested to be involved in host/pathogen interactions include ACPs, PPs of the types 2A, 2B, and 2C, and PTPs (e.g. Archambaud et al. 2005, Fox et al. 2001, Reilly et al. 1996, Remaley et al. 1985b, Saha et al. 1985, Sue-A-Quan et al. 1995, Takekawa et al. 1998, Warmka et al. 2001, Yamamori et al. 2000, Gilbert et al. 2007).

Intracellular and extracellular ACP activity has been reported in *P. marinus* (Volety and Chu 1995, 1997). Attempts to localize ACP activity by transmission electron microscopy revealed activity around the nucleus, which led to the conclusion that ACPs might be involved in cell cycle regulation (Volety and Chu 1997). However, the histological staining protocol used would detect most phosphatase activity and lacks specificity for the enzyme of interest (e.g. a secreted ACP). Later, Chu et al. (1998b) reported that more virulent strains have higher ACP activity than less virulent strains.

Except for the preliminary work on ACP activities and the sequence of a putative protein phosphatase 1 (PP1) that appears to be constitutively expressed at the transcriptional level (Coss and Vasta, unpublished data, Gauthier 1998), phosphatases of *P. marinus* have not been characterized in detail. Here the

identification of several putative phosphatase genes is reported, including a PP2C-like gene that was designated as *PmPP2C2*. Gene expression studies using polyclonal antibodies raised against rPmPP2C2 were performed to assess its role in intracellular survival and host preference of *P. marinus*. In addition, the extracellular ACP activity of *P. marinus* was further characterized and partially purified in an attempt to identify and characterize their genes.

MATERIALS AND METHODS

Perkinsus **cultures**

P. marinus TxSc (ATCC 50849), *P. marinus* CB5D4, *P. andrewsi* (ATCC 50807), and *Perkinsus* sp. (*M. mercenaria*) were maintained at 28 ℃ in DMEM:HAM's F12 (1:2) medium supplemented 5% (v/v) FBS and 15 ppt ASW (Crystal Sea Marine Mix, Marine Enterprises International, Inc., Baltimore, MD, USA) following Gauthier and Vasta (1995).

Protein extractions

Cultures of *P. marinus* TxSc, and *P. marinus* CB5D4 were centrifuged at 400 x g for 20 min. The culture supernatant was collected, sterilized by passing through Acrodisc® syringe filters with a 0.2 µm Supor® membrane (Pall Corporation, Ann Arbor MI, USA), and stored at -20 ℃ until further analysis. Cell pellets were washed once in 0.5 M NaCl and resuspended in extraction buffer (50 mM Tris Cl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM PMSF). The

slurry was transferred to round bottom polystyrene or glass tubes to which glass beads were added. The samples were vortexed for 1 min at maximum speed at room temperature. Alternatively, cells were osmotically lysed in low salt buffer (25 mM Tris Cl or 25 mM phosphate buffer, pH 7.2, 0.5% (v/v) Triton X-100, 1 mM PMSF).

Total cell extracts were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 14,000 x g for 30 min at 4 ℃ to remove cell debris. Cell extracts were stored at -20 ℃ until further analysis.

Measurement of protein concentration

If not otherwise stated, protein concentrations were measured using the bicinchoninic acid (BCA) protein assay from Pierce Biotechnology (Pierce) following the manufacturer's recommendations.

Polyacrylamide gel electrophoresis

Samples were subjected to continuous or discontinuous polyacrylamide gel electrophoresis (PAGE) under denaturing and non-denaturing conditions. For continuous PAGE, precast Ready gel Tris HCl gels containing a gradient of 10-20% acrylamide from Biorad (Bio-Rad Laboratories, Hercules, CA, USA) were used. Electrophoresis was performed in a Mini Protean III cell (Bio-Rad Laboratories) under constant voltage of 200 V. For discontinuous PAGE, gels were prepared following the method of Laemmli (1970). Briefly, for denaturing PAGE, separating gels contained 10% to 12% polyacrylamide in resolving gel

buffer (375 mM Tris Cl, 0.1% (w/v) SDS, pH 8.8). Stacking gels contained 4% polyacrylamide, in stacking gel buffer (125 mM Tris Cl, 0.1% (w/v) SDS, pH 6.8).

Gels were run at a constant voltage of 200 V at 10 °C on a Hoefer SE 250 vertical electrophoresis unit (Hoefer Inc., San Fransisco, CA, USA) using a electrophoresis buffer containing 196 mM glycine, 50 mM Tris Cl pH 8.3, 0.1% (w/v) SDS.

The composition of stacking and separating gels as well as the electrophoresis buffer used for non-denaturing PAGE were identical to those used in denaturing PAGE except that SDS was omitted.

Detection of phosphatase activity

To detect phosphatase activities standard colorimetric assays were adapted to a 96 well plate format. Briefly, to each well 20 µl sample and 180 µl of the adequate reaction buffer that contained 2 mg/ml disodium-p-nitrophenol phosphate (pNPP) (Sigma-Aldrich) were added. The plate was incubated at 37 °C for 30 min. The reaction was stopped by adding 80 µl of a 2 M NaOH solution.

For acid phosphatase detection, the reaction was conducted in 0.5 M citrate buffer (pH 4.5). Protein phosphatase activity was assessed in 50 mM Tris Cl (pH 7.4), supplemented with 1mM $MnCl_2$, $CaCl_2$ and $MgCl_2$, and alkaline phosphatase activity was assessed using 50 mM Tris Cl (pH 8.0). The amount of p-nitrophenol (pNP) released was measured colorimetrically at a wavelength of 405 nm using a SpectraMax 340 plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). As a reference, serial diluted pNP (Sigma-Aldrich) (50

nmol to 5 nmol) was used. The assays were blanked against the buffers that contained the samples to be tested. A unit was defined as nmol pNP produced per hour.

Optimization of an acid phosphatase "in-gel" assay

To detect acid phosphatase activity in polyacrylamide gels, an assay reported by Allen et al. (1963) and developed to detect ACP activity in starch gels was optimized as followed. 800 mU of potato ACP type II and *P. marinus* spent culture medium with 3 U of ACP activity were run on 10% polyacrylamide gels under non-denaturing conditions. The polyacrylamide gels were rinsed in double distilled H_2O and incubated at room temperature for 2 h under constant agitation in either 0.1 M acetate buffer (pH 4.8) or 0.5 M citrate buffer (pH 4.5). Both buffer systems contained 1mg/ml α-naphtylphosphate (Sigma-Aldrich) and 1 mg/ml fast garnet GBC (Sigma-Aldrich). After incubation, gels were rinsed in double distilled H_2O and scanned at a resolution of 300 to 600 dpi.

Determination of the pH optimum

The phosphatase activities of filter sterilized spent culture medium collected from a late log phase *P. marinus* culture were assessed using the modified colorimetric assay described above, except that the samples were incubated in 0.5 M citrate buffers ranging from pH 2.5 to 6.0.

Effect of sodium-(L+)-tartrate on the extracellular acid phosphatase activity of *Perkinsus marinus*

ACP activities of filter-sterilized spent culture medium collected from a late log phase *P. marinus* culture were assessed as described above except that the samples were incubated in 0.5 M citrate buffer (pH 4.5) containing 2 mg/ml p-nitrophenol and 100 µM, 250 µM, 500 µM, 1 mM, 2.5 mM, 5 mM, 10 mM, or 20 mM sodium-(L+)-tartrate, respectively. Phosphatase activities were compared to phosphatase activities of culture supernatant incubated in the same buffer without sodium-(L+)-tartrate.

Partial purification of *Perkinsus marinus* extracellular acid phosphatase activity

Filter-sterilized spent culture medium obtained from late log phase *P. marinus* culture was concentrated 70-fold by ultrafiltration through a Amicon® PM polyethersulfone membrane with a molecular weight cut-off of 10,000 (Millipore, Billerica, MA, USA). The concentrated medium was dialyzed exhaustively against 20 mM Tris Cl (pH 8.0). The dialyzed supernatant was applied to a Q-sepharose column and eluted at 100 mM NaCl, 150 mM NaCl and 1 M NaCl (100 ml each). Fractions (20 ml) were analyzed for ACP activity and protein concentration. Active fractions were pooled, dialyzed exhaustively against 20 mM Tris Cl (pH 8.0), and subsequently applied to a MonoQ HPLC column and eluted at a flow rate of 1 ml/min with a linear gradient from 100 mM to 300mM NaCl. All fractions (1 ml) were collected and analyzed for ACP activity and protein concentration. Active fractions were pooled, concentrated, and size-

separated on a superose 12 HR column at a flow rate of 100 µl/min. All fractions were collected and analyzed for ACP activity and protein concentration. The fraction with the highest ACP activity was analyzed for purity by SDS PAGE and Coomassie stain.

Anionexchange chromatography using the MonoQ HPLC column and size exclusions were performed on an ÄKTApurifier™ system (Amersham Bioscience, General Electric Company, Fairfield, CT, USA).

SDS PAGE and non-denaturing PAGE analysis was performed on aliquots of the pooled active fractions from both anion exchange chromatography steps and active fractions obtained from the gelfiltration step. Gels were either stained for protein using Coomassie Brilliant Blue (R250) or BioSafe Coomassie (Bio-Rad Laboratories) or analyzed for ACP activity using the optimized "in-gel" assay described above. Coomassie stained SDS polyacrylamide gels were scanned at a resolution of 600 dpi. The digitalized images were analyzed using the software NIH Image 1.62 (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). To account for possible loading differences in each lane, band intensities were standardized to the intensity of a prominent band at approximately 45 kDA.

Identification and characterization putative phosphatase genes

To identify putative *P. marinus* phosphatase genes, a preliminary EST database (PerkDB; Kissinger, Robledo, and Vasta, unpublished data) with approximately 2,500 sequences from an ongoing functional genomic project was searched for putative phosphatase sequences. Based on selected EST

sequences primers were designed to PCR amplify *P. marinus* TxSc cDNA sequences from λ-zap cDNA libraries. PCR products were separated by electrophoresis on 1% agarose gels and visualized by staining with EtBr (0.5 µg/ml). Amplicons were gel-purified using the QIAquick Gel Extraction kit (QIAGEN), cloned into *E. coli* JM109 using the Promega pGEMII vector system (Promega Corporation) and sequenced. 3' ends of putative phosphatase cDNAs were obtained by PCR of the λ-zap cDNA libraries using gene-specific primers and the primers T7 (sense 5'-GTA ATA CGA CTC ACT ATA GGG C-3') and T3 (antisense 5'- AAT TAA CCC TCA CTA AAG GG-3') (Stratagene, La Jolla, CA, USA). 5' ends were obtained by RNA ligase-mediated rapid amplification of 5´ cDNA ends (5' RLM-RACE) (Invitrogen). PCR products were size-separated, gel-purified, cloned and sequences as described above. cDNA sequences were assembled by fragment assembly using GCG (GCG Wisconsin Package, v10.2, Accelrys Software Inc., Burlington, MA) and translated using either Translate (available at http://us.expasy.org) or Map and ExtractPeptide (GCG Wisconsin Package, v10.2, Accelrys Software Inc., Burlington, MA). Molecular weight (MW), isoelectric point (pI), and cellular localization of the deduced aa sequences were predicted using the web based tools Compute pI/MW (available at http://us.expasy.org), TargetP v.1.0 (Emanuelsson et al. 2000), SignalP v.3.0 (Bendtsen et al. 2004), and WoLF PSORT (Horton et al. 2006). Similarity searches were performed using protein-protein BLAST (BLASTP) and BLASTX analysis (Altschul et al. 1990). In addition, deduced aa sequences were

analyzed for possible conserved domains by searching the Conserved Domain Database (CDD) v2.10 at the NCBI (Marchler-Bauer et al. 2005).

To obtain genomic sequences, the TIGR db *Perkinsus marinus* database (www.tigr.org) was searched with partial cDNA sequences using BLASTN with default parameters (Altschul et al. 1990). In addition, partial genomic sequence was obtained by PCR of genomic *P. marinus* DNA with gene-specific primers, cloning and sequencing. Sequences were either pair-wise aligned using ClustalW with default parameters (Thompson et al. 1994) or GAP (GCG Wisconsin Package, v10.2, Accelrys Software Inc.) with default parameters. When necessary, contigs were assembled with Sequencher v.4.2 (Gene Codes Corp., Ann Arbor, MI, USA), by fragment assembly using GCG (GCG Wisconsin Package, v10.2, Accelrys Software Inc.), or with the Staden Package v1.6.0. Genomic sequences were aligned with the respective cDNA sequences by fragment assembly (GCG Wisconsin Package, v10.2, Accelrys Software Inc.), or with the Staden Package v1.6.0 and adjusted by eye.

Recombinant expression of a putative protein phosphatase 2C of *Perkinsus marinus*

The CDS of a putative protein phosphatase 2C gene from *P. marinus* (hereafter referred to as *PmPP2C2*) was inserted in a pET30 Ek/Lic vector using the Ek/Lic cloning kit (NOVAGEN, EMD Biosciences, Inc., Madison, WI). Briefly, the CDS was PCR amplified from a λ-zap cDNA library using the primers rPP2C2-F1 (sense 5'-GAC GAC GAC AAG ATG TCG GGT CTA ATC CGG CCC-3') and rPP2C2-R2 (antisense 5'-GCC ATA CCA CTA GTG GTT GAT AAA

CCG GGC TTC TCC TC-3'). Both primers contain the adapter sequence for enterokinase ligation independent cloning (Ek/Lic) (underlined). The PCR reaction mixture consisted of 5 mU TaKaRa *Ex Taq*™ DNA polymerase (TaKaRA Bio Inc.), the proprietary Ex Taq™ reaction buffer (contains 2 mM Mg^{2+} at final concentration, TaKaRa Bio Inc.), 200 µM of each dNTP, and 10 pmole of each primer. As a positive control, 50 ng of genomic *P. marinus* DNA was used. In the negative PCR control, the DNA template was substituted by double distilled sterile H_2O. Cycling conditions were 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, with a final extension at 72 °C for 7 min. PCR products were size-separated, gel-purified, cloned and sequenced as described above. Sequences were pair-wise aligned with cDNA sequence of *PmPP2C2* using GAP (GCG Wisconsin Package, v10.2, Accelrys Software Inc.). One clone containing the CDS and the adapters for insertion into the pET-30 Ek/Lic vector was PCR amplified with the primers rPP2C2-F1 and rPP2C2-R2 using KOD DNA polymerase (Novagen, EMD Biosciences). The PCR reaction mixture consisted of reaction buffer (120 mM Tris-HCl, 10 mM KCl, 6 mM $(NH4)_2SO_4$, 0.1% Triton X-100, 0.001% BSA, pH 8.0), 1 mM $MgCl_2$, 200 µM each of dNTP, 0.4 µM of each primer and 1 U KOD DNA polymerase. The cycling conditions were as follows. 25 cycles of 98 ℃ for 15 sec, 63 ℃ for 2 sec, and 72 ℃ for 20 sec. The PCR products were separated on 1.5% agarose gels prestained with 0.5 µg/ml EtBr and amplicons of expected size cored and gel-purified. Gel-purified products were inserted in a pET30Ek/Lic vector by Ek/Lic (Novagen, EMD Biosciences) following the manufacturer's recommendations.

To verify the sequence and junctions, plasmids were transformed into *E. coli* NovaBlue competent cells (Novagen, EMD Biosciences) according to the manufacturer's recommendation and sequenced. For recombinant expression, verified constructs were transformed into *E. coli* BL21 (DE3) using standard protocols.

To express rPmPP2C2, *E. coli* BL21 (DE3) carrying the expression construct were incubated in LB supplemented with Kanamycin at 37 ℃ to an OD_{600} of 0.4 to 1. Bacteria were placed at 22 ℃ and expression induced by adding different amounts of isopropyl 1-thio-D-galactopyranoside (IPTG) (0.1 to 1 mM final concentration) to the bacterial suspension. Bacteria were incubated at either 22 ℃ or 37 ℃ for 2 h. Cells were collected by centrifugation at 5,000 to 10,000 x g for 5 min at room temperature. Supernatants were discarded and cell pellets stored at -20 ℃. Cell extracts and insoluble fractions were prepared and purified using BugBuster® (Novagen) following the manufacturer's recommendation. Expression and localization of rPmPP2C2 was assessed by SDS-PAGE as described above.

Purification of recombinant expressed *Perkinsus marinus* protein phosphatase 2C2

To purify rPmPP2C2, insoluble fractions were obtained as described above, solubilized in 6 M guanidine HCl, 50 mM phosphate buffer, 300 mM NaCl (pH 7.0), dialyzed against 8 M urea, 50 mM Tris Cl (pH 7.5) and size-separated by continuous SDS-PAGE as described above. Gels were stained with 300 mM $CuCl_2$ for 5 min. Protein bands of interest (approximately 54 kDa in size) were

cut, gel slices destained in 250 mM EDTA and 250 mM Tris Cl (pH 7.4), and equilibrated in electroelution buffer (0.1% (w/v) SDS, 25 mM Tris Cl, 192 mM glycine, pH 8.3). Gel slices were minzed and proteins electroeluted into Centricon® YM 10 centrifugal filter devices (Millipore) using a Centriluter® Micro-Electroeluter (Millipore) for 2 h at a constant voltage of 200 V. Electroeluted fractions were pooled and concentrated by centrifugation using Centricon® plus-20 and YM 10 centrifugal filter devices at conditions recommended by the manufacturer (Millipore). Protein concentration was measured by the BCA assay (Pierce). To assess purity, electroeluted proteins were separated by continuous SDS-PAGE and gels stained with Coomassie.

Generation of polyclonal antisera against recombinant expressed *Perkinsus marinus* protein phosphatase 2C2

To generate antisera against rPmPP2C2, two New Zealand white female rabbits were immunized with purified rPmPP2C2 by a commercial vendor (Open Biosystems, Huntsville, AL, USA). Pre-bleeds and crude sera collected at 28 d, 56 d, and 70 d post primary immunization were obtained and stored at -20 °C. The titer of the crude antisera was assessed by the vendor using an enzyme-linked immunosorbent assay (ELISA).

Purification of immunoglobulin G and anti-rPmPP2C2 antibodies

The immunoglobulin G (IgG) fraction was purified from the crude antisera by protein A-Sepharose affinity chromatography following standard protocols. Briefly, a protein A-Sepharose 4B matrix (Invitrogen) was packed into a Poly-

Prep column (Bio-Rad Laboratories) and equilibrated in phosphate buffer saline (PBS; 20 mM phosphate buffer, 150 mM NaCl, pH 7.2). Three column volumes of crude antiserum were diluted in equal volume PBS, applied to the column and used to resuspend the matrix. The mixture was incubated in the column for 1 h at room temperature or, alternatively, for 16 h at 4 °C under constant agitation. After the matrix settled, the flow through was collected. The column was washed with PBS and wash fractions monitored by absorption at a wave length of 280 nm (A_{280}). After the A_{280} reached the baseline, bound IgGs were eluted by applying 100 mM acetic acid in 150 mM NaCl. Eluates were collected in fractions of one half of the column volume in collection tubes containing 50 µl of 1.9 M Tris base/ml eluate. Eluate fractions containing protein (as judged by A_{280}) were pooled and dialyzed against PBS containing 0.02% (w/v) sodium azide at 4 °C. Purified IgGs were preabsorbed against total cell extracts of *E. coli* BL21(DE3) strains transformed with an expression construct containing the CDS of PmAPX2 and against fresh DMEM:HAM's F12 (1:2) culture medium.

Specific IgGs against rPmPP2C2 (anti-rPmPP2C2 antibodies) were purified from preabsorbed IgGs by rPmPP2C2-agarose affinity chromatography using the same procedure described above.

Coupling of recombinant *Perkinsus marinus* protein phosphatase 2C2 to agarose matrices

Recombinant PmPP2C2 (solubilized in 4 M guanidine HCl, 100 mM phosphate buffer, 0.05% sodium azide, pH 7.5) was coupled to 4% cross-linked beaded agarose that has been activated to form aldehyde functional groups.

Activated agarose beads (AminoLink® Coupling Gels) were purchased from Pierce. Coupling was conducted according to the manufacturer's recommendations.

Western blotting

Western blot analysis was conducted to validate the antisera, purified IgGs and anti-rPmPP2C2 antibodies, to assess the localization of putative PmPP2C2 and its possible association to observed ACP activities, and to monitor the expression pattern of putative PmPP2C2. Briefly, proteins separated on non-denaturing or denaturing polyacrylamide gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Micron Separations Inc. Westboro, MA, USA) by electroblotting following Ursitti et al. (1995). Prior to the transfer, PVDF membranes were activated in 100% methanol for 5 min and equilibrated in Towbin buffer (25 mM Tris base, 192 mM glycine, 20% (v/v) methanol) for 20 min at room temperature. Non-denaturing polyacrylamide gels were incubated in SDS containing electrophoresis buffer for 20 min and subsequently equilibrated in Towbin buffer for 20 min at room temperature. Denaturing polyacrylamide gels were equilibrated in Towbin buffer without preincubation in SDS containing electrophoresis buffer. Electrotransfer was performed in a TE 22 Mini Transfer Tank unit (Hoefer) at 400 mA for 1 h at 10 °C using Towbin buffer as transfer buffer. PVDF membranes were blocked in blocking buffer (PBS, 0.05% (v/v) Tween 20 (PBST), supplemented with 3% (w/v) skim milk) over night at 4 °C under constant agitation. After blocking, PVDF membranes were incubated in blocking buffer containing either antiserum raised against rPmPP2C2, purified

IgGs that were preabsorbed against *E. coli* cell extracts and DMEM:HAM's F12 (1:2) medium, or anti-rPmPP2C2 antibodies for 1 h at room temperature and washed 5 times 15 min each in PBST. Blots were then incubated for 1 h at room temperature in the dark in blocking buffer containing anti-rabbit IgG conjugated to HRP (Pierce). Blots were washed 5 times 15 min each, and transferred to PBS for 5 min. Blots were developed for 5 min with SuperSignal West Pico substrates from Pierce and exposed to autoradiographic film (Pierce CL-XPosure film) for up to 20 min.

Validation of the antibodies and immunolocalization of the putative *Perkinsus marinus* protein phosphatase 2C2

To validate the antiserum, purified IgGs, and anti-rPmPP2C2 antibodies and to assess the localization of putative PmPP2C2, spent culture medium and cell extracts of *P. marinus* CB5D4 and TxSc (50 ug protein each) were separated on SDS-polyacrylamide gels. Proteins were blotted onto PVDF membranes that were probed with crude antisera and purified IgGs (at a dilution of 1:1000). In addition, blots containing *P. marinus* cell extracts and culture supernatant were probed with anti-rPmPP2C2 antibodies (at a dilution of 1:100 and 1:200). HRP-conjugated goat-anti rabbit antibodies were used at dilutions of 1:20,000 (for blots probed with crude antisera and purified IgGs) and 1:5,000 (for blots probed with anti-rPmPP2C2 antibodies).

Assessment of the possible association of the putative *Perkinsus marinus* protein phosphatase 2C2 to acid phosphatase activities

Spent culture medium and total cell extracts of late log phase *P. marinus* cell cultures were separated on non-denaturing polyacrylamide gels. Gels run in parallel were blotted onto PVDF membranes, stained for ACP activity, and for protein with Coomassie. Blots were probed with crude antiserum at a dilution of 1:1000 and HRP conjugated goat-anti rabbit antibody at a dilution of 1:10,000.

Evaluation of the expression pattern of the putative *Perkinsus marinus* protein phosphatase 2C2

To evaluate the gene expression pattern of PmPP2C2, trophozoites of *P. marinus* CB5D4 were exposed to sera collected from *C. virginica* and *M. mercenaria*.

Experimental cultures were obtained by seeding 1L DMEM:HAM's F12 (1:2) culture medium with *P. marinus* CB5D4 at a final cell density of 7.7×10^6 cells/ml ($O.D._{600} = 0.5$). The cultures were grown to late log phase (six days) in a shaking incubator at 140 rpm at 28 °C. Cells were pelleted by centrifugation (400 x g) for 10 min at room temperature and washed twice in 30 ppt ASW (Crystal Sea Marine Mix, Marine Enterprises International, Inc.). Cell pellets were resuspended in 30 ppt ASW to a cell density of 2.25×10^9 cells/ml and 3.5 ml aliquots added to the respective experimental medium (60 ml each).

Prior to seeding, the pH of experimental media was adjusted with 50 mM HEPES buffer to pH 7.6. The osmolality of the media was measured with a

Vapro® Vapor Pressure Osmometer 5520 (Wescor Inc., Logan, UT, USA) and adjusted to an osmolality between 742 mOsm/kg and 745 mOsm/kg.

Experimental media were sera obtained from *M. mercenaria* with a osmolality of 742 mOsm/kg and a protein concentration of 0.5 mg/ml, and *C. virginica* with a osmolality of 742 mOsm/kg and a protein concentration of 13.5 mg/ml. As controls, standard DMEM:HAM's F12 (1:2) with a osmolality of 832 mOsm/kg, ASW, and, to account for possible effects of proteins on the gene expression of *P. marinus*, ASW supplemented with 0.5mg/ml and 13.5mg/ml with osmolalities of 744 mOsm/kg, 745 mOsm/kg, and 745 mOsm/kg, respectively, were used.

To prepare sera, hemolymph of 74 *M. mercenaria* and 80 *C. virginica* that tested negative for *Perkinsus* species with the genus-specific PCR-based assay (Robledo et al. 2002) using modified PCR conditions described in Chapter 3 was collected from the adductor mussel and pooled. Hemocytes were pelleted by centrifugation at 400 x g at 4 °C for 20 min, and the pellet resuspended in 1 ml cell free hemolymph. Hemocytes were lysed by several passes through a syringe with a 21 gauge needle. Cell debris was removed by centrifugation at 14,000 x g for 30 min at 4 °C and the supernatant added to the respective cell free hemolymph. The protein concentration of the sera was estimated using the BCA assay (Pierce). All media were filter-sterilized.

P. marinus trophozoites were maintained in the experimental media for 48 h at 28 °C at constant agitation (100 rpm). Four to eight ml aliquots were removed at 30 min, 2 h, 6 h, 12 h, 24 h, and 48 h, respectively. At each time

point cells were collected as follows. Cells were pelleted by centrifugation at 400 x g at room temperature for 10 min and washed twice in 30 ppt ASW. In addition, prior to exposure to the experimental media, untreated *P. marinus* cells and cells washed twice in ASW were collected. Cell pellets were either immediately frozen at -80 ℃ or resuspended in Tri® Reagent (Sigma-Aldrich) for rRNA extraction prior to freezing. Culture supernatants were collected, passed through Acrodisc® syringe filters with a 0.45 µm HT Tuffryn® membrane (Pall Corporation) to remove possible cells, and stored at -80 ℃.

RESULTS

Determination of the pH optimum

To determine the pH optimum of the extracellular phosphatase activity found in *P. marinus* culture supernatant, culture supernatants were incubated in citrate buffer at varying pH values (2.5 to 5.5). The activity was highest at pH 4.5 (Figure 24). In addition, to detect possible alkaline phosphatase activity, *P. marinus* culture supernatant was incubated in Tris Cl buffer (pH 8.0). However, under alkaline conditions, no activity was observed.

Under optimal pH conditions, culture supernatant of *P. marinus* cultures grown to late log phase (8 d) exhibited an ACP activity of 6.9 U/10^6 cells.

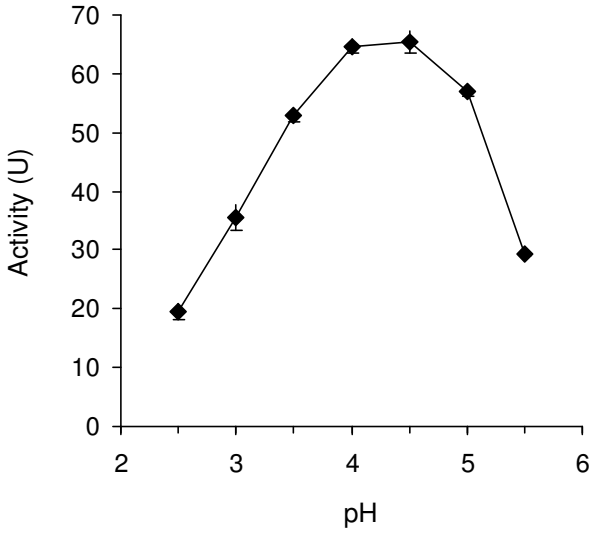


Figure 24. Effect of pH on the extracellular ACP activity of Perkinsus marinus. One Unit (U) is defined as one nmol pNP produced in one h at 37 ℃. Error bars represent the standard deviation.

Effect of sodium-(L+)-tartrate on the extracellular acid phosphatase activity of *Perkinsus marinus*

ACPs that are proposed to inhibit ROS production by mammalian neutrophils are considered to be resistant to sodium-(L+)-tartrate. I therefore investigated its effects on the ACP activity observed in *P. marinus* culture supernatant. Sodium-L(+)-tartrate showed a dose dependent inhibitory effect on the *P. marinus* secreted ACP (sACP) activity. 0.1 mM sodium-(L+)-tartrate inhibited *P. marinus* sACP by 13%, and 0.5 mM sodium-(L+)-tartrate by 33%. At concentrations of sodium-(L+)-tartrate above 10 mM, the *P. marinus* sACP activity was greatly reduced (Figure 25).

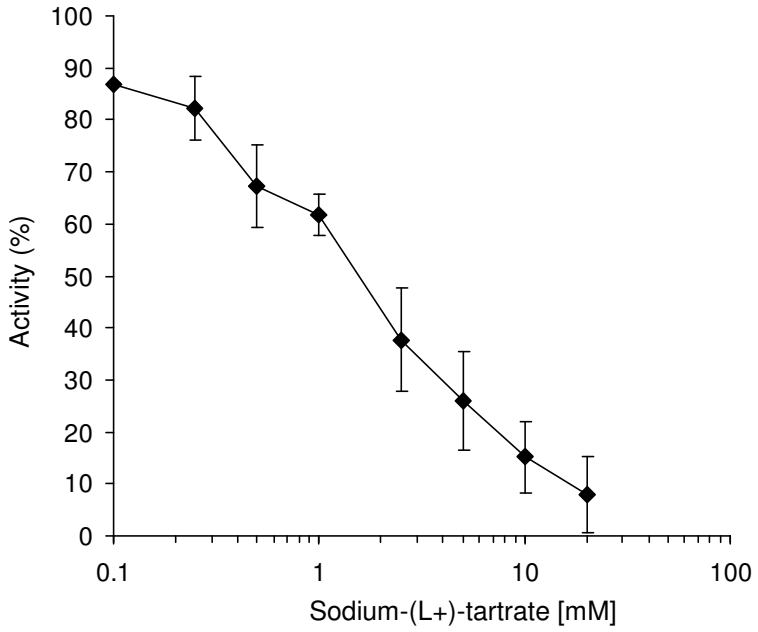


Figure 25. Effect of sodium-(L+)-tartrate on the ACP activity observed in *P. marinus* culture supernatant. Culture supernatant of *P. marinus* was incubated in the presence of different concentrations of sodium-(L+)-tartrate (0.1 to 20 mM) and its effect on the ACP activity compared to the ACP activity in control samples (no sodium-(L+)-tartrate added). Error bars represent the standard deviation.

Optimization of an acid phosphatase "in-gel" assay

To detect ACP activities in non-denaturing polyacrylamide gels, an assay designed for detection of ACP activities in starch gels (Allen et al. 1963) was optimized. Under the conditions described by Allen et al. (1963), ACP activity was observed in the control (potato ACP type II). Only weak activity was observed in active fractions of *P. marinus* culture supernatant eluted from an anion exchange column (Figure 26 B). However, if the acetate buffer (0.1 M, pH 4.8) that is used for the detection of ACP activities in starch gels was replaced by

0.5 M citrate buffer (pH 4.5) - the same buffer that is used in the microtiter plate assay - ample ACP activity was observed in both samples (Figure 26 C).

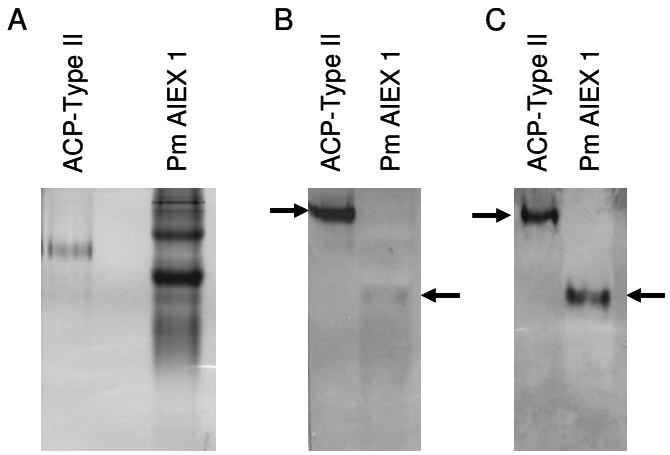


Figure 26. Optimization of an ACP "in-gel" assay. Proteins that have ACP activity appear as dark bands (arrows). **A.** Non-denaturing polyacrylamide gel stained with Coomassie. **B.** Non-denaturing polyacrylamide gel stained for ACP activity using 0.1 M acetate buffer (pH 4.8) as reaction buffer. **C.** Non-denaturing polyacrylamide gel stained for ACP activity using 0.5 M citrate buffer (pH 4.5) as reaction buffer. ACP II: 800 mU of potato ACP type II; PmAIEX1: 3 U of an active fraction of *P. marinus* culture supernatant eluted from an anionexchange column.

Partial purification of *Perkinsus marinus* extracellular acid phosphatase activity

To purify ACP activity observed in *P. marinus* culture supernatant, concentrated culture supernatant dialyzed against 20 mM Tris Cl (pH 8.0) was first loaded onto a Q-sepharose column. Under these conditions, the ACP activity was retained on the column and eluted at 100 mM NaCl and 150 mM (Figure 27). The activity in the fractions collected from the first anion exchange increased by 2.6-fold and contained 80% of the starting activity (Table 8). Subsequently, the active fraction was loaded onto a MonoQ column and eluted using a salt gradient from 100 mM NaCl to 300 mM NaCl. The ACP activity

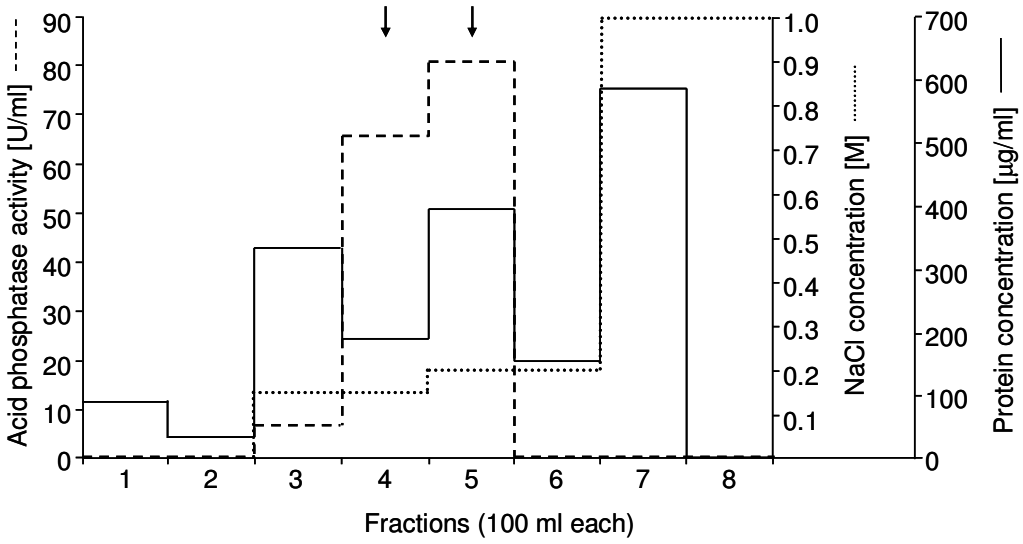


Figure 27. Q-Sepharose anion exchange chromatography using a discontinuous salt gradient. Active fractions (arrows) were collected and further purified.

Table 8. Summary of the partial purification of the secreted ACP activity. AIEX-1: Q-sepharose anionexchange chromatography; AIEX-2: MonoQ anionexchange chromatography; SEC: Size exclusion chromatography.

Purification step	Total activity (kU)	Total Protein (mg)	Specific activity (kU/mg protein)	Purification fold	Yield (%)
Culture supernatant	18.06	185.20	0.098	1.0	100
AIEX-1	14.54	58.10	0.250	2.6	80
AIEX-2	10.72	8.30	1.280	13.0	56
SEC (B-07)	0.22	0.15	1.512	15.6	1

eluted as a single peak between 150 mM and 200 mM NaCl (Figure 28). The activity increased 13-fold compared to the starting material, and the active fractions contained 59% of the starting activity (Table 8). Size exclusion

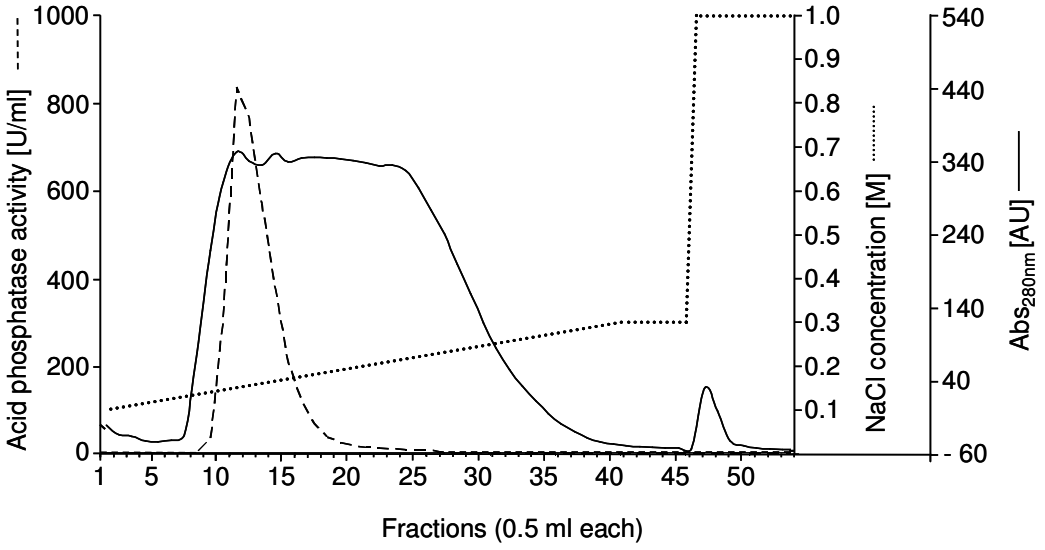


Figure 28. MonoQ anion exchange chromatography using a linear salt gradient. Active fractions were pooled and further purified.

chromatography using a Sephadex HR 12 column resulted in major activity peak that did not coincide with the major protein peak. In addition, a minor activity of smaller molecular weight was observed (Figure 29). In the most active fraction, the activity increased further (16-fold compared to the starting material), however, the yield was low (1% of the starting activity) (Table 8). The collected fractions from the purification steps were subjected to discontinuous non-denaturing and SDS PAGE. Each sample analyzed contained 25 µg protein. Staining of non-denaturing gels for ACP activity using the optimized "in-gel" assay showed that with each purification step the intensity of the active band increased (Figure 30 A), while an equal amount of protein was loaded. Coomassie stain of non-denaturing and SDS-containing polyacrlyamide gels suggested that the complexity of the samples was somewhat reduced (Figure 30 B, C). However, the fraction collected from the size exclusion step that had the

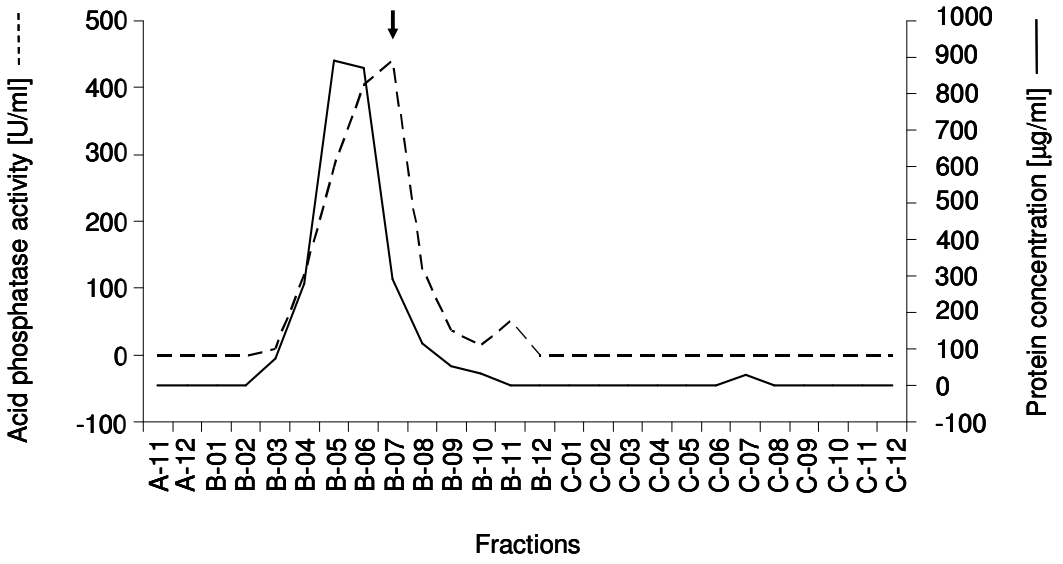


Figure 29. Size exclusion chromatography using a superose 12 HR column. For further analysis only the fraction with highest ACP activity was collected (arrow).

highest fold increase in specific activity (Table 8), still contained several major proteins (Figure 30 C). To possibly identify the protein associated with the ACP activity, the active fractions were subjected to SDS-PAGE. The gel stained for proteins by Coomassie and scanned to obtain densitograms of each fraction (Figure 30 D). After normalization of the densitograms to a prominent band of around 45 kDa, one band (band 6) of approximately 25 kDa showed an increase in density between the fractions at a ratio comparable to the increase in specific activity of the fractions (Figure 30 C, D).

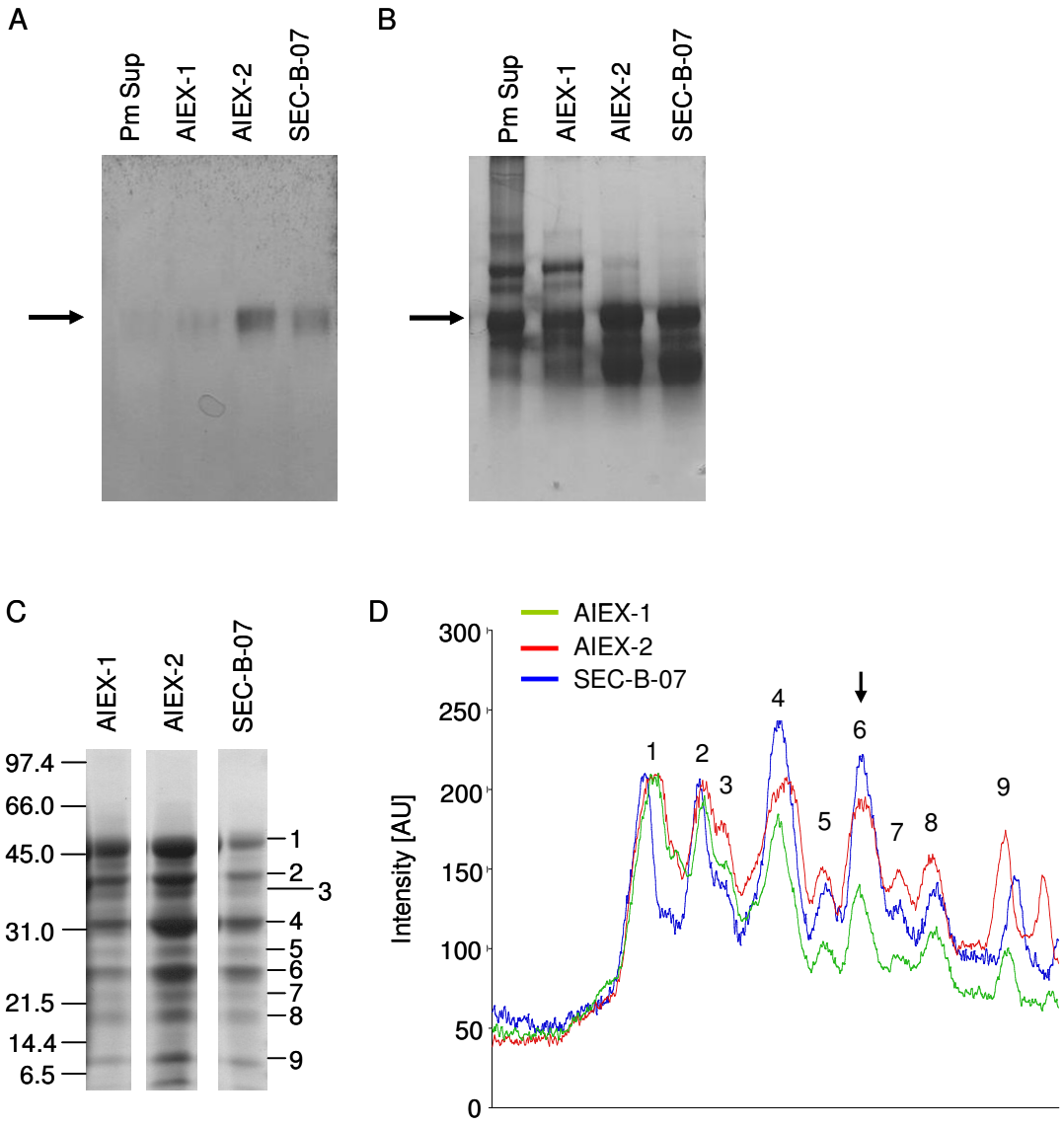


Figure 30. Partial purification of the secreted ACP activity and densitometry scan of active fractions. **A.** Detection of ACP activity in the active fractions collected from the different purification steps. Arrow indicates ACP activity in the samples. **B.** Coomassie stain of the active fractions separated on non-denaturing gels. Arrow indicates the position of the activity observed in A. **C.** Coomassie stain of active fractions separated on SDS containing polyacrylamide gels. Size markers are indicated on the left. Visible protein bands were numbered from 1 to 9 on the right. **D.** Densitometry scan of C. Note that one band (arrow) shows a density increase between the samples at a ratio comparable to the increase of specific activity of the samples. PmSup: culture supernatant of a *P. marinus* culture; AIEX-1: active fraction obtained from the first anion exchange step; AIEX-2: active fraction collected from the second anion exchange step; AU, arbitrary units; SEC-B-07, active fraction from the size exclusion column.

Identification and characterization of putative phosphatase genes

To identify putative phosphatases that may play a role in host preference and intracellular survival, a preliminary EST database generated by an ongoing functional genomic project in collaboration with J. Kissinger (University of Georgia, Athens) was searched for phosphatases. The EST database contained approximately 2,500 sequences obtained from two λ-zap cDNA libraries of *P. marinus* TxSc trophozoites that have been grown either in standard DMEM:HAM's F12 (1:2) medium or in medium supplemented with *C. virginica* serum.

Five putative phosphatase sequences were identified: one PP2B-like gene, two PP2C-like genes, one DSP-like gene, and a haloacid dehydrogenase (HAD) phosphatases-like gene (Table 9). The genes were designated *PmPP2B*, *PmPP2C1* and *PmPP2C2*, *PmDSP*, and *PmHAD*, respectively. cDNA

Table 9. EST sequences with similarities to phosphatases.

EST	Designation	Hit (BLASTX)	E-value
PMBAE34TH	*PmPP2B*	Protein phosphatase 2B-like (human)	$8.9e^{-18}$
PMBA602TH	*PmPP2C1*	Protein phosphatase 2C-like (human)	$2.7e^{-14}$
PMAA901TH	*PmPP2C2*	Protein phosphatase 2C-like (*Nicotiana tabacum*)	$9.9e^{-7}$
PMBAG08TH	*PmHAD*	HAD superfamily phosphatase (bacterial)	$6.7e^{-6}$
PMBAA35TH	*PmDSP*	Dual specificity phosphatase (*Xenopus laevis*)	$4.0e^{-5}$

sequences were obtained from all five putative phosphatase genes by 5' RLM-RACE and by PCR of the two λ-zap cDNA libraries with gene-specific primers and the primer T3 and T7. All five cDNA sequences contained in-frame stop

codons at the 3' end. Polyadenylated tails were observed in the cDNA sequences of *PmDSP, PmHAD,* and *PmPP2C2.* None of the cDNA sequences contained a stop codon upstream of the first putative methionine. A search of translated cDNA sequences against the CDD located at the NCBI revealed that the cDNAs of *PmDSP* and *PmHAD* contained only partial conserved domains of DSPs and HAD phosphatases, respectively, and thus were not further analyzed. Conversely, deduced aa sequences of the *PmPP2B* and the two *PmPP2C* genes contained the complete catalytic domains of the PPM (represented by the catalytic domain of PP2A) and PPP (represented by the catalytic domain of PP2C) families, respectively. Additional 5' RLM-RACE with different gene-specific primers for *PmPP2B* and the two *PmPP2C* genes did not reveal any sequences further upstream of the initially obtained sequences for *PmPP2B* and the two *PmPP2C*s, suggesting together with the presence of complete conserved domains that the 5' ends of the three cDNAs were complete.

The cDNA sequence of *PmPP2B* was 1,788 bp long and contained a CDS of 1,560 bp that encodes a putative PP2B of 520 aa with a calculated pI of 6.39 and a MW of 59 kDa. The cDNA sequence of *PmPP2C1* was 1,310 bp long with a CDS of 1,218 bp encoding for a putative PP2C1 of 406 aa with a calculated pI of 4.58 and a MW of 44 kDa. The cDNA sequence of *PmPP2C2* was 1,500 bp long and its CDS of 1,344 bp encodes a putative PP2C of 449 aa with a calculated pI of 7.99 and MW of 49 kDa. Analysis with SignalP, TargetP, and WoLF PSORt of the three deduced aa sequences suggested that PmPP2B is cytosolic. PmPP2C1 was predicted to be targeted to the mitochondria, however,

the reliability score was low. PmPP2C2 was predicted to contain a 19 aa N-terminal signal peptide and thus to be secreted.

Genomic DNA sequences of *PmPP2B* and the two *PmPP2C* genes were obtained by searching TIGR db *Perkinsus marinus* (www.tigr.org) with partial cDNA sequences with BLASTN using default parameters. The *PmPP2B* and *PmPP2C1* cDNA sequences showed high similarities to one or more contigs of the genome sequence database. In both cases, the highest BLAST hit contained the respective cDNA sequence. In contrast, the cDNA sequence of *PmPP2C2* aligned with two contigs in the genome sequence database with its 5' or 3' end, respectively, leaving a gap. The missing genomic DNA sequence was obtained by PCR on genomic *P. marinus* DNA with specific primers that flanked the gap. Alignment of each cDNA sequences with the respective genomic DNA sequences revealed that the *PmPP2B* gene is organized in 18 exons. *PmPP2C1* is organized in two exons, and *PmPP2C2* in 11 exons (Figure 31).

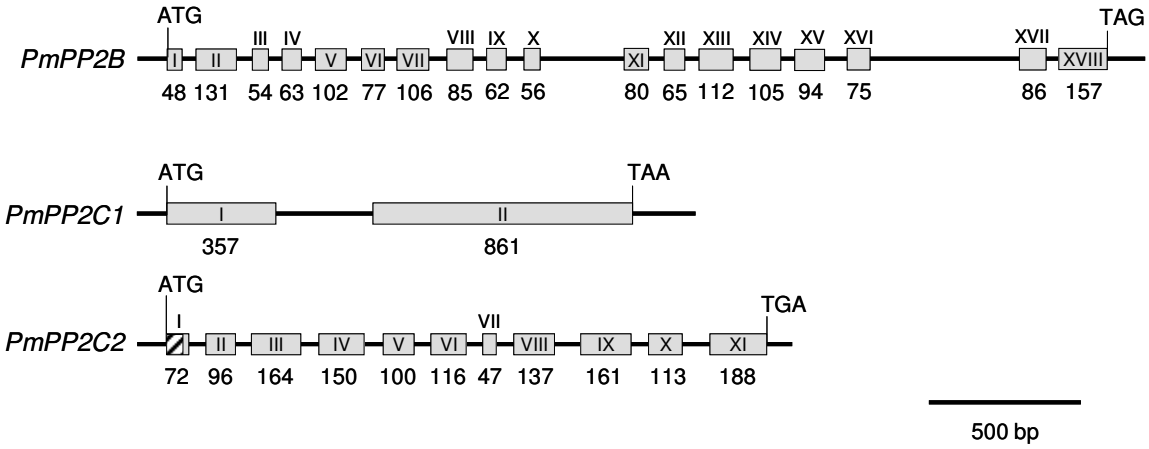


Figure 31. Gene organization of *PmPP2B*, *PmPP2C1*, and *PmPP2C2*. Roman numerals indicate exon number, and Arabic numerals the size of the respective exon in bp. ▨: Putative signal peptide of *PmPP2C2*.

Recombinant expression of a putative protein phosphatase of *Perkinsus marinus*

Computational analysis of three putative PPs of *P. marinus* suggested that *PmPP2C2* is secreted. To further characterize the gene and to obtain polyclonal antibodies, the complete CDS including the putative signal peptide was cloned into a pET30 Ek/Lic vector that contains a 6 x Histidine tag. The construct was transformed into *E. coli* BL21(DE3) for expression. Initial expression experiments using standard conditions (induction of expression with 1 mM IPTG at 37 °C) resulted in expression of a protein of expected size (approximately 55 kDa), however, the recombinantly expressed protein aggregated and was insoluble (Figure 32). To optimize expression, I therefore tested whether inductions using different IPTG concentrations (0.1 mM, 0.25 mM, 0.75 mM, and 1 mM) and lower temperatures (25 °C) would lead to the production of soluble rPmPP2C2. Under all induction conditions tested, the produced protein remained insoluble, as shown for inductions using 0.1 mM and 1 mM IPTG (Figure 32).

Western blot analysis of *Perkinsus marinus* cell extracts and culture supernatants

Cell extracts and culture supernatants of *P. marinus* TxSc and CB5D4 were subjected to SDS-PAGE and gels electroblotted. Antiserum raised against rPmPP2C2 recognized two major bands in *P. marinus* TxSc and five in *P. marinus* CB5D4 (Figure 33 A). One band had the expected size of the putative PmPP2C2 (approximately 49 kDa; arrow in Figure 33 A). However, the intensity

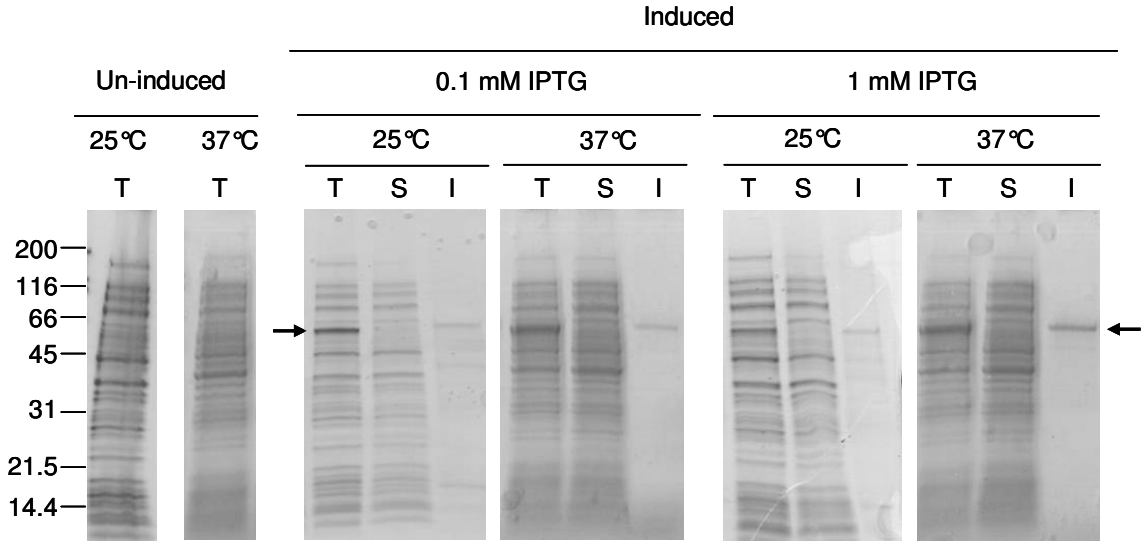


Figure 32. Recombinant expression of PmPP2C2. rPmPP2C2 was expressed in *Escherichia coli* BL21(DE3) (arrow). However, under the conditions used (induction with 0.1 mM or 1 mM IPTG at 25 ℃ and 37 ℃), rPmPP2C2 remains insoluble. T: total cell extract, S: soluble fraction; I: insoluble fraction.

of the bands in the *P. marinus* TxSc cell extract was weak compared to *P. marinus* CB5D4. The affinity purified IgGs recognized three major proteins in cell extracts of both *P. marinus* strains, with estimated molecular weights of 57 kDa, 49 kDa, and 43 kDa (Figure 33 B). The intensity pattern of the bands differed between the two *P. marinus* strains. In *P. marinus* TxSc, the strain that has been in culture for more than a decade, the 57 kDa had the highest density, whereas in *P. marinus* CB5D4, a strain that was isolated in 2002, the 49 kDa band was most intense. When both cell extracts were tested with immunopurified anti-rPmPP2C2 IgGs, only one band of approximately 49 kDa was observed in *P. marinus* CB5D4. In *P. marinus* TxSc, an additional faint band of higher molecular weight was observed (Figure 33 C).

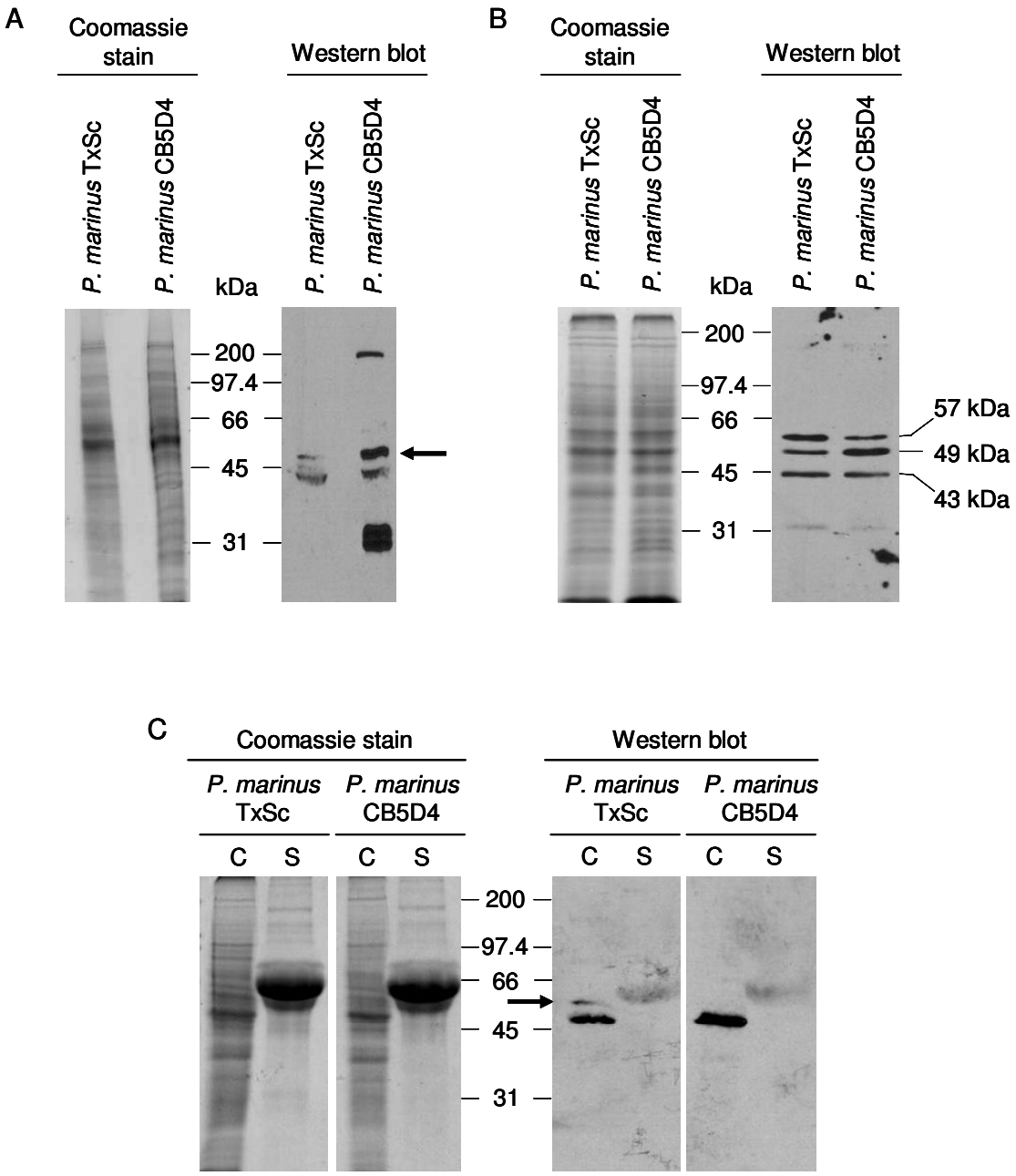


Figure 33. Western blot analysis of cell extracts and culture supernatant of *Perkinsus marinus* TxSc and CB5D4. Cell extracts and culture supernatants were subjected to SDS PAGE using discontinuous gels (10% polyacrylamide, Panel A and C) or gradient gels (10-20% polyacrylamide, Panel B). **A.** Western blot analysis with crude antiserum. Note that one band in both cell extract samples has the size of the putative PmPP2C2 (arrow). **B.** Western blot analysis with affinity purified IgGs. **C.** Western blot analysis with anti-rPmPP2C2 antibodies. Note the additional faint band in *P. marinus* TxSc cell extract samples (arrow). C: cell extract; S: culture supernatant.

Culture supernatants of both *P. marinus* strains were tested with crude antiserum and with immunopurified anti-rPmPP2C2 IgGs. Neither the crude antiserum nor the immunopurified anti-rPmPP2C2 IgGs recognized proteins in the culture supernatants (shown for the immunopurified anti-rPmPP2C2 IgGs, Figure 33 C).

To assess whether the putative PmPP2C2 is associated with ACP activity, *P. marinus* CB4D5 cell extract and culture supernatant were subjected to non-denaturing PAGE, and the gels stained for proteins with Coomassie, for ACP activity, and tested with crude antisera. In culture supernatants, only one ACP activity was observed. Cell extracts showed two ACP activities. The activity with higher electrophoretic mobility co-migrated with the activity observed in the culture supernatant (Figure 34). Western blot analysis revealed one band in the culture supernatant. However, this band did not co-migrate with proteins that

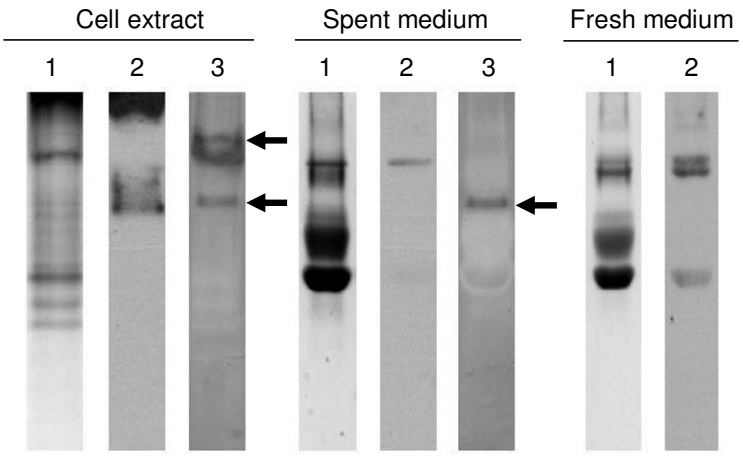


Figure 34. Western blot analysis of *Perkinsus marinus* CB5D4 cell extract and culture supernatant subjected to non-denaturing PAGE. Gels were stained for protein with Coomassie, ACP activity or electroblotted. Blots were probed with antiserum. 1: Coomassie stain; 2: Western blot; 3: "in gel assay" for ACP activity. Arrows indicate ACP activity.

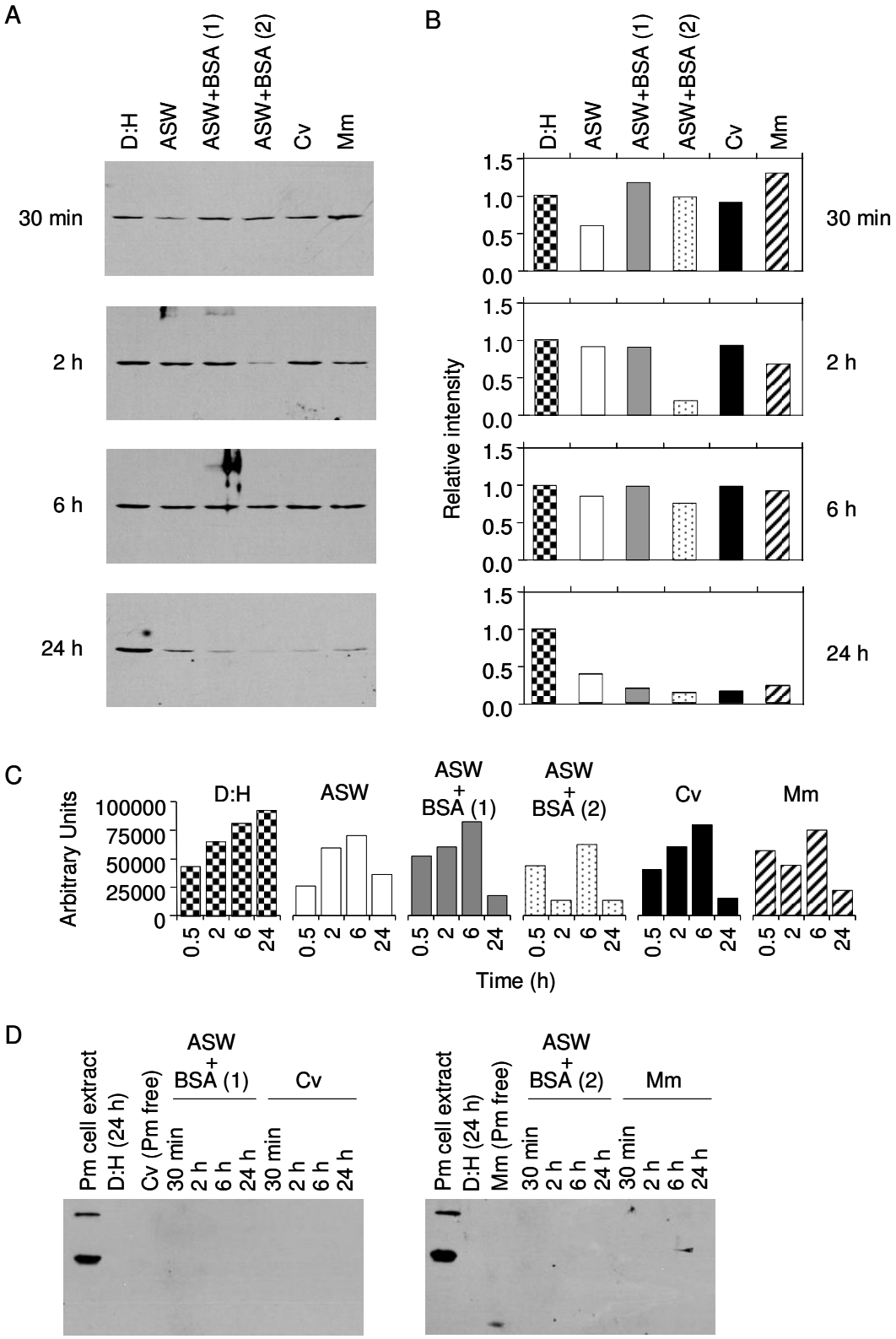
exhibited ACP activity in the culture supernatant. Similarly, in cell extracts one band was recognized that did not co-migrate with ACP activity (Figure 34).

Expression pattern of the *Perkinsus marinus* protein phosphatase 2C2

To evaluate the effect of host sera on the expression of the putative PmPP2C2, *P. marinus* CB5D4 trophozoites were exposed for 48 h to sera collected from *C. virginica* and *M. mercenaria*, and to ASW, ASW supplemented with 0.5 mg/ml BSA, and ASW supplemented with 13.5 mg/ml BSA. As a control, *P. marinus* trophozoites were maintained in standard DMEM:HAM's F12 (1:2) nutrient culture medium. For the present study, Western blot analysis was performed on cell extracts collected at 30 min, 2 h, 6 h, and 24 h after exposure using immunoaffinity-purified anti-rPmPP2C2 IgGs (Figure 35). Even though the results obtained are preliminary, general observations can be made. Overall, no differences were observed between the protein levels in cell extracts of trophozoites exposed to the experimental media and cells maintained in standard DMEM:HAM's F12 (1:2) medium for the first 6 h of exposure. An increase protein levels was observed in all treatments. However, after 24 h, protein levels decreased in *P. marinus* trophozoites minted in the experimental media. In

Figure 35 (next page). Western blot analysis of *Perkinsus marinus* CB5D4 cell extracts exposed to ASW, ASW and BSA (13.5 mg/ml and 0.5 mg/ml), and serum from *Crassostrea virginica* and *Mercenaria mercenaria*. Forty microgram of cell extract was subjected to SDS-PAGE and analyzed by Western blot with anti-rPmPP2C2 antibody. Times of exposure to the respective treatments are on the left (panel A) and right (panel B). **A.** Western blots of cell extracts. **B.** Densitometry scans of Western blots. Intensities of the signals are expressed relative to the signal observed from *P. marinus* cells grown DMEM:HAM's F12 (1:2). **C.** Change of the signal intensity over time. **D.** Western blots of selected media. ASW + BSA (1): ASW supplemented with 13.5 mg/ml BSA; ASW + BSA (2): ASW supplemented with 0.5 mg/ml BSA. Cv: *C. virginica* serum; D:H: DMEM:HAM's F12 (1:2) medium; Mm: *M. mercenaria* serum.

Figure 35


A
D:H
ASW
ASW+BSA (1)
ASW+BSA (2)
Cv
Mm
30 min
2 h
6 h
24 h
B
Relative intensity
1.5
1.0
0.5
0.0
C
Arbitrary Units
100000
75000
50000
25000
0
ASW + BSA (1)
ASW + BSA (2)
0.5
2
6
24
Time (h)
D
Pm cell extract
D:H (24 h)
Cv (Pm free)
Mm (Pm free)
30 min
2 h
6 h
24 h

contrast, the protein level of PmPP2C2 in cells maintained in standard DMEM:HAM's F12 (1:2) medium increased further (Figure 35 B, C).

To test, whether the decrease of intracellular protein levels after 24 h of exposure was due to secretion of the protein, culture supernatants of *P. marinus* cultures exposed to the two host sera and the ASW supplemented with 0.5 mg/ml and 13.5 mg/ml BSA were analyzed with a different batch of immunoaffinity-purified anti-rPmPP2C2 IgGs at a dilution of 1:100. As controls, culture supernatants and cell extract of cells maintained in DMEM:HAM's F12 (1:2) culture medium, both after 24 h exposure, were used. Under the condition used, the immunoaffinity-purified anti-PmPP2C2 IgGs used recognized a protein of expected size and an additional protein of approximately 94 kDa. Release of PmPP2C2 was not observed (Figure 35 D).

After 24 h exposure, the viability of the cells was between 98.5% to 100%, regardless of treatment and no differences in cell numbers were observed (not shown).

DISCUSSION

Perkinsus marinus is a parasite of a variety of bivalves, including *C. virginica*, its type host, and *M. mercenaria*. However, *in vitro* studies on the proliferation rate of *P. marinus* trophozoites exposed to plasma of different bivalves (Anderson 2001, Gauthier and Vasta 2002) and the observations on its distribution and prevalence in two bivalve hosts presented in Chapter 3 suggest that it may have host preference towards *C. virginica*.

P. marinus is avidly phagocytosed by oyster hemocytes that usually produce ROSs to kill potential pathogens. However, *P. marinus* apparently succeeds to either scavenge ROSs rapidly or to suppress their metabolic production (Anderson 1999, Volety and Chu 1995). ROS scavengers include antioxidant enzymes such as SODs and peroxidases. Studies on the antioxidant pathway suggest that *P. marinus* contains at least two iron containing SODs, and, instead of having CAT, it possesses two APXs (Ahmed et al. 2003, Gauthier 1998, Schott and Vasta 2003, Schott et al. 2003a, Schott et al. 2003b, Wright et al. 2002, Chapter 4). Enzymes that have been implicated to interfere with the production of ROSs include ACPs. Indeed, exposure of mammalian neutrophils to purified secreted ACPs (sACPs) of the bacterial pathogen *F. tularensis* and membrane bound ACPs (mACPs) of *Leishmania* species prior to stimulation of ROS production by N-formyl-methionylleucyl-phenylalaninexy or phorbol 12-myristate 13-acetate reduced or inhibited ROS production (Reilly et al. 1996, Remaley et al. 1984, Remaley et al. 1985b). The mechanisms for how phosphatases suppress the production of ROSs have not been elucidated. NADPH oxidase activation involves a protein kinase C (PKC). PKC is activated by an increase in intracellular Ca^{2+} levels that result in the translocation of the PKC to the membrane where it associates with diacylglycerol. Therefore, it has been proposed that phosphatases could deplete secondary messengers that are involved in the release of Ca^{2+} and lead to the activation of PKC, in particular phosphatidylinositol phosphate and/or inositol 1,4,5-trisphosphate (reviewed in Dowling et al. 1992). Alternatively, phosphatase could inhibit the assembly of the

NADPH oxidase. The NADPH oxidase is a membrane-associated complex. In its inactive state, some components are cytosolic and need to be translocated to the membrane. Translocation and assembly requires phosphorylation of serine residues of one of the subunits, namely $p47^{phox}$ (De Leo et al. 1996). Thus, it is conceivable that dephosphorylation of these residues may inhibit the assembly of the NADPH oxidase and consequently its activation. However, both models require the translocation of pathogen enzymes into the cytosol of the host cell. Other possibilities are that phosphatases dephosphorylate extracellular receptors that are involved in signaling pathways leading to the activation of the NADPH oxidase (Reilly et al. 1996), or that phosphatases affect the signalling pathways directly.

Characterization and purification of an extracellular acid phosphatase activity in *Perkinsus marinus*

In *P. marinus*, extracellular ACP activities have been reported that appear to be higher in more virulent *P. marinus* isolates (Chu et al. 1998b, Volety and Chu 1997). In the present study, the presence of an extracellular ACP activity in *P. marinus* TxSc was confirmed that has an pH optimum at 4.5 and is comparable to other virulent protistan parasites (Table 10).

Volety and Chu (1997) suggest that the ACP activity may be upregulated by high temperature and low salinity. The authors report lower extracellular ACP activity in culture supernatant from *P. marinus* cultures grown in DMEM:HAM's F12 (1:2) following Gauthier and Vasta (1995) at a osmolality of 840 mOsm/kg, which corresponds to a salinity of sea water of approximately 30 ‰, compared to

Table 10. Comparison of extracellular ACP activities of *Perkinsus marinus* and kinetoplastid parasites. ACP activities for the kinetoplastid parasites were reported by Shakarian et al. (2003) and are expressed as U/10^6 cells.

Parasite	Strain	ACP activity (U/10^6 cells)
P. marinus	TxSc (ATCC 50849)	6.9
C. fasciculata	ATCC 50083	2.5
C. luciliae	ATCC 30258	3.7
L. donovani	1S-CL2D	14.7
L. donovani	WR657	1.7
L. donovani	WR684	4.5
L. infantum	FVM100JL	1.4
L. mexicana	M379	1.8
L. tropica	WR664	5.5
L. tropica	WR683	5.3

400 to 570 mOsm/kg, which correspond to salinities of sea water of approximately 14 ‰ and 20 ‰, respectively. The authors argue that higher salinities may be stressful for *P. marinus* and may result in a decreased growth rate. However, in contrast, Gauthier and Vasta (1995) report optimal growth of *P. marinus* in medium with a osmolality of approximately 900 mOsm/kg (32 ‰).

The ACP activities reported to inhibit ROS production in mammalian neutrophils belong to the histidine ACP family and are considered to be tartrate-resistant. Therefore, the effect of tartrate on the ACP activity observed in culture supernatants was investigated. Tartrate inhibited the *P. marinus* ACP activity in a dose dependent manner, however, its sensitivity towards tartrate was lower

compared to tartrate-sensitive ACP activities of other pathogens. For example, extracellular *P. marinus* ACP activity was inhibited by 13% by 0.1 M sodium-(L+)-tartrate. At this tartrate concentration, ACP activities from *Entamoeba histolytica* that are considered tartrate-sensitive are inhibited by more than 33% (Aguirre-García et al. 2000). Similarly, 0.5 M sodium-(L+)-tartrate inhibited extracellular *P. marinus* ACP activity by 33%, whereas sACPs of *L. donovani* that are considered tartrate-sensitive are inhibited by 80-85% (Remaley et al. 1985a). In contrast, tartrate-resistant membrane bound ACP (mACP) activities of *L. donovani* are only inhibited by 6% at this concentration. Structural analysis of a human prostatic acid phosphatase that was crystallized in the presence of a tartrate derivative suggested that tartrate inhibits ACPs by binding to the active site (LaCount et al. 1998). Binding of the tartrate derivative appeared to be dependent not only on the topology of the active site structure, but also on the presence of key residues in the active sites that determine the strength of the tartrate binding (LaCount et al. 1998). Thus, the observation that the sensitivity of *P. marinus* ACP activities to tartrate was lower compared to other tartrate-sensitive ACP activities may be due to a unique structure and amino acid composition of the active site of the *P. marinus* extracellular ACP. However, it also cannot be ruled out that *P. marinus* may possess more than one extracellular phosphatase activity, some of which would be sensitive to tartrate and the other(s) tartrate-resistant. Indeed, size exclusion chromatography on ACP enriched fractions suggested the presence of a major ACP activity and a minor ACP activity with a smaller molecular weight.

To further characterize the *P. marinus* extracellular ACP activity, I attempted its purification using a purification scheme involving two subsequent anion exchange chromatography steps followed by a size exclusion step. The fraction with the highest ACP activity collected from the size exclusion showed an 16-fold increase in specific activity, but SDS-PAGE analysis revealed a complex protein banding pattern in this sample. The complexity of the size exclusion sample suggests formation of protein aggregates of the observed ACP activity with other secreted products. However, until the protein(s) possibly associated with the ACP activity have been identified and purified, this remains speculation.

A possible additional approach to further purify the extracellular ACP activity of *P. marinus* includes affinity chromatography. Ligands for affinity chromatography that have been used to purify ACPs of mammals include substrate analogues such as phosphonic acids and tartrate derivatives (Dissing et al. 1979, Van Etten and Saini 1978). Preliminary analysis of the effects of phosphonic acids on *P. marinus* sACP activities, showed that they do not inhibit the sACP activity of *P. marinus* (data not shown). Thus, phosphonic acids appear not be suitable ligands for the purification of *P. marinus* ACPs. In contrast, the use of tartrate derivatives as ligands warrants further investigation, since *P. marinus* sACP activity is inhibited by sodium-(L+)-tartrate. However, if the ACP activity forms complexes with other secreted proteins, as suggested by the complexity of the size exclusion sample, affinity chromatography may also not contribute to further purify the extracellular ACP activity of *P. marinus*.

To obtain further information on the ACP activity, the fractions collected at the different purification steps were analyzed by densitometry. After normalization, analysis of the respective densitograms revealed that the intensity of a band with an approximate MW of 25 kDa increased at a rate comparable to the increase of specific activity between the different fractions, suggesting that the protein associated with the ACP activity may have a MW of 25 kDa. If this holds true, the excreted *P. marinus* ACP activity would fall in between two classes of ACPs, the low molecular weight ACPs (14-18 kDa) and the high molecular weight ACPs (40-60 kDa).

Identification and characterization of putative phosphatase genes

Since I was unable to purify or identify the ACP observed in the culture supernatants by biochemical means at this point, I chose to pursue a genomic approach taking advantage of an ongoing functional genome project (PerkDB, Kissinger, Robledo, and Vasta). Within this project an EST database containing approximately 2,500 sequences was generated that was searched for phosphatases and specifically for histidine ACPs. Sequences with similarities to histidine ACPs were not found in the EST database, however, five other putative phosphatase sequences were identified, including one PP2B and two PP2C. The phosphatase-like genes were designated as *PmPP2B*, *PmPP2C1*, and *PmPP2C2*, respectively. Computational analysis of the full CDS suggested that the proteins encoded by the three genes had a MW of 59 kDa, 44 kDa, and 49 kDa, and a pI of 6.39, 4.58, and 7.99, respectively. Thus, at least the putative PmPP2B and PmPP2C1 would bind to an anion exchange resin at pH 8.0, which

were the conditions used for the purification of the ACP purification. Whether PmPP2C2 would bind is questionable and needs to be tested.

Using subcellular localization and topology predictions with TargetP, WoFL PSORt, and SignalP suggested PmPP2B to be cytosolic. PmPP2C1 is possibly targeted to the mitochondria, however, the reliability score that indicates the difference to the next best prediction, indicated that the prediction was not robust. In contrast, PmPP2C2 was predicted to be secreted and to contain a putative 19 aa N-terminal signal peptide.

Recombinant expression and preliminary functional analysis of the putative *Perkinsus marinus* protein phosphatase 2C2

Due the predicted localization of PmPP2C2, I focused on further characterizing *PmPP2C2* and recombinantly expressed the full length CDS of this gene including its putative signal sequence in *E. coli* BL21(DE3). The recombinantly expressed protein remained insoluble under all condition tested, rendering functional analysis difficult. Insolubility of recombinant expressed proteins may be due to several reasons, including to improper folding of the protein in *E. coli*. Strategies to circumvent the formation of insoluble proteins include the co-expression of chaperons, or the use of strains that target the recombinant expressed protein to the periplasmic space. Alternatively, other expression systems such as yeast based systems may be more appropriate for the recombinant expression of PmPP2C2 in order to receive a fully functional protein that is targeted to the correct subcellular localization. Functional analysis of PmSOD2 in heterologous systems support this notion. Attempts to express

PmSOD1 that contains a mitochondrial target sequence in an *E. coli* SOD mutant strain failed to complement the *E. coli* mutant (Schott, Pecher, and Vasta, unpublished data). In contrast, PmSOD1 expression in a *S. cerevisiae* SOD2 mutant resulted in a protein that not only complemented the yeast mutant, but was also targeted to the mitochondria, as predicted (Schott and Vasta 2003).

To raise antibodies against rPmPP2C2, rPmPP2C2 was purified by electroelution from SDS-polyacrylamide gels. rPmPP2C2 remained soluble in SDS-PAGE running buffer. Crude antiserum reacted with multiple proteins from cell extracts of *P. marinus* TxSc and CB5D4, whereas affinity purified IgGs recognized three proteins, one of which had the MW expected for PmPP2C2. Immunopurified antibodies against rPmPP2C2 (anti-rPmPP2C2 antibodies) reacted with one protein of expected size in *P. marinus* CB5D4 cell extracts. Interestingly, an additional protein was weakly recognized by the antibodies in *P. marinus* TxSc cell extracts. It could possibly stem from residual carryover of cell debris, but this possibility remains to be tested. In one experiment, however, an additional band at approximately 94 kDa was observed in *P. marinus* CB5D4 cell extracts. For this particular experiment, a different batch of purified antibody was used at a higher concentration (1:100 instead of 1:200), which could explain the additional band as being non-specific, however, that needs to be investigated. As mentioned above, the putative PmPP2C2 contains a putative signal sequence that suggests its secretion. Western blot analysis of spent culture media with either the crude antiserum or the anti-rPmPP2C2 antibody did not react with proteins in culture supernatants. These results suggest that

PmPP2C2 is not secreted or, if it is, not in detectable amounts under conditions at which extracellular ACP activity is observed. To assess whether PmPP2C2 is associated with any ACP activity, cell extracts and culture supernatants were subjected to non-denaturing PAGE. Gels were either stained for proteins with Coomassie, for ACP activity, or analyzed by Western blotting using crude antiserum. As expected, one ACP activity was observed in culture supernatants. In cell extracts, two ACP activities could be detected, one of which co-migrated with the extracellular activity. However, proteins recognized by the crude antiserum did not co-migrate with any of the observed activities, suggesting that the putative PmPP2C2 is not associated with ACP activities observed.

Assessment of the expression pattern of the *Perkinsus marinus* protein phosphatase 2C2

One aim of the presented thesis work was to identify possible factors of *P. marinus* that are involved in its observed host preference and its virulence. Therefore, the expression pattern of PmPP2C2 in *P. marinus* CB5D4 trophozoites was assessed. Members of PP2Cs exhibit a broad range of physiological functions and are implicated in virulence of certain pathogens (Archambaud et al. 2005, Luan 2003). They are involved in the regulation of gene expression by either regulating the translocation of transcription factors such as NF-κB, or by dephosphorylation of elongation factors (Archambaud et al. 2005, de Nadal et al. 2001, Mamoun and Goldberg 2001, Prajapati et al. 2004). In *T. gondii* a PP2C-like protein has been shown to be translocated to the host nucleus, possibly modulating the host response to *T. gondii* infection and/or

altering the infected cell's behavior to accommodate *T. gondii* (Gilbert et al. 2007). Members of the PP2C family also have been shown to be involved in regulating stress responses (Barford et al. 1998). In *S. cerevisiae*, for example, a PP2C has been reported to deactivate a kinase cascade that is activated by osmotic stress (Maeda et al. 1994).

P. marinus CB5D4 trophozoites were challenged with serum obtained from *C. virginica*, and *M. mercenaria*, or maintained in ASW only, and ASW supplemented with BSA concentrations similar to protein concentrations observed in the two sera. The osmolality of all experimental media was around 745 mOsm/kg. Levels of PmPP2C2 expression were compared to expression levels in trophozoites maintained in the standard culture medium that had a salinity of 832 mOsm/kg. Preliminary Western blot analysis of cell extracts from *P. marinus* trophozoites that have been exposed to host sera or ASW treatments for 30 min, 2h , 6h, and 24 h did not reveal differences of PmPP2C2 levels. In general, PmPP2C2 protein levels increased over the first 6 h and then decreased after 24 h exposure. This observation suggests that PmPP2C2 is unlikely to be involved in determining host preference or virulence. Interestingly, in contrast to trophozoites maintained in ASW and host sera PmPP2C2 levels of trophozoites maintained in standard culture medium did not decrease after 24 h. Exposure to ASW alone in this experiment may have imposed at least two different stresses on *P. marinus*: osmotic stress due to lower salinity and starvation due to deprivation of nutrients. Both stressors may have contributed to the observed decrease in PmPP2C2 levels. Similarly, since it is not known whether *P. marinus*

is able to feed on components present in host sera, osmotic stress and nutrient deprivation may also be the cause of the PmPP2C2 reduction observed. Additional adverse effects of sera components on *P. marinus* may also contribute to the observed reduction. However, the following two observations suggest that the decrease in PmPP2C2 protein levels was not a direct response to osmotic stress, but possibly to starvation. First, if it were a direct response to osmotic stress, a fast response during the first 2 h would be expected. Secondly, PmPP2C2 protein levels increased in all treatments during the first 6 h of exposure, which hints to constitutive expression and translation of PmPP2C2. It is of note that the cell numbers after 24 h exposure and the viability as judged by neutral red staining between the different treatments were not different. Thus, whether the decrease of PmPP2C2 protein levels is related to a general reduction of metabolic activity remains to be explored. Alternatively, PmPP2C2 in the ASW and host sera exposed trophozoites may have been excreted. However, analysis of culture supernatants did not reveal secretion or release of PmPP2C2.

In conclusion, the observation that changes in PmPP2C2 levels are similar between the treatments with sera obtained from two different hosts over the first 24 h suggests that PmPP2C2 is not involved in the determination of host preference and the physiological function of PmPP2C2 in *P. marinus* remains elusive.

CHAPTER 6

SUMMARY DISCUSSION

Characterization of *Perkinsus* species, and assessment of their distribution and host preference

Perkinsus species are ubiquitous endoparasitic protists of a variety of mollusk species. Seven *Perkinsus* species are currently accepted and some have been reported in more than one host species, supporting the assertion that these species have a broad host range (reviewed in Villalba et al. 2004), but their host specificity or host preference have not been described in detail. To assess the host range, host specificity, or host preference, it is imperative to have the ability to distinguish between *Perkinsus* species, especially if the *Perkinsus* species in question are sympatric such as *P. chesapeaki* (type host *M. arenaria*; McLaughlin et al. 2000), *P. andrewsi* (type host *M. balthica*; Coss et al. 2001b), and *P. marinus* (type host: *C. virginica*; Mackin et al. 1950) that are sympatric in Chesapeake Bay, USA, or *P. mediterraneus* (type host: *Ostrea edulis* Casas et al. 2004) and *P. olseni* (syn. *P. atlanticus*; type host: *Haliotis ruber*; Lester and Davis 1981) that are both observed in the Mediterranean Sea, and most recently *P. honshuensis* (type host: *Venerupis philippinarum*; Dungan and Reece 2006) and *P. olseni*, both infecting *V. philippinarum* collected from Honshu Island, Japan.

Specific assays based on the PCR technology have been developed for the genus *Perkinsus*, and the species *P. marinus*, *P. olseni*, and *P. andrewsi* (Coss et al. 2001b, de la Herrán et al. 2000, Marsh et al. 1995, Robledo et al. 2000, Robledo et al. 2002, Yarnall et al. 2000) but are lacking for *P. chesapeaki*, *P. mediteraneus,* and *P. honshuensis*.

The development and optimization of culture methods for *Perkinsus* species in the early 1990s (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, La Peyre et al. 1993) enabled the establishment of clonal lines of the parasite and facilitated the development of specific PCR-based diagnostic assays. In a few cases, species-specific diagnostic assays have been developed without the availability of clonal cultures, as demonstrated for *P. olseni* for which diagnostic assays were established before it was successfully cultured (de la Herrán et al. 2000, Marsh et al. 1995, Robledo et al. 2000, Robledo et al. 2002, Yarnall et al. 2000). The discriminatory power of the above mentioned molecular methods also allowed the rigorous identification and characterization of *Perkinsus* species, strains, and isolates.

The relevance of this aspect became apparent during the *Perkinsus* infection distribution study reported herein. While validating an assay developed for a *Perkinsus* isolate from the hard clam *M. mercenaria* [*Perkinsus* sp. (*M. mercenaria*)], cross-amplification of *P. andrewsi* was observed, and attempts to elucidate the cause of this apparent lack of specificity concerning both *Perkinsus* species/isolates led to the discovery of a second distinct rRNA gene unit (designated as rRNA-B) in the *P. andrewsi* genome (Pecher et al. 2004). While

Perkinsus sp. (*M. mercenaria*) only had one unit, its rRNA gene unit shared high sequence similarities to the rRNA-B unit of *P. andrewsi*, suggesting it may be a variant of *P. andrewsi* (Chapter 2). As discussed in Chapter 2 of this dissertation, this finding not only adds new complexity to the use of rRNA sequences for taxonomic analysis and species designations based on molecular characters, but also underscores the need for rigorous characterization of the complete rRNA gene locus. The latter is best exemplified with *P. chesapeaki.* For this species, the SSU and ITS1-5.8S-ITS2 region have been characterized (Kotob et al. 1999a, Kotob et al. 1999b). Based on the available sequences, *P. chesapeaki* appears to be closely related to *P. andrewsi* and *Perkinsus* sp. (*M. mercenaria*). However, the IGS sequence that may be more informative to distinguish between strains is not available for *P. chesapeaki*. Unfortunately, further molecular characterization of *P. chesapeaki* is not possible, since a *bona fide* type culture is not available. Thus, it is difficult to address the relationship of *P. chesapeaki* to the other two *Perkinsus* isolates.

In contrast, a hapantotype for *P. andrewsi* and *P. honshuensis* were deposited and the cultures are available (Coss et al. 2001b, Dungan and Reece 2006). One should hope that type cultures of the other two *Perkinsus* species (*P. qugwadi* and *P. mediteraneus*) will be established shortly, and made available in the near future.

This dissertation work compares, for the first time, the distribution of *Perkinsus* species, *P. marinus*, *P. andrewsi*, and *Perkinsus* sp. (*M. mercenaria*) in two host species, *C. virginica* and *M. mercenaria*, collected along the North

American Atlantic coast from Virginia to Maine by using established PCR-based assays (Chapter 3).

PCR is a powerful diagnostic tool. However, especially when DNA is extracted from environmental samples or particular tissues, PCR is prone to inhibition mainly due to co-purification of inhibitory agents such as humic acids or pigments that can result in either complete amplification inhibition or reduced sensitivity, and thus lead to false negative results. During the initial screening, surprisingly low *Perkinsus* infection prevalence was observed in *M. mercenaria* samples. Routine validation of the negative PCR results suggested PCR amplification inhibition in hard clam DNA samples that was not encountered in oyster DNA samples. After a laborious modification and optimization of the reaction conditions, this inhibition of PCR amplification could be overcome by the use of a specialty *Taq* DNA polymerase and the addition of BSA and DMSO (Chapter 3). Thus, a modified PCR protocol has been developed and is presented in this dissertation work that allows reliable PCR amplification from hard clam samples. However, it is of note that while the modified PCR procedure does remove PCR amplification inhibition in hard clam samples, it does not overcome the amplification inhibition observed in scallop DNA extracts (Pecher and Vasta, unpublished data), which underscores the importance of validation and optimization of PCR conditions for each sample type used.

Results from the present study confirmed previous observations that *Perkinsus* species have high prevalence in *C. virginica* populations in some areas. However, the previous studies assessed the presence of *Perkinsus*

infections using diagnostic assays that do not discriminate between *Perkinsus* species (i. e. the FTM assay), thus infections cannot be attributed solely to *P. marinus*. In contrast, by using specific assays, the present study was able to show that *P. marinus* was indeed the most prevalent *Perkinsus* species in *C. virginica*. Since *P. marinus* is a recognized pathogen of *C. virginica* that has contributed to mass mortalities in the past, this finding has important implications for shellfish farmers and state regulators. Regardless of discrepancies between high *Perkinsus* prevalence, infection density and low host mortality rates that were observed in some areas (Ford 1996), the chances are high that the infectious agent was indeed *P. marinus*, and not another *Perkinsus* species with possible less pathogenicity towards *C. virginica*. This risk justifies efforts and precautions to avoid further spreading of a potentially devastating shellfish parasite in and from these areas.

Environmental factors play an important role in controlling *Perkinsus* infections. Temperature and salinity are the main factors that affect the distribution, abundance, density, and severity of *Perkinsus* infections (Andrews 1988, Andrews and Hewatt 1957, Andrews and Ray 1988, Chu and La Peyre 1993a, Chu et al. 1993, Chu 1996, Hewatt and Andrews 1956). Increased temperature and salinity positively correlate with increased *Perkinsus* infection abundance and densities, and higher oyster mortalities (Chu and La Peyre 1993a, Chu et al. 1993, Hewatt and Andrews 1956). Thus, due to the projected world wide warming trends, severe *Perkinsus* infection outbreaks associated with mass mortalities in *C. virginica* populations may be observed more frequently in

the northeastern Atlantic coast of the United States. However, the presence of host strains with higher resistance to *P. marinus* that also may have contributed to the observed discrepancy between high *Perkinsus* prevalence, infection density and low host mortality rates cannot be ruled out, and this warrants further investigation. If this assertion holds true, attempts can be made to select and breed *C. virginica* strains that would potentially be more resistant to *Perkinsus* infections, and thereby benefit shellfish farmers and restoration efforts. This may also prove useful for comparative studies to better understand the interaction(s) between *P. marinus* and *C. virginica*. In addition, in accordance with studies on the proliferation of *P. marinus* that suggested a preference of *P. marinus* (Anderson 2001, Gauthier and Vasta 2002) for *C. virginica*, findings presented in the present work provided strong evidence that indeed *P. marinus* has a host preference towards its type host, *C. virginica*.

Identification of factors potentially involved in virulence and host preference

A further objective of my dissertation work was to identify and characterize *P. marinus* factors that may be involved in the proposed host preference. Factors that have been suggested contribute to virulence and thus potentially to host preference of *Perkinsus* species, include ACPs, antioxidant enzymes, and proteases (Brown and Reece 2003, Gauthier 1998, La Peyre et al. 1996, La Peyre et al. 1995b, Volety and Chu 1997, Wright et al. 2002). Proteases are thought to be involved mainly in necrotic activity observed in *P. marinus* infections, and have been reported to suppress some immune functions of

oysters such as hemocyte migration and lysozyme production (Garreis et al. 1996 Anderson 1996), and two serine protease-like genes have been identified (Brown and Reece 2003). In contrast, antioxidant enzymes and ACPs have been proposed to be responsible for the reported interference of the oxidative burst of oyster hemocytes usually observed during phagocytosis, by either scavenging generated ROSs or inhibiting their production (Anderson 1999, Volety and Chu 1995). Our laboratory previously identified and characterized in detail two SOD genes that encode functional iron type SODs, one of which may be involved in virulence of *P. marinus* (Gauthier 1998, Schott and Vasta 2003, Schott et al. 2003b, Wright et al. 2002, Asojo et al. 2006). Peroxidases such as CATs were not described. Further characterization of the antioxidant pathway of *P. marinus* presented here suggested the lack of CAT genes and activity in *P. marinus* (Chapter 4). Instead, through genomic approaches, putative APX gene sequences were identified. The presence of APX activity was confirmed biochemically and two APX genes isolated and further characterized (Schott et al. 2003a, Chapter 4, Schott and Vasta, unpublished).

In addition, it was proposed that *P. marinus* could also modulate the ROS response of oyster hemocytes by interfering with the production of ROSs (Anderson 1999, Volety and Chu 1997). In other pathogen systems, ACP have been proposed to inhibit ROS production (Reilly et al. 1996, Remaley et al. 1984, Saha et al. 1985). While extracellular ACP activity has been observed by me and others in *P. marinus*, the gene(s) associated with ACP activity have not been identified. To identify ACP genes, two different approaches were taken. First,

the purification of the protein(s) responsible for the ACP activity was attempted, which was only partially achieved, possibly due to a low abundance of the protein in the culture supernatant. Low yields observed in the last purification step (1%) corroborate this notion.

An additional approach to enrich for ACP activity of *P. marinus* includes affinity chromatography. However, if the ACP activity forms complexes with other secreted proteins, as suggested by the complexity of samples subjected to size exclusion (Chapter 4), affinity chromatography may also not contribute to the further purification of the extracellular ACP activity of *P. marinus*.

Secondly, a genomic approach was taken to identify possible ACP genes and a preliminary EST sequence database with approximately 2,500 sequences was searched for putative acid phosphatase genes. No ACP genes could be identified in this particular EST database, but other phosphatase genes including a PP2C-like gene with a predicted signal sequence were found. PP2Cs may be involved in virulence by either directly modulating host responses or regulating the expression of their own virulence genes (Archambaud et al. 2005, Gilbert et al. 2007). Further analysis of the putative PP2C (designated as PmPP2C2), however, neither confirmed its secretion nor its involvement of PmPP2C2 in host preference. Thus, the physiological function of PmPP2C2 remains elusive.

That ACP genes have not been identified in the EST database search may not be surprising, because the size of the database was limited. Indeed, a preliminary search of a second EST database containing more than 31,000 sequences that was generated in collaboration with TIGR for aiding in the

annotation of the *P. marinus* genome enabled the identification of three sequences that possess sequence signatures of histidine ACPs (Pecher and Vasta, unpublished data). These sequences are promising candidates for further investigation, especially since the ACPs proposed to inhibit ROS production, belong to histidine ACPs.

In conclusion, despite continued efforts and success with selected candidate virulence factors, the full spectrum of *P. marinus* mechanisms for intracellular survival is yet to be fully elucidated. With the *P. marinus* genome sequencing project coming to conclusion, alternative approaches for the identification of potential virulence genes and factors, such as microarray analysis and comparative proteomics, will become more feasible.

Furthermore, recently *P. marinus* was successfully transfected for the first time (Robledo and Vasta, unpublished), and optimization of the transfection system is underway. With this tool in hand, functional analysis of already identified and characterized genes such as the putative PmPP2C2 or putative virulence genes identified by microarray studies and proteomics will be possible.

Even though the full importance of the ACP activity and the biological function of the putative PmPP2C2 remain elusive, both the biochemical and genomic approaches implemented throughout this research represent a template strategy that can serve as a model for processing all additional phosphatase sequences that have been identified - or that may be identified in the future - in the EST and genome databases. This will enable the research community to obtain information about their structural, functional, and evolutionary aspects in

the context of host/parasite interactions, with particular focus on intracellular survival.

APPENDICES

A. *PmPP2B* cDNA

Nucleotides are numbered with reference to the initiator ATG (underlined). The nucleotide coding sequence is in uppercase, the 5' and 3' untranslated sequences (UTRs) in lower case. The corresponding aa sequence is written underneath. A * denotes a stop codon.

```
-36 tcgtgcgccgttaacgcggaaattgctcttccccca<u>ATG</u>GTGAAAGCATCGCCACCGGAC
    S  C  A  V  N  A  E  I  A  L  P  P  M  V  K  A  S  P  P  D

 25 TGGCAGTGTCTCAGGAAGCATCTGTTCGCGGAGGGTAGACTGTATATAGAGTCAATCACG
    W  Q  C  L  R  K  H  L  F  A  E  G  R  L  Y  I  E  S  I  T

 85 AGGATCGTCAATGAACTTATACGGCTGTGTACGGCAGAGGCTAATGTTGTCAAAGTGAAG
    R  I  V  N  E  L  I  R  L  C  T  A  E  A  N  V  V  K  V  K

145 GACCCGGTTACAGTAGTAGGAGATATACATGGACAGTACTATGACCTTATCAAGCTACTG
    D  P  V  T  V  V  G  D  I  H  G  Q  Y  Y  D  L  I  K  L  L

205 GAAGTCGGGGGAGATCCAGCCACAACTCAATATCTCTTCCTCGGAGACTATGTTGACAGA
    E  V  G  G  D  P  A  T  T  Q  Y  L  F  L  G  D  Y  V  D  R

265 GGCAGCTACAGTGTGGAAGTACTACTACTTTTATATGCGTTGAAGATCAACTACCCCAAG
    G  S  Y  S  V  E  V  L  L  L  L  Y  A  L  K  I  N  Y  P  K

325 ACTATAACGTTACTACGAGGAAATCACGAGTGTAGACAAATGACTGCTTTTTTTAATTTC
    T  I  T  L  L  R  G  N  H  E  C  R  Q  M  T  A  F  F  N  F

385 CGAGACGAATGCGAGTACAAGTACAACCTAACTGTGTACGAGCTGTTTATGGAGTCGTTC
    R  D  E  C  E  Y  K  Y  N  L  T  V  Y  E  L  F  M  E  S  F

445 GACTGTCTGCCGCTGGCCGCTGTCGTTAACGGGAAGTTCCTGTGTATTCACGGTGGTTTG
    D  C  L  P  L  A  A  V  V  N  G  K  F  L  C  I  H  G  G  L

505 TCTCCTGAGCTCAACACACTCGCTGACTTTAACAAAATTAACCGATTTCAAGAACCTCCG
    S  P  E  L  N  T  L  A  D  F  N  K  I  N  R  F  Q  E  P  P

565 AGACAAGGCCTTTTCTGTGACATACTCTGGAGTGACCCTGAGGAGGAGAAGGAGGGTGTT
    R  Q  G  L  F  C  D  I  L  W  S  D  P  E  E  E  K  E  G  V

625 GCGGTGTTCAAGTCGAAGGATAGGACCTTTGTTCCCAACGATGTACGCGGCTGTAGCTTC
    A  V  F  K  S  K  D  R  T  F  V  P  N  D  V  R  G  C  S  F

685 CTGTATACGTATGAAGCTGCAACGAATTTCTTGAAGAAGAATTCGTTATTGTCGGTTATT
    L  Y  T  Y  E  A  A  T  N  F  L  K  K  N  S  L  L  S  V  I

745 CGAGCCCATGAAGCTCAGCTGGAGGGATATAAAATGCACAGAACTAACGAGGCCACCGGC
    R  A  H  E  A  Q  L  E  G  Y  K  M  H  R  T  N  E  A  T  G
```

```
 805 TTTCCCTCGGTCATCACCATTTTCTCGGCACCTAATTACTGTGATGTGTATAATAACAAG
     F  P  S  V  I  T  I  F  S  A  P  N  Y  C  D  V  Y  N  N  K

 865 GGCGCGGTTTTGAGATTTGAGAACAACACGTTGAACATTTTACAGTTCAATTTTTCCAAG
     G  A  V  L  R  F  E  N  N  T  L  N  I  L  Q  F  N  F  S  K

 925 CATCCGTACCACTTGCCCAACTTTATGGACGTCTTTGCGTGGTCAATGCCTTTTGTCGCA
     H  P  Y  H  L  P  N  F  M  D  V  F  A  W  S  M  P  F  V  A

 985 GAGAAAATTACGGAGATGTTATTTTACGTATTAAATCCTCAGAATGAGTCTGGCAGTTAC
     E  K  I  T  E  M  L  F  Y  V  L  N  P  Q  N  E  S  G  S  Y

1045 ACCCGTGCTCCCGATGTTACCGATGAAGATCTCCCACAGCTTCCCGAAGCCGATCTGAAA
     T  R  A  P  D  V  T  D  E  D  L  P  Q  L  P  E  A  D  L  K

1105 CAGTTGAAATTGGCCCGTCTGTCGAGTTTGTCTGACGAACAGAATAAATCGGTATCACTT
     Q  L  K  L  A  R  L  S  S  L  S  D  E  Q  N  K  S  V  S  L

1165 GCTGCCCGTTTGCATCAGCATATGATGGCCGAAGGGGCTAAGGTTATCGAGCAAAGTAGT
     A  A  R  L  H  Q  H  M  M  A  E  G  A  K  V  I  E  Q  S  S

1225 AGTAGTTGCGGTAAAGGTCGGGCAGCTGAGGCGGCGGGTGGTAAGGCGATATCGAAGGAG
     S  S  C  G  K  G  R  A  A  E  A  A  G  G  K  A  I  S  K  E

1285 AAGGCCGATCGTATGAGGAAGAAGATTCAAACTGTGAGTCGGATGATGCTTATGTTCAAG
     K  A  D  R  M  R  K  K  I  Q  T  V  S  R  M  M  L  M  F  K

1345 ACCTTACGCGAGGAGAACGAGACCATCATCAACCTCAAAGGCGTTTGCCCTGGGCACAGG
     T  L  R  E  E  N  E  T  I  I  N  L  K  G  V  C  P  G  H  R

1405 TTGGCACCTGGTCTGCTACTGTCGGGCCGCGATGCGCTGCAAAACGAGTTGGAGAAGTTC
     L  A  P  G  L  L  L  S  G  R  D  A  L  Q  N  E  L  E  K  F

1465 ACGAAGGCTCGAGCCATGGATTTAGAAAATGAAAGAATGCCGACCGAGAAGGAGGTCGAT
     T  K  A  R  A  M  D  L  E  N  E  R  M  P  T  E  K  E  V  D

1525 AGCCAGCCCCATTTCCATCGTGGAATGTCATCATCATAGtggaaagggtcttgcccactt
     S  Q  P  H  F  H  R  G  M  S  S  S  *  W  K  G  S  C  P  L

1585 gggggttcctctcgaggggtcatcgaagccctctctttagctcgtgtaggattattagct
     G  G  S  S  R  G  V  I  E  A  L  S  L  A  R  V  G  L  L  A

1645 gcatcatcatcatcgtcgtcgttgtctatgaattctagtatgatattggaagcgttgggc
     A  S  S  S  S  S  S  L  S  M  N  S  S  M  I  L  E  A  L  G

1705 tcgtgtggactgttcatataattggttttgtagttctgtaaaaagggaaaaaaaa
     S  C  G  L  F  I  *  L  V  L  *  F  C  K  K  G  K  K
```

B. *PmPP2C1* cDNA

Nucleotides are numbered with reference to the initiator ATG (underlined). The nucleotide coding sequence is in uppercase, the 5' and 3' UTRs in lower case. The corresponding aa sequence is written underneath. A * denotes a stop codon.

```
-30 ggtttgtggtgttttcctacattttacgcaATGGGTGGGTTACTGTCTCACCCCGTTACC
    G  L  W  C  F  P  T  F  Y  A  M  G  G  L  L  S  H  P  V  T

 31 GCCGTCCATCTACAACGACGTGCCAACGACAAGTTCCAATGCGGTGTCGCAACTCTACAG
    A  V  H  L  Q  R  R  A  N  D  K  F  Q  C  G  V  A  T  L  Q

 61 GGTTGGAGGATCTCTCATGAAGATGCGCACTGCATAGACTTGGACTGGGGTTCAACTCAT
    G  W  R  I  S  H  E  D  A  H  C  I  D  L  D  W  G  S  T  H

121 GAAGAAGGCTTCTTTGCTGTACTCGATGGTCATACTGGTGATGACGCTGCCGAGTTCGGA
    E  E  G  F  F  A  V  L  D  G  H  T  G  D  D  A  A  E  F  G

181 TCAAAGGAGTTACCTAAGCAGCTAGACGAGTCGGCTGGAGATCCCGAGGACCGCACTGTA
    S  K  E  L  P  K  Q  L  D  E  S  A  G  D  P  E  D  R  T  V

241 CAAGGCGTACAGGCCGGTTTCTTAGCCACTGATCAGGCGCTGAGAGAGACTCATTCTGAG
    Q  G  V  Q  A  G  F  L  A  T  D  Q  A  L  R  E  T  H  S  E

301 GCCGGCGCTGTTGTTGTGGCTTCCATTGTGACGCCTCTTGGCAACGGAAAATACAAGGTT
    A  G  A  V  V  V  A  S  I  V  T  P  L  G  N  G  K  Y  K  V

361 CGCCTCATGAACGCTGGTGATTCCCGCGCATTGGTATTCTCGGGTAAGAACGTTGACATG
    R  L  M  N  A  G  D  S  R  A  L  V  F  S  G  K  N  V  D  M

421 ACTAAGTTGGAGAATGCCCAAGAGGACGTAACGAAGCAGCTATGCAATGATGATAGCACT
    T  K  L  E  N  A  Q  E  D  V  T  K  Q  L  C  N  D  D  S  T

481 GAGGCTAGCGGTGATAAGGGTATATTCGGTGGAGGGGACATGCTGGGCTCAAAGAAGAAT
    E  A  S  G  D  K  G  I  F  G  G  G  D  M  L  G  S  K  K  N

541 CTAGCGTCTCTCACTGACGCTGACGTCGGAATTTTGTTGGCCACTAAGGACCATAAGCCT
    L  A  S  L  T  D  A  D  V  G  I  L  L  A  T  K  D  H  K  P

601 GATGATCCCGAGGAAAAGGCTCGGATTGAAGAAGCTGGTGGTTTTGTGAGCACAGACGAA
    D  D  P  E  E  K  A  R  I  E  E  A  G  G  F  V  S  T  D  E

661 CCACCTCGGCTTTGTGGTGTGTTGGCCCTATCCAGAGGCCTTGGCGACTTCGCGTATAAA
    P  P  R  L  C  G  V  L  A  L  S  R  G  L  G  D  F  A  Y  K

721 GACGACGCGGATCTTCCAGCTGATAAGCAAAAGTGCATTGCTGTGCCTGATGTTAGTGAA
    D  D  A  D  L  P  A  D  K  Q  K  C  I  A  V  P  D  V  S  E

781 GTTATCGTTGAAGCTGGCGACTGGGTAGTATTGGCTTGTGATGGTGTGTTTGACGTTATG
    V  I  V  E  A  G  D  W  V  V  L  A  C  D  G  V  F  D  V  M

841 TCCAACGAGGATGTCGCGCGAGAAGTTATGGTACGAGCTCATGCTGGGGAGGATCTGGCT
    S  N  E  D  V  A  R  E  V  M  V  R  A  H  A  G  E  D  L  A

901 GAGGTTGCCGCTGAAATATTACAACGTTGTCTCAACCTACTGGATTTCCAGGACAATATG
    E  V  A  A  E  I  L  Q  R  C  L  N  L  L  D  F  Q  D  N  M

961 ACTTGTATGATTATTAGAGTCGGCACGACTGAGCGTGGTCTGGAGACTGAAGTCCCAGAA
    T  C  M  I  I  R  V  G  T  T  E  R  G  L  E  T  E  V  P  E
```

```
1021 GAAACACAAGAGGAACTCCAGTTTGGTGGCTATTCAGACCTCCATACATTGGCTCCGGGA
     E  T  Q  E  E  L  Q  F  G  G  Y  S  D  L  H  T  L  A  P  G

1081 ATGGCAGAAAAGTATCAGACTTTCTTCAGGAGAGCTGGATTCAGTAAAAATCCCGCAGCC
     M  A  E  K  Y  Q  T  F  F  R  R  A  G  F  S  K  N  P  A  A

1141 TGCAATACGTGTTCAAAAGTGTATCCGCATATGGCGCAATGTCCGTTGTAAaacgccgta
     C  N  T  C  S  K  V  Y  P  H  M  A  Q  C  P  L  *  N  A  V

1201 atatggccgagtgacctgtcaaaagaaaggc
     I  W  P  S  D  L  S  K  E  R
```

C. *PmPP2C2* cDNA

Nucleotides are numbered with reference to the initiator ATG (underlined). The nucleotide coding sequence is in uppercase, the 5' and 3' UTRs in lower case. The corresponding aa sequence is written underneath. A * denotes a stop codon.

```
-36 ttttcctcctgggttgcctttaattggccctcagtgATGTCGGGTCTAATCCGGCCCGCG
    F  S  S  W  V  A  F  N  W  P  S  V  M  S  G  L  I  R  P  A

 25 TTTGTGTGGATGCCGGCGGCAGTTGTAGCGGCTGTAAAGGTTGCAGAGGTCGCCCGTCGA
    F  V  W  M  P  A  A  V  V  A  A  V  K  V  A  E  V  A  R  R

 85 ACGCGATGTGAGCAGGGAGCCCCTCAGGGATCGGCTGTTGTTGCTCCTGTTACAGGGGCA
    T  R  C  E  Q  G  A  P  Q  G  S  A  V  V  A  P  V  T  G  A

145 GAAGGCCCTCAGCTTCTCATGCCTGTTGCCCAGGCAACATCAGCACGGCCGCTGCCAAGC
    E  G  P  Q  L  L  M  P  V  A  Q  A  T  S  A  R  P  L  P  S

205 GCTCGGATTGGCTGCCATCAATACGCGGCTAACTCCCCTTGTGAAGATCGACTAGCATTA
    A  R  I  G  C  H  Q  Y  A  A  N  S  P  C  E  D  R  L  A  L

265 CAACAGTTCCCGGGTGGAACGCTCTGTGCATGTGTTTTCGATGGGCATGGAGGCTGGCAG
    Q  Q  F  P  G  G  T  L  C  A  C  V  F  D  G  H  G  G  W  Q

325 GTCGCTGAGTACCTCCGTGGTCATCTACCATCGCTGTTGGCCAGTCGACTCCCTCACAAG
    V  A  E  Y  L  R  G  H  L  P  S  L  L  A  S  R  L  P  H  K

385 TCTGGGCATATAGACTCTCGTACAATTGAGTCGGCATGCAAGGAGGCATTTATGGTGGCG
    S  G  H  I  D  S  R  T  I  E  S  A  C  K  E  A  F  M  V  A

445 GATTCTGAGTTGGAAAAGCACGCACGGGAAGCTCAGAAACTGGGTTTTTCCCAGACTGTG
    D  S  E  L  E  K  H  A  R  E  A  Q  K  L  G  F  S  Q  T  V

505 AAAACTGGCGCCTGTGGATTAGCACTGCTCATCACACAGACCAGCCTGGTGGTAGCGAAT
    K  T  G  A  C  G  L  A  L  L  I  T  Q  T  S  L  V  V  A  N

565 GCTGGAGACTGTAAGGCGGTGCTTTATAGAGATCAGCGTCCTGCATTGCCGTTGAATATG
    A  G  D  C  K  A  V  L  Y  R  D  Q  R  P  A  L  P  L  N  M

625 CAGCATAACGCCAGCGATGTACGAGAGCAGAGACGGCTCGAGCTCGAACATCCTAATGAG
    Q  H  N  A  S  D  V  R  E  Q  R  R  L  E  L  E  H  P  N  E

685 AACAATGTTGTTAGGTGTAAGAAAGAGTGGCATGAACCAGTAGTCGTGGCCGTTCCCAAG
    N  N  V  V  R  C  K  K  E  W  H  E  P  V  V  V  A  V  P  K

745 AGCGGCTGGCTGGCCGTGAAGTCGTGGTTGGGCTACCCCGTCGAGCTAGAGAGATTGGAA
    S  G  W  L  A  V  K  S  W  L  G  Y  P  V  E  L  E  R  L  E

805 CATGCTACCAAGTACTCGGGCTGTTATGTCAAGGGCCGTCTCCAGCCGACTCGAAGTTTT
    H  A  T  K  Y  S  G  C  Y  V  K  G  R  L  Q  P  T  R  S  F

865 GGTGATTTCTATCTCAAGTCGGCCGAGTTTCTCTTTAACCATGCATCTGGGCGGAACTTC
    G  D  F  Y  L  K  S  A  E  F  L  F  N  H  A  S  G  R  N  F

925 CTACCACCTCCTGACCCAAAGAGTTCTGCTCATACACTTACTCAACCACTGCAGCACTCC
    L  P  P  P  D  P  K  S  S  A  H  T  L  T  Q  P  L  Q  H  S
```

```
 985 TTCCCCTACATCACTAGCGAGCCCGAGGTGATGGTATATCCACGACATGAGGATGACAAG
     F  P  Y  I  T  S  E  P  E  V  M  V  Y  P  R  H  E  D  D  K

1045 TTCATAATATTGGGCAGTGATGGTCTGTGGGATAATGTGACTGATGAAGAAGCTGTAGGG
     F  I  I  L  G  S  D  G  L  W  D  N  V  T  D  E  E  A  V  G

1105 TTCGTGAGGCGACTACTTTTACAAGAAAACAGCAGTTGGAGTGCCAACTCTGTTGCAGAG
     F  V  R  R  L  L  L  Q  E  N  S  S  W  S  A  N  S  V  A  E

1165 GCGCTGACAGGAGAGGTGTTGAGTCGAGCAGCGAAGAAGAGCTCCAAGAGCTTAGCGGAG
     A  L  T  G  E  V  L  S  R  A  A  K  K  S  S  K  S  L  A  E

1225 CTCCAGGCTCTCCCACAGGGCAACCAACGTCGGAGGCTCCATGATGACATTTCGGTCTGT
     L  Q  A  L  P  Q  G  N  Q  R  R  R  L  H  D  D  I  S  V  C

1285 ATAATCGATCTTCGTCCGATCAAGGATCATTCGTCTACAGTCGGCCATACCACTAGTGGT
     I  I  D  L  R  P  I  K  D  H  S  S  T  V  G  H  T  T  S  G

1345 TGAagctgtactcctgatggtggatatggtcgtgggagatagtgcaggtcttgtttcagt
     *  S  C  T  P  D  G  G  Y  G  R  G  R  *  C  R  S  C  F  S

1405 ctcagtctaaatgcaccaacaaatagtacatgtcttaaacttttttgaacgtatagcatc
     L  S  L  N  A  P  T  N  S  T  C  L  K  L  F  *  T  Y  S  I

1465 aaaaaaaaaaaaaaaaa
     K  K  K  K  K
```

D. *PmPP2B* gene sequence

Nucleotides are numbered with reference to the initiator ATG at exon 1. Coding sequences are highlighted with the corresponding aa sequence written underneath. Untranslated sequences at the 5' and 3' end of the cDNA sequence are in lower case. Note that the gene sequence downstream of the coding sequence is not complete. Also, the gene sequence upstream of the coding sequence is not shown in its entire length. Only approximately 1 kb is shown.

```
-1048 AGGATAAAAAGCATAAGAAGGATAAGAAGCATAAGAGGAGCGGTTATAGTAGTAGCAGCA
 -988 GTAGCAGTAGCAGTGACTGAGTAGATTTGTTCCAAGAAGAGTGGTATCAGCTTTTAATTT
 -928 GAGACGATGAAAATGGCTGCTGCATATTTACACTAAGAATCAATCACAATTTTTTATACA
 -868 AGCGTGTTGTGCTATGTATCATGCACGATGTTTCGTTTTAGTATGTGCGAAATAGACGCT
 -808 TATTCGTTCTAGCGGGCGCTCTGAATAGTGGGAATATGATCATTCGATGCGGCCTGCATT
 -748 GTGAGTATCGAGATTTATATGGTTTTTCGGTTGGAGCGCCAGGAGTGGGGTTTAAATTTC
 -688 CCGGAGGATTGCCAAATGGTGGAATTATGGTTTGGCTCGCCCCGGTGGTAAGTTTGGGAC
 -628 AGTGGAAGGATGTATTCCGAATAAGATGAGGGAATTGTGGTAAGGGGCATAAGACCGCTG
 -568 GGGAGGGGTTAATGATCTCCTTCAGCTCGTATCAATCGCTCGACTATGACCATACACTTT
 -508 GTTGCATTACTCCCAAGGCTCGCTTTAGTGTTTTATCAAGGAAATATTGTCGCTCTCAGC
 -448 GAGGGGGAAATCGTTGGTATCCACACTGGCAATATAGTAGGGATGGGAAGTCGCTGGAAG
 -388 TAACCCTTTTTATGAGGGCCTTGGACTGACAGTATGGGTCCTCTCACGTAACCTCTGGTC
 -328 GATATCGACTGTTACTAACCTATGGAATTTCAACTGAATTCTATTGAGTGTCTCGATTTG
 -268 ACATGCTTCTGGTGCTACTGTTACTACTTGGTTTTCTGGGTGGGAATTACTCGCAATACA
 -208 ATTATTTTTGGACCTGCATTACTCTGAAGAGTCAAAGGCCTGCAGTATTTGAGTATCCCA
 -148 TATGGAGCCCTTGGCAGACCCACTGCATGACCGCAGAGTTCGTACCGTAACTCCGCCACC
  -88 TCGTGCGGTAGGTGGAGGGAACTAACACGGCTCGGCAGGCTTGTGATGTTTTTACTCAgc
  -28 cgttaacgcggaaattgctcttcccccaATGGTGAAaGCATCGCCACCGGACTGGCAGTG
                              M  V  K  A  S  P  P  D  W  Q  C
   33 TCTCAGGAAGCATCTGGTGAGGTCAATATAGCATTGACTGTAGTTATGGTCAGTTTTAGT
       L  R  K  H  L              (intron 1)                    F
   93 TCGCGGAGGGTAGACTGTATATAGAGTCAATCACGAGGATCGTCAATGAACTTATACGGC
        A  E  G  R  L  Y  I  E  S  I  T  R  I  V  N  E  L  I  R  L
  153 TGTGTACGGCAGAGGCTAATGTTGTCAAAGTGAAGGACCCGGTTACAGTAGTAGGAGATA
        C  T  A  E  A  N  V  V  K  V  K  D  P  V  T  V  V  G  D  I
  213 TACATGGACAGTAGCAGTGCAAAGGAGTTGGTGATTCTGGCCTGTAGTAGAATGGTAGGT
        H  G  Q               (intron 2)                       Y
```

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273 ACTATGACCTTATCAAGCTACTGGAAGTCGGGGGAGATCCAGCCACAACTCAGTACGCCC
      Y  D  L  I  K  L  L  E  V  G  G  D  P  A  T  T  Q

333 GGGAGGGTTTATGCAACTTTTACGTGTACATTAGATATCTCTTCCTCGGAGACTATGTTG
       (intron 3)                        Y  L  F  L  G  D  Y  V  D

393 ACAGAGGCAGCTACAGTGTGGAAGTACTACTACTTTTGTAATAGGGTCTCATGTCGGCTC
      R  G  S  Y  S  V  E  V  L  L  L  L      (intron 4)

453 ACTTGACTTGCCCATGGTAATCAGATATGCGTTGAAGATCAACTACCCCAAGACTATAAC
                             Y  A  L  K  I  N  Y  P  K  T  I  T

513 GTTACTACGAGGAAATCACGAGTGTAGACAAATGACTGCTTTTTTTAATTTCCGAGACGA
     L  L  R  G  N  H  E  C  R  Q  M  T  A  F  F  N  F  R  D  E

573 ATGCGAGTAAGACTTTGTTCAGTCGAATCTTCCACTGAGAATCCCCGTTGGGTGTAGGTA
     C  E                      (intron 5)                        Y

633 CAAGTACAACCTAACTGTGTACGAGCTGTTTATGGAGTCGTTCGACTGTCTGCCGCTGGC
     K  Y  N  L  T  V  Y  E  L  F  M  E  S  F  D  C  L  P  L  A

693 CGCTGTCGTTAACGGTAGTTCTCTCAGGCCGGGTCGAAGCTATTGTATTGTTTAGGGAA
     A  V  V  N  G                 (intron 6)                  K

753 GTTCCTGTGTATTCACGGTGGTTTGTCTCCTGAGCTCAACACACTCGCTGACTTTAACAA
     F  L  C  I  H  G  G  L  S  P  E  L  N  T  L  A  D  F  N  K

813 AATTAACCGATTTCAAGAACCTCCGAGACAAGGCCTTTTCTGGTGAGTTCAGCGGCGGTG
     I  N  R  F  Q  E  P  P  R  Q  G  L  F  C

873 GTGGTTGTTGCCTCTGCTGGCTTTGATGAGCTGTGGTTTAGTGACATACTCTGGAGTGAC
          (intron 7)                           D  I  L  W  S  D

933 CCTGAGGAGGAGAAGGAGGGTGTTGCGGTGTTCAAGTCGAAGGATAGGACCTTTGTTCCC
    P  E  E  E  K  E  G  V  A  V  F  K  S  K  D  R  T  F  V  P

993 AACGATGTAAGTCATGGGTGTTTTCGACATTGTTAAGCTGGGCTACAGGTACGCGGCTGT
    N  D              (intron 8)                     V  R  G  C

1053 AGCTTCCTGTATACGTATGAAGCTGCAACGAATTTCTTGAAGAAGAATTCGTAGGATGGT
     S  F  L  Y  T  Y  E  A  A  T  N  F  L  K  K  N  S

1113 CGGGTGTCAGGGCTTCCTTTCGGACAACCCGGGTTCTATTCTTGGTATAGGTTATTGTCG
                  (intron 9)                              L  L  S

1173 GTTATTCGAGCCCATGAAGCTCAGCTGGAGGGATATAAAATGCACAGTAGGACAACTAAA
     V  I  R  A  H  E  A  Q  L  E  G  Y  K  M  H  R

1233 GTGTCGTGATGTTTATAATCTACCTGCGTCGCGAAATGTTCGAACGAAAAGACTATTCAC

1293 CCCCTCTCTATCGGGGTTTTTCCATCCTTGTGAGCTACTGATGAGAAACCCAAGAGCAGT

1353 GGGCTGATCGATATTTGAACCTTAGCACTGTATTAGATGGAATTGACGATCATTGAAATT
                          (intron 10)

1413 GCTCATTGAATGGTATGAAGTGTAGAATATAACGTCCAACTCCGGCTAGGATAACATTGA

1473 AAGCTCCTTGGTGTGCATTCAGGAACTAACGAGGCCACCGGCTTTCCCTCGGTCATCACC
                             T  N  E  A  T  G  F  P  S  V  I  T

1533 ATTTTCTCGGCACCTAATTACTGTGATGTGTATAATAACAAGGTGATAAACAGCCGATTT
     I  F  S  A  P  N  Y  C  D  V  Y  N  N  K
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1593 GAAAACACTTCCGTGGGCATGCGTGTCTTCTTAGGGCGCGGTTTTGAGATTTGAGAACAA
               (intron 11)           G  A  V  L  R  F  E  N  N

1653 CACGTTGAACATTTTACAGTTCAATTTTTCCAAGCATCCGTAAGTGGTCGTCGATCTTGA
      T  L  N  I  L  Q  F  N  F  S  K  H  P       (intron 12)

1713 GGTTGATATTATGAATTCCTACAGGTACCACTTGCCCAACTTTATGGACGTCTTTGCGTG
                             Y  H  L  P  N  F  M  D  V  F  A  W

1773 GTCAATGCCTTTTGTCGCAGAGAAAATTACGGAGATGTTATTTTACGTATTAAATCCTCA
      S  M  P  F  V  A  E  K  I  T  E  M  L  F  Y  V  L  N  P  Q

1833 GAATGAGTCTGGCAGTGTAGGTTTGTGAAATTTTATCACACTCGCTCGCTTGACGCGAAA
      N  E  S  G  S                    (intron 13)

1893 ATGTATTTTAGTACACCCGTGCTCCCGATGTTACCGATGAAGATCTCCCACAGCTTCCCG
               Y  T  R  A  P  D  V  T  D  E  D  L  P  Q  L  P  E

2053 AAGCCGATCTGAAACAGTTGAAATTGGCCCGTCTGTCGAGTTTGTCTGACGAACAGGTAG
       A  D  L  K  Q  L  K  L  A  R  L  S  S  L  S  D  E  Q

2113 GGCCAATGCGAACGATGTGATGTGTTCATTGCAGTTCCTTTTCAGAATAAATCGGTATCA
               (intron 14)                        N  K  S  V  S

2173 CTTGCTGCCCGTTTGCATCAGCATATGATGGCCGAAGGGGCTAAGGTTATCGAGCAAAGT
     L  A  A  R  L  H  Q  H  M  M  A  E  G  A  K  V  I  E  Q  S

2233 AGTAGTAGTTGCGGTAAAGGTAGTTGGTGGGCAGTTGTTGGCAGTCTTGGAGGGGTTGTA
     S  S  S  C  G  K  G                 (intron 15)

2293 TGGGGCGGCACAGTACGGTGGTCCTCTCTCAGGTCGGGCAGCTGAGGCGGCGGGTGGTAA
                                        R  A  A  E  A  A  G  G  K

2353 GGCGATATCGAAGGAGAAGGCCGATCGTATGAGGAAGAAGATTCAAACTGTAATAGGGAG
      A  I  S  K  E  K  A  D  R  M  R  K  K  I  Q  T

2413 TAACAGAGACTTTTTAACTAAGCGTAGTTGTTGGTTGTGGATTAGCAGTAGTCTGTTTAC

2473 AACATTGATGATTATTGAACGCATATTTCCGAGCTTATGATGCATGTGTGTCAACAATGG

2533 CTTCAGGGCGAGGAAAAATGGTGTAGTAGATAGAATAGTAGCTGGTTTGGTAAATGAATA

2593 ACGATATGACACGTGAAGTGGATGTAGTAGTTTAACGGCTTGTAGTATGCATACTGATGG
                                  (intron 16)

2653 TTTGAGACTGTCTGCCGTAATGTGGGATAGGTCTCGGGTTCGAATCCCAGGTCCGGTATT

2713 TTTCATGCCGGATCCGTACTTTGACGCTTCCCTTAGTATAGTCGTCAACTAGGCTCCCGA

2773 CCAGGTCGCTTGCTGTTTTCAAACCCTTGGAATGATTCGACTTGCGATATATAGTCCCGC

2833 TCGCATCGCATAGTATTTATCTCCGCTATGAAAAATACCGGCTCCCGCTTATCCTTTATA

2893 GGTGAGTCGGATGATGCTTATGTTCAAGACCTTACGCGAGGAGAACGAGACCATCATCAA
      V  S  R  M  M  L  M  F  K  T  L  R  E  E  N  E  T  I  I  N

2953 CCTCAAAGGCGTTTGCCCTGGGCACAGGTGAGATCAGGAAATGCTCGAATTATGGTATGG
      L  K  G  V  C  P  G  H  R           (intron 17)
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3013 GACCTCGTCAGGTTGGCACCTGGTCTGCTACTGTCGGGCCGCGATGCGCTGCAAAACGAG
                L  A  P  G  L  L  L  S  G  R  D  A  L  Q  N  E

3073 TTGGAGAAGTTCACGAAGGCTCGAGCCATGGATTTAGAAAATGAAAGAATGCCGACCGAG
     L  E  K  F  T  K  A  R  A  M  D  L  E  N  E  R  M  P  T  E

3153 AAGGAGGTCGATAGCCAGCCCCATTTCCATCGTGGAATGTCATCATCATAGtggaaaggg
     K  E  V  D  S  Q  P  H  F  H  R  G  M  S  S  S  *

3213 tcttgcccacttgggggttcctctcgaggggtcatcgaagccctctctttagctcgtgta

3273 ggattattagctgcatcatcatcatcgtcgtcgttgtctatgaattctagtatgatattg

3333 gaagcgttgggctcgtgtggactgttcatataattg
```

E. *PmPP2C1* gene sequence

Nucleotides are numbered with reference to the initiator ATG at exon 1. Coding sequences are highlighted with the corresponding aa sequence written underneath. Untranslated sequences at the 5' and 3' end of the cDNA sequence are in lower case.

```
-687 TCGGAGATCTGACGATATAGCAAATGTACTTCCATCGAGTGCGGAGAGCAAATCTTACCC
-627 TCCCAATTTTCTCCATTAGCAATACGACGATAATCGTCAGCTGTGAGAAAGCCGGAACGG
-567 AGACCTCTTACGAGACCCTCTCCGAGACCATCATCGATGTTGAAGTAAACGAGATCAGCC
-507 ATGGGAAATATAGCAGTACCAGGACAACTACAAAACCAACAATCTAGTACAGTCACCAAT
-447 TCTTCGCGCTGCGGTCAAAGATATTGCGTACCGATTTGCTCAAGTTTCTCACTCGCACGC
-387 TTAAAATAGCGCTGCAGACAAGTTCATAGAAAAACACGCAAACGATGATACCAACATGGA
-327 GTAGATCGCGCTTTTGGCGCACTAACGACACCATGTTGTACTATGGACATGATGACGGCT
-267 ATGCTCTAGCCAATACAATGGAGATCGGAGTAGAGACCCAGCGGCGAACCGGAGGCGCTG
-207 CTCAGCGTTGGCTGAGCTCATCTTGCTATCTCGAAAAAGTGTCCTTTGGAACATTGTGCA
-147 GTAGCGCGCGCCATTGTAATCGATCCTTTTTGCTCAGCACGTGGCAACGCATTGAGTTCA
 -87 AGGTTCGTCTCGTGAACTTCAGTGGTTGCTTATTTGAGTACACTCTCTTCCTTGGAAggt
 -27 ttgtggtgttttcctacattttacgcaATGGGTGGGTTACTGTCTCACCCCGTTACCGCC
                              M  G  G  L  L  S  H  P  V  T  A
  34 GTCCATCTACAACGACGTGCCAACGACAAGTTCCAATGCGGTGTCGCAACTCTACAGGGT
     V  H  L  Q  R  R  A  N  D  K  F  Q  C  G  V  A  T  L  Q  G
  94 TGGAGGATCTCTCATGAAGATGCGCACTGCATAGACTTGGACTGGGGTTCAACTCATGAA
     W  R  I  S  H  E  D  A  H  C  I  D  L  D  W  G  S  T  H  E
 154 GAAGGCTTCTTTGCTGTACTCGATGGTCATACTGGTGATGACGCTGCCGAGTTCGGATCA
     E  G  F  F  A  V  L  D  G  H  T  G  D  D  A  A  E  F  G  S
 214 AAGGAGTTACCTAAGCAGCTAGACGAGTCGGCTGGAGATCCCGAGGACCGCACTGTACAA
     K  E  L  P  K  Q  L  D  E  S  A  G  D  P  E  D  R  T  V  Q
 274 GGCGTACAGGCCGGTTTCTTAGCCACTGATCAGGCGCTGAGAGAGACTCATTCTGAGGCC
     G  V  Q  A  G  F  L  A  T  D  Q  A  L  R  E  T  H  S  E  A
 334 GGCGCTGTTGTTGTGGCTTCCATTGTAGGACTAAGGGGATCTCTATTGTGATCGTAGTTC
     G  A  V  V  V  A  S  I
 394 TATACAGTAGAAGGATCGTAGTACTGTGTAACGCAGTGTATAGATGTAAATGCTTCCTGC

 454 AGTGTAAAAGGTTTTAACAAAAGTGGAGTACAATGAATGTGTCCATCGATGATTCACTTC

 514 CGCTTAGCTCGAAGTCGAATTTTTATAGAATTTCTCAGTCTAGAAATCTACGTTTGGCAG
                                  (intron 1)
 574 CTGTTTGAACATGAAATTCGAGTGTTATAATTGGGTAACTATCATAATGTTGACACTAGT
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 634 CAGCTTGCCATTGGCTTTTGCCGCATACATGATTAAATTCGTATAGGGAAGCTCTTTATC

 694 GTAGACTGTGCTCAGGTGACGCCTCTTGGCAACGGAAAATACAAGGTTCGCCTCATGAAC
                 V  T  P  L  G  N  G  K  Y  K  V  R  L  M  N

 754 GCTGGTGATTCCCGCGCATTGGTATTCTCGGGTAAGAACGTTGACATGACTAAGTTGGAG
     A  G  D  S  R  A  L  V  F  S  G  K  N  V  D  M  T  K  L  E

 814 AATGCCCAAGAGGACGTAACGAAGCAGCTATGCAATGATGATAGCACTGAGGCTAGCGGT
     N  A  Q  E  D  V  T  K  Q  L  C  N  D  D  S  T  E  A  S  G

 874 GATAAGGGTATATTCGGTGGAGGGGACATGCTGGGCTCAAAGAAGAATCTAGCGTCTCTC
     D  K  G  I  F  G  G  G  D  M  L  G  S  K  K  N  L  A  S  L

 934 ACTGACGCTGACGTCGGAATTTTGTTGGCCACTAAGGACCATAAGCCTGATGATCCCGAG
     T  D  A  D  V  G  I  L  L  A  T  K  D  H  K  P  D  D  P  E

 994 GAAAAGGCTCGGATTGAAGAAGCTGGTGGTTTTGTGAGCACAGACGAACCACCTCGGCTT
     E  K  A  R  I  E  E  A  G  G  F  V  S  T  D  E  P  P  R  L

1054 TGTGGTGTGTTGGCCCTATCCAGAGGCCTTGGCGACTTCGCGTATAAAGACGACGCGGAT
     C  G  V  L  A  L  S  R  G  L  G  D  F  A  Y  K  D  D  A  D

1114 CTTCCAGCTGATAAGCAAAAGTGCATTGCTGTGCCTGATGTTAGTGAAGTTATCGTTGAA
     L  P  A  D  K  Q  K  C  I  A  V  P  D  V  S  E  V  I  V  E

1174 GCTGGCGACTGGGTAGTATTGGCTTGTGATGGTGTGTTTGACGTTATGTCCAACGAGGAC
     A  G  D  W  V  V  L  A  C  D  G  V  F  D  V  M  S  N  E  D

1234 GTCGCGCGAGAAGTTATGGTACGAGCTCATGCTGGGGAGGATCTGGCTGAGGTTGCCGCT
     V  A  R  E  V  M  V  R  A  H  A  G  E  D  L  A  E  V  A  A

1294 GAAATATTACAACGTTGTCTCAACCTACTGGATTTCCAGGACAATATGACTTGTATGATT
     E  I  L  Q  R  C  L  N  L  L  D  F  Q  D  N  M  T  C  M  I

1354 ATTAGAGTCGGCACGACTGAGCGTGGTCTGGAGACTGAAGTCCCAGAAGAAACACAAGAG
     I  R  V  G  T  T  E  R  G  L  E  T  E  V  P  E  E  T  Q  E

1414 GAACTCCAGTTTGGTGGCTATTCAGACCTCCATACATTGGCTCCGGGAATGGCAGAAAAG
     E  L  Q  F  G  G  Y  S  D  L  H  T  L  A  P  G  M  A  E  K

1474 TATCAGACTTTCTTCAGGAGAGCTGGATTCAGTAAAAATCCCGCAGCCTGCAATACGTGT
     Y  Q  T  F  F  R  R  A  G  F  S  K  N  P  A  A  C  N  T  C

1534 TCAAAAGTGTATCCGCATATGGCGCAATGTCCGTTGTAAaacgccgtaatatggccgagt
     S  K  V  Y  P  H  M  A  Q  C  P  L  *

1594 gacctgtcaaaagaaagGTTGGAAGGATCATAAGAAGGTTTGTAAGGCAGTGAAGGGCAA

1654 CGTGAATAGTGTAACTCCCACATCTGTGAATGATTCGGCTGTTCAGAGTGGCGGTCGTAA

1714 AGGTCATAATCATAGTGGCCATATCAAGAGACGTTGAGTCCAGCATGACTGCTGTCGTTT

1774 GGTGGTGAAGGGTCGCGGTAATTACTTTTATGTAGTCTCCTGTACATATTACTTTCTACC

1834 GTGTGCTCGGTGAGTTCACTCATCATCTTGGCGAGTTTTGTTGGTTAAGTTGCTTTGGTG

1894 ATGATAATAGTTGTTGTAGTGAACTACCGCTTGCACAATACACTGTAGATGATTAATTTC

1954 TAGTCTTGTGTGTGAAGTTTTAATACTACTTGGGGAGAGGATTTGTGATGGTTGTGGATG

2014 TATGGGCTCAGCCTGCTGATAGTGCCTTGCGGCACTTAGTACCAGAAGCCGTCAAACTCT

2074 TCGAGAGATCGCATTCCCCGATGGTCCAATGGTAGGGCTACTGAAATTGGTCACTGAGTT

2134 TTTTGTCACTGCGCCACGAAATTGCCGTCCTTAGGGGAGACACTCCTTTGACTCCTACGA
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2194 GGCTGTTGGGGATGCTAGATGAGGCCGGCATCGATCAGGTGAGTTGGTTTCAATACTGGA

2254 AGAGGTCGTGGCATAGCTGCCCCATTTAGAGAATTCGGTCTCACTGAGGAATATATTGCT

2314 GTGCCATAGCCGCCCCATCTAGAGAATTTGGTCTGACTGAGGCATATACTGCCGTGGCAT

2374 AGGCGCCCCATGGGAAGACATTTGTCGTTCTGAGGCATATCTTGGTATGGCATAGCCGCC

2334 CCATCGGAAGACATTGGTCGTTCTGAGGCATACCTTGGTATGGCATAGCTGCCCCATCGG

2394 AAGACATTGGTCGTTCTGAGGCATATCTTGGTATGGCATAGCCGCCCCATCGGAAAACGT

2454 TGGTCGTTGTGAGGCATACCTTGGTATGGCATAGCCGCCCCGTGTGAATATTCCGAACTT

2514 ACTGCAGCAAACATGAAAAAGTGCGGTCATCCTTTACGCTTTGACCCGTCGACTGATGAT

2574 AGTGATGTAGTCTCTTGTACTATGTTCAGTACTGACCCTACTACTACTTATCATTATAGT

2614 AGGATATTATAGTAATACTCTATAGCGTTATCAGCTTTATA

F. *PmPP2C2* gene sequence

Nucleotides are numbered with reference to the initiator ATG at exon 1. Coding sequences are highlighted with the corresponding aa sequence written underneath. Untranslated sequences at the 5' and 3' end of the cDNA sequence are in lower case. Note that the gene sequence upstream and downstream of the coding sequence is not shown in its entire length. Only approximately 1 kb shown. The predicted signal sequence is underlined.

```
-997 TCAGGTAGTGATGGTAAGACCGTGGGTACTCGGTGGGGGGCCTGTTATGTTAAGGGAAGA
-937 CTCCAGCCAACCCGAGCGTTGGGAGACTTCTATTTTAAAGTCAGTGCACTTTAGATTGCC
-877 AACTTGTGATAGTTGTAGCAGGACATGGACATTGCGAGAGCGGCGAGAGTAGCTCCTTAT
-817 CCTCATGAACGTAGCGATGACGATGGTAAAAGTCGACCTAAGGTTCAGTTCTCGCAGTGG
-757 TTGTTCTAGAGAACCCTTTCCCGCCCTTCACTCCACCTTATGTATCAGCAGTACCCGATG
-697 TTCGTACAGAGTGTAGGGACCCGACTCGGGACATGTAGGCATCTGCTGGGTCGATGCTAG
-637 GCCAGCATGATAACACAGGTTCTTGATAATGGCTTCTGATGGCCTTTGGGACTACTTGCC
-577 GGATCCTGAGATCGTCTCAACAGCGGTAGATGTATTTGAACGGGAAGGGCCACAGGCAGC
-517 AGCAGAAGCCCTAGTATCACGAGTATGGGGAGAATGTGGTCTAACGTGTGTGTGTGGTAG
-457 TGGGTGCGGTGATAGGTCATTGACCAAGCCGCTCGGTCAGCGAAATTAACTCCGAACGTT
-397 GTTCGACGTATGCAACCTGGCAGAACTAGGAGGAATATACATGATGACGTCACAGTAGTG
-337 ATTGTAAAGCTGTAGGTCTTCGCGGCGTTTGCTTGTCTGAAGTGGCATCAAGACAACCAT
-277 TGCACTCTTACTATGAATATTGTTTATATTACTATTAGCATGTTTGGATTAGCGATTGGC
-217 GAAAACGGTTCCCTGCATCTGCAGTGGGAGCCATCGTGATTGTCGCTTCTATTGGCTCCG
-157 GTCGCAGTGTGTGGTTACAGAGAGTCACTCAAGTCTCTCAACACGTGAACCCACTACTAC
 -97 TTGTGTTGCACTAGGGCAGGGTCGCTGTTGTTCTTTGGACACTGGTTCACAATTGAGTCA
 -37 TttttcctcctgggttgcctttaattggccctcagtgATGTCGGGTCTAATCCGGCCCGC
                                          M  S  G  L  I  R  P  A
  24 GTTTGTGTGGATGCCGGCGGCAGTTGTAGCGGCTGTAAAGGTTGCAGAGGTATGCTCCCT
      F  V  W  M  P  A  A  V  V  A  A  V  K  V  A  E
  84 GCAGGGTCTTGCCTTAGCAGCAATCATTGGTATAGGTCGCCCGTCGAACGCGATGTGAGC
           (intron 1)                    V  A  R  R  T  R  C  E  Q
 144 AGGGAGCCCCTCAGGGATCGGCTGTTGTTGCTCCTGTTACAGGGGCAGAAGGCCCTCAGC
       G  A  P  Q  G  S  A  V  V  A  P  V  T  G  A  E  G  P  Q  L
 204 TTCTCATGCCTGTAGGTCACAGAAAGGCTACTGTGGATACCCGTCTCCGTTGGTCAGGTT
       L  M  P                    (intron 2)                 V
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264 GCCCAGGCAACATCAGCACGGCCGCTGCCAAGCGCTCGGATTGGCTGCCATCAATACGCG
    A  Q  A  T  S  A  R  P  L  P  S  A  R  I  G  C  H  Q  Y  A

324 GCTAACTCCCCTTGTGAAGATCGACTAGCATTACAACAGTTCCCGGGTGGAACGCTCTGT
    A  N  S  P  C  E  D  R  L  A  L  Q  Q  F  P  G  G  T  L  C

384 GCATGTGTTTTCGATGGGCATGGAGGCTGGCAGGTCGCTGAGTATTGATATGCTCGTTTT
    A  C  V  F  D  G  H  G  G  W  Q  V  A  E

444 TGAGCCGTCGCAGTAGAAACACTGACCAGGTACCTCCGTGGTCATCTACCATCGCTGTTG
      (intron 3)                 Y  L  R  G  H  L  P  S  L  L

504 GCCAGTCGACTCCCTCACAAGTCTGGGCATATAGACTCTCGTACAATTGAGTCGGCATGC
    A  S  R  L  P  H  K  S  G  H  I  D  S  R  T  I  E  S  A  C

564 AAGGAGGCATTTATGGTGGCGGATTCTGAGTTGGAAAAGCACGCACGGGAAGCTCAGAAG
    K  E  A  F  M  V  A  D  S  E  L  E  K  H  A  R  E  A  Q  K

624 TAAGGCATGTCACGCCAGGTCCGTAGTTCGTGAGGGCCTTACTATTACCTCTTTCAGACT
                        (intron 4)                              L

684 GGGTTTTTCCCAGACTGTGAAAACTGGCGCCTGTGGATTAGCACTGCTCATCACACAGAC
     G  F  S  Q  T  V  K  T  G  A  C  G  L  A  L  L  I  T  Q  T

744 CAGCCTGGTGGTAGCGAATGCTGGAGACTGTAAGGCGGTAAGTCATGGAAGTCGTGGATG
     S  L  V  V  A  N  A  G  D  C  K  A              (intron 5)

804 AGAGGACCAGTCCTTTGCCCTATAAGGTGCTTTATAGAGATCAGCGTCCTGCATTGCCGT
                               V  L  Y  R  D  Q  R  P  A  L  P  L

864 TGAATATGCAGCATAACGCCAGCGATGTACGAGAGCAGAGACGGCTCGAGCTCGAACATC
      N  M  Q  H  N  A  S  D  V  R  E  Q  R  R  L  E  L  E  H  P

924 CTAATGAGAACAATGTTGTTAGGTAATTGGTACGGCTCTCAGGTGGTGTGCATATTTGTT
      N  E  N  N  V  V  R              (intron 6)

984 CTCTTCCTAGGTGTAAGAAAGAGTGGCATGAACCAGTAGTCGTGGCCGTTCCCAAGAGTG
               C  K  K  E  W  H  E  P  V  V  V  A  V  P  K  S

1044 AGGTTCTCTATGGGCACCATATATCGCTTGAATTGCATGTCCTTCAGGCGGCTGGCTGGC
           (intron 7)                                G  W  L  A

1104 CGTGAAGTCGTGGTTGGGCTACCCCGTCGAGCTAGAGAGATTGGAACATGCTACCAAGTA
      V  K  S  W  L  G  Y  P  V  E  L  E  R  L  E  H  A  T  K  Y

1164 CTCGGGCTGTTATGTCAAGGGCCGTCTCCAGCCGACTCGAAGTTTTGGTGATTTCTATCT
      S  G  C  Y  V  K  G  R  L  Q  P  T  R  S  F  G  D  F  Y  L

1224 CAAGGTAACCCCGTCTATCGACCCTAGAGCATCTTGGTGTTTTCCCAATGGCTGGGGCAT
      K                  (intron 8)

1284 TCCTCACTTGACGTTTTGTTCTGTGGTCAGTCGGCCGAGTTTCTCTTTAACCATGCATCT
                                 S  A  E  F  L  F  N  H  A  S

1344 GGGCGGAACTTCCTACCACCTCCTGACCCAAAGAGTTCTGCTCATACACTTACTCAACCA
     G  R  N  F  L  P  P  P  D  P  K  S  S  A  H  T  L  T  Q  P

1404 CTGCAGCACTCCTTCCCCTACATCACTAGCGAGCCCGAGGTGATGGTATATCCACGACAT
     L  Q  H  S  F  P  Y  I  T  S  E  P  E  V  M  V  Y  P  R  H

1464 GAGGATGACAAGTAGACACGTCGTTGTGCGAGAGGCGAGAATTGCAGGTTGGCTAACGCT
     E  D  D  K              (intron 9)

1524 GGCAGGTTCATAATATTGGGCAGTGATGGTCTGTGGGATAATGTGACTGATGAAGAAGCT
          F  I  I  L  G  S  D  G  L  W  D  N  V  T  D  E  E  A
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1584 GTAGGGTTCGTGAGGCGACTACTTTTACAAGAAAACAGCAGTTGGAGTGCCAACTCTGGT
     V  G  F  V  R  R  L  L  L  Q  E  N  S  S  W  S  A  N  S  V

1644 AGGCTGTGACTAGAGTGAGGGAGAATCGTCTGAGACGGAATAGTTTGGAATTATGGTCTC
                          (intron 10)

1704 CATGGTCGTTTTCATTCTCAGTTGCAGAGGCGCTGACAGGAGAGGTGTTGAGTCGAGCAG
                           A  E  A  L  T  G  E  V  L  S  R  A  A

1764 CGAAGAAGAGCTCCAAGAGCTTAGCGGAGCTCCAGGCTCTCCCACAGGGCAACCAACGTC
       K  K  S  S  K  S  L  A  E  L  Q  A  L  P  Q  G  N  Q  R  R

1824 GGAGGCTCCATGATGACATTTCGGTCTGTATAATCGATCTTCGTCCGATCAAGGATCATT
       R  L  H  D  D  I  S  V  C  I  I  D  L  R  P  I  K  D  H  S

1884 CGTCTACAGTCGGCCATACCACTAGTGGTTGAagctgtactcctgatggtggatatggtc
       S  T  V  G  H  T  T  S  G  *

1944 gtgggagatagtgcaggtcttgtttcagtctcagtctaaatgcaccaacaaatagtacat

2004 gtcttaaacttttttgaacgtatagcatcGCCTCTGGCAGGCTTGTGTTTTAGTGGTGGA

2064 AAAACGTACAGAAGTGCCCTACTTTGCATTGTGTCGAGATGGCTGCGTCTATGCTAGCAG

2124 CGACCCATTATAAGAATCAGGCAGGTGGAGCCTCCAGTCCTGAAGTAGTGGGGGGGACGC

2184 TCTGCTGCCACCAGTGTTGAAATATATGTTCACTTTCCTTAGCTGAGCTCAGGAGTTGAC

2244 TCACTAGTCATAGGAGTTAATATAGGAATTGGGTTAAAAGTATACAGCAACCTGCAGGAA

2304 TTTCTTCAGAATATTCATATTGTCACCAACGCATGTGGTTGACCATGTGCATATTGCCAC

2364 CTCAACGTGACAATACTGGTAGTAGCTATCTTACATTAGGAGTCATGTGATGGAGGACCT

2424 GCTACGGGTGGTACTCGAACAGGAGACGCTGGAGATGCGGGGGCAGCAGCACGAGGTGTT

2484 GACGGGGCGACTAAGACGAACACTATGGGGTCTTGGGAGTCGGCAGAGTAGTGAAGCCGA

2544 TGCTTGGCCGACTAAGGCGTGCTGAGGGATTCTTTTCAGACAGCGGCAACAGAGCATACG

2604 CCTATACAACTTGGCTCGGTTTGTGTTCGAGATATGCTACACCTTGGATCTACCTGAAGA

2664 GGTGACACGGTTCATGGGTTGTCTAGTCTGATGGCGGTCTTGACAGGTTACGGCCACTGC

2724 AATGCTTTATTTGCATTCGGTATTGGATAAGATGAGAATGAAGGACCTTTCGGATGATCG

2784 ATATGTAATGCTTATGCTTAGTCGATGCTTCTTTGGGCAACTCCCTTCTGTTTTAGCTGA

2844 TAGCAATAGCGTCCATCGTGCTGGCGTGTAAGGTGTGTGAGGACAGATTGCCTAGGTCAC
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G. List of published and submitted papers

Quesenberry, M. S., Saito, K., Krupatkina, N. D., Robledo, J. A. F., Drgon, T., **Pecher, W. T.**, O'Leary, N., Alavi, M., Miller, T., Schneider, R. E., Belas, R., Deeds, J. R., Place, A. R., Zohar, Y., Vasta, G. R. 2002. Bioassay for ichthyocidal activity of *Pfiesteria piscicida*: Characterization of a culture flask assay format. *J Appl Phycol* **14**: 241-54.

Schott, E. J., **Pecher, W. T.**, Okafor, F., Vasta, G. R. 2003. The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species. *Exp Parasitol* **105**: 232-40 (Reprint see Appendix I. 1).

Pecher, W. T., Robledo, J. A. F, Vasta, G. R. 2004. Identification of a second rRNA gene unit in the *Perkinsus andrewsi* genome. *J Eukaryot Microbiol* **51**: 234-45 (Reprint see Appendix I. 2).

Pecher, W. T., Alavi, M. R., Schott , E. J., Robledo J. A. F., Roth L., Berg, S. T., Vasta, G. R. Assessment of the northern distribution range of selected Perkinsus species in eastern oysters (*Crassostrea virginica*) and hard clams (*Mercenaria mercenaria*) using PCR-based detection assays. *J Parasitol* submitted.

H. List of contributions to scientific meetings

Quesenberg, M. S., Saito, K., Krupatkina, D. N., O'Leary, N., **Pecher, W. T**., Alavi. M., Miller, T., Schneider, R., Belas, M. R., Deeds, J. R., Place, A. R., Zohar, Y., Vasta, G. R. Fish bioassay for *Pfiesteria piscicida* toxicity: Characterization of a culture flask assay format. CDC National meeting on *Pfiesteria.* Atlanta, GA, October 2000.

Pecher, W. T., Robledo, J. A. F., Coss, C. A., Vasta, G. R. Further molecular characterization of *Perkinsus andrewsi* and related isolates. Annual Meeting of the National Shellfisheries Association. Mystic, CT, April 14-18, 2002.

Schott, E. J., Robledo, J. A. F., **Pecher, W. T**., Okafor, F., Vasta, G. R. The antioxidant system of *Perkinsus marinus*. Annual Meeting of the National Shellfisheries Association. Mystic, CT, April 14-18, 2002.

Pecher, W. T., Robledo, J. A. F., Coss, C. A., Vasta, G. R. Identification of a second rRNA gene unit in the *Perkinsus andrewsi* genome. The 10th International Congress of Parasitology Associations. Vancouver, BC, August 4-9, 2002.

Saito, K., Quesenberry, M. S., Krupatkina, D. N., Robledo, J. A. F., Drgon, T., **Pecher, W. T.**, O'Leary, N., Alavi, M., Miller, T., Schneider, R. E., Belas, R., Deeds, J. R., Place, A. R., Zohar, Y., Vasta, G. R. Flask bioassay for ichthyocidal activity of *Pfiesteria piscicida*. Xth International Conference on Harmful Algae, St. Pete Beach, FL, USA, October 21-25, 2002.

Pecher, W. T., Robledo, J. A. F., Schott, E. J., Vasta, G. R. Application of genus-, species-, and strain-specific molecular probes to the detection of *Perkinsus* spp. in the Atlantic coast of USA. International Conference on Shellfish Restoration. Charleston, SC, November 20-24, 2002.

Pecher, W. T., Robledo, J. A. F., Coss, C. A., Vasta, G. R. Further molecular characterization of *Perkinsus andrewsi* and a related isolate. International Conference on Shellfish Restoration. Charleston, SC, November 20-24, 2002.

Schott, E. J., Robledo, J. A. F., **Pecher, W. T.**, Okafor, F., Vasta. G. R. The antioxidant pathway of *Perkinsus marinus*: functional analysis and localization of two iron superoxide dismutases. International Conference on Shellfish Restoration. Charleston, SC, November 20-24, 2002.

Pecher, W. T., Schott, E. J., Robledo, J. A. F., Vasta G. R. Assessment of the epizootiology of *Perkinsus* spp. at the Atlantic Coast of the USA using genus-, species-, and strain-specific molecular probes. Annual Meeting of the National Shellfisheries Association, New Orleans, LA, April 13-17, 2003.

Schott, E. J., Robledo, J. A. F., **Pecher, W. T.**, Okafor, F., Vasta, G. R. The antioxidant pathway of *Perkinsus marinus*: functional analysis and localization of two iron superoxide dismutases. Annual Meeting of the National Shellfisheries Association, New Orleans, LA, April 13-17, 2003.

Pecher, W. T., Robledo, J. A. F., Vasta, G. R. *Perkinsus andrewsi* has a second rRNA gene unit. 11th East Coast Protozoology Conference. University of Maryland, Baltimore County, Catonsville, MD, June 6-8, 2003.

Schott, E. J., Robledo, J. A., **Pecher, W. T.**, Okafor, F., Silva, A., Vasta, G. R. The antioxidant system of *Perkinsus marinus*. 11th East Coast Protozoology Conference. University of Maryland, Baltimore County, Catonsville, MD, June 6-8, 2003.

Schott, E. J., Robledo, J. A., **Pecher, W. T.**, Vasta, G. R. Antioxidant activities of the protistan parasite *Perkinsus marinus*. Molecular Parasitology Meeting XIV. Marine Biological Laboratory, Woods Hole, MA, September 14-18, 2003.

Pecher, W. T., Alavi, M., Schott, E. J., Robledo, J. A. F., L. Roth, Berg, S. T., Hancock, H. A., Vasta, G. R. Assessment of the distribution of *Perkinsus* spp. along the Mid and North Atlanic Coast using molecular probes. 79th Annual meeting of the American Society of Parasitologists. Philadelphia, PA, July 24-28, 2004.

Schott, E. J., **Pecher, W. T.**, Robledo, J. A. F., Vasta, G. R. Ascorbate-dependent peroxidase activity in the alveolate parasite, *Perkinsus marinus*. Annual meeting of the American Society of Parasitologists. Philadelphia, PA, July 24-28, 2004.

Pecher, W. T., Vasta, G. R. Isolation and partial characterization of selected phosphatases of the protistan parasite *Perkinsus marinus.* Molecular Parasitology Meeting XV. Marine Biological Laboratory, Woods Hole, MA, September 19-23, 2004.

Schott, E. J., **Pecher, W. T.**, Robledo, J. A. F., Vasta, G. R. Ascorbate peroxidase and the antioxidant repertoir of *Perkinsus marinus*. Molecular Parasitology Meeting XV. Marine Biological Laboratory, Woods Hole, MA, September 19-23, 2004.

Vasta, G. R., Alavi, M., Schott, E. J., Saito, K., Tasumi, S., **Pecher, W. T.** *Perkinsus* spp. and *Bonamia* spp. infections in the Asian Oyster (*Crassostrea ariakensis*) maintained in a fully contained aquaculture setting. Annual Meeting of the National Shellfisheries Association. Philadelphia, PA, April 11-14, 2005.

Schott, E. J., Robledo, J. A. F., Alavi, M. R., Saito, K., Tasumi, S., **Pecher, W. T.**, Vasta, G. R. *Perkinsus* spp. and *Bonamia* spp. infections in *Crassostrea ariakensis* maintained in a fully contained aquaculture setting. Annual Meeting of the National Shellfisheries Association. Philadelphia, PA, April 11-14, 2005.

Pecher, W. T., Alavi, M., Schott, E. J. , Robledo, J. A. F., Vasta, G. R. Application of molecular probes to assess the distribution of *Perkinsus* spp. in the eastern oyster *Crassostrea virginica* along the Mid- and North Atlanic Coast of North America. International Marine Biotechnology Conference. St John's, Newfoundland & Labrador, Canada, June 7-12, 2005.

Pecher, W. T., Vasta, G. R. Characterization of two putative protein phosphatase genes of the protistan oyster parasite *Perkinsus marinus*. Molecular Parasitology Meeting XVI. Marine Biological Laboratory, Woods Hole, MA, September 11-15, 2005.

Pecher, W. T., Vasta, G. R. Characterization of a protein phosphatase 2C-like gene of *Perkinsus marinus*, a protistan parasite of the eastern oyster, *Crassostrea virginica*. Molecular Parasitology Meeting XVII. Marine Biological Laboratory, Woods Hole, MA, September 10-14, 2006.

Pecher, W. T., Alavi, M., Robledo, J. A. F., Schott, E. J., Roth, L., Berg, S. T., Hancock, H. A., Vasta, G. R. Assessment of the distribution of *Perkinsus* species in the eastern oyster *Crassostrea virginica* and hard clam *Mercenaria mercenaria* along the Mid- and North Atlantic-coast of North America using molecular probes. Aquaculture 2007. San Antonio, TX, February 26 - March 2, 2007.

I. Reprints of the published papers relevant to the dissertation work

I. 1. Reprint of Schott et al. 2003

THE PROTISTAN PARASITE *PERKINSUS MARINUS* IS RESISTANT TO SELECTED REACTIVE OXYGEN SPECIES [5]

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ABSTRACT

The parasite *Perkinsus marinus* has devastated natural and farmed oyster populations along the Atlantic and Gulf coasts of North America. When viable *P. marinus* trophozoites are engulfed by oyster hemocytes, the typical accumulation of reactive oxygen species (ROS) normally associated with phagocyte activity is not observed. One hypothesis to explain this is that the parasite rapidly removes ROS. A manifestation of efficient ROS removal should be a high level of resistance to exogenous ROS. We investigated the *in vitro* susceptibility of *P. marinus* to ROS as compared to the estuarine bacterium *Vibrio splendidus*. We find that *P. marinus* is markedly less susceptible than *V. splendidus* to superoxide and hydrogen peroxide (H_2O_2), but equally sensitive to hypochlorite. Viable *P. marinus* trophozoites degrade H_2O_2 *in vitro*, but lack detectable catalase activity. However, extracts contain an ascorbate dependent peroxidase activity that may contribute to H_2O_2 removal *in vitro* and in vivo.

Keywords: Alveolata; Hydrogen peroxide; Hypochlorite; Oxidative stress; Peroxidase; *Perkinsus marinus*; Superoxide; *Vibrio splendidus*

Abbreviations used: ASA, ascorbic acid; ASW, artificial sea water; CAT, catalase; CFU, colony forming unit; ROS, reactive oxygen species; SOD, superoxide dismutase; XO, xanthine oxidase.

1. INTRODUCTION

The intracellular protistan parasite *Perkinsus marinus* is responsible for drastic declines in the eastern oyster (*Crassostrea virginica*) populations along the Atlantic and Gulf coasts of North America (Perkins and Menzel, 1967). Worldwide, there are multiple *Perkinsus* species associated with diseases of economically important bivalves (Azevedo, 1989).

P. marinus shares structural and molecular characteristics with both the Apicomplexa and the Dinozoa (Goggin and Barker, 1993; Levine, 1978; Siddall et al., 1997), and recent molecular analyses indicate that *Perkinsus* is basal to, but not within, the dinoflagellate lineage (Saldarriaga et al., 2003), and a new phylum, the Perkinsozoa, has been established to include the genera *Perkinsus*, *Parvilucifera*, and *Cryptophagus* (Brugerolle, 2002; Norén et al., 1999). Some oyster species, such as *Crassostrea gigas*, are apparently resistant to *P. marinus* infections (Meyers et al., 1991), but are susceptible to other pathogens, such as the estuarine bacterium *Vibrio splendidus* (Lacoste et al., 2001), although the molecular basis of these differences is not understood.

Perkinsus marinus infections likely occur after ingestion of trophozoites or zoospores by filter feeding, followed by their engulfment by oyster hemocytes, and systemic dissemination (Perkins, 1996). Intrahemocytic survival and proliferation of *P. marinus* is believed to rely on its ability to abrogate the deleterious effects of reactive oxygen species (ROS) typically generated by the host hemocyte upon phagocytosis of biotic or abiotic particles. Like neutrophils of vertebrates, oyster hemocytes produce superoxide by an NADPH oxidase

complex associated with the plasma membrane (Takahashi and Mori, 2000). In turn, superoxide is converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs), and in the presence of chloride ion, H_2O_2 is the substrate for the production of hypochlorite (HOCl), in a reaction catalyzed by myeloperoxidase (MPO) (Anderson et al., 1992; Greger et al., 1995). Cytotoxic effects of ROS include peroxidation of lipids, breakage of DNA strands, and inactivation of enzymes, especially those containing Fe–S centers (Birnboim and Sandhu, 1997; Brawn and Fridovich, 1981; Gardner and Fridovich, 1991). Compared to superoxide, H_2O_2 is relatively stable and able to diffuse rapidly across membranes (Ohno and Gallin, 1985). Hypochlorite is well known for its ability to damage membranes and cause cell rupture, as well as damage DNA and proteins (Dukan et al., 1999; Vissers et al., 1994). Although various molecules are efficient scavengers of HOCl (e.g., arginine, thiol-containing proteins, ascorbate;Ferrante et al., 1987; Hu et al., 1993), there is no enzymatic mechanism for its destruction.

Engulfment of live *P. marinus* trophozoites by oyster hemocytes does not result in the accumulation of appreciable quantities of ROS (Nakamura et al., 1985; Perkins, 1996; Volety and Chu, 1995), and limited parasite killing is observed (La Peyre et al., 1995). In contrast, phagocytosis of either heat-or osmotically killed *P. marinus* cells elicits a robust ROS response, suggesting that labile factors from live *P. marinus* are responsible (Anderson, 1999). Two hypotheses, not mutually exclusive, can be proposed: (a) *P. marinus* actively inhibits the ability of hemocytes to generate ROS, or (b) *P. marinus* degrades the

host-generated ROS at a rate sufficient to prevent their accumulation and detection. If the latter is true, it would be expected that *P. marinus* is relatively tolerant to *in vitro* exposure to ROS. To investigate this possibility, we undertook experiments to examine the relative sensitivity of *P. marinus* to ROS, as compared to that of *V. splendidus*, which is associated with disease in the pacific oyster, *C. gigas* (Lacoste et al., 2001).

2. MATERIALS AND METHODS

2.1. Reagents and cell culture

Arginine, ethidium bromide, H_2O_2, xanthine, NaOCl, fluorescein diacetate, neutral red, and Aspergillus niger catalase were obtained from Sigma (St. Louis, MO); xanthine oxidase was obtained from Calbiochem (La Jolla, CA). *P. marinus* strain Texas (ATCC 50849), was grown at 28 ℃ in DMEM:Hams F12 (Sigma) with 5% fetal bovine serum (Paragon, Baltimore, MD) and 15 ppt artificial seawater (ASW; Instant Ocean, Mentor, OH) (Gauthier and Vasta, 1993). Bacterial strains *V. splendidus* (ATCC 33125) and *Vibrio salmonicida* (ATCC 43839) were maintained on LB agar (Difco, Detroit MI) at 22 ℃.

2.2. Reactive oxygen exposures

Cultured *P. marinus* trophozoites, washed once in PBS500 (500 mM NaCl, 20 mM NaPO4, pH 7.2) and suspended to a final OD600 of 0.025, were exposed to ROS for 1 h in a total volume of 0.8 ml in 24-well tissue culture

plates. All ROS-generating reagents were also diluted in PBS500. Exposure to superoxide-generating xanthine oxidase (XO) was accomplished by aliquoting 0.4 ml of a trophozoite suspension (at OD_{600} of 0.05) into triplicate wells, followed by 0.2 ml of 5 mM xanthine, then 0.2 ml of the appropriate XO stocks to yield final concentrations of 100, 200, 500, and 1000 U/ml. Confirmation of superoxide production by X/XO was achieved using the water-soluble tetrazolium WST-1 (Dojindo Molecular Technologies, Gaithersburg, MD), at 430 nm, following the method of Tan and Berridge (2000). By this method the rate of WST-1 reduction was linear from 5 to 110, and a 25 mU/ml reaction created 5.3 lM/min superoxide (results not shown). The theoretical yield of superoxide from 25 mU/ml is 25 lM/min; however, our assay conditions differ from those reported elsewhere (Bortolussi et al., 1987). Exposure of trophozoites to H_2O_2 and HOCl were similarly conducted by mixing 2X stocks of trophozoites with 2X solutions of either H_2O_2 or HOCl. ROS exposures were terminated by pelleting cells by centrifugation at 450g, followed by two washes with sterile ASW. Trophozoites exposed to HOCl were washed first with ASW containing the radical scavenger arginine (1 mM), then washed again with ASW. Cells were then suspended in culture medium and allowed to rest for 1 h before removing samples for viability assessments.

Vibrio splendidus were grown overnight on LB agar, harvested, and washed once in PBS500, then exposed to superoxide-generating xanthine oxidase (X/XO) activity in a manner similar to that described by Bortolussi et al. (1987). Briefly, 50 µl of a suspension of bacteria at a calculated OD_{600} of 0.01

was aliquoted into triplicate wells of a 96-well microtiter plate, followed by 25 µl of 5 mM xanthine, then 25 µl of a XO stock to yield final concentrations of 0, 5, 25, 100, and 200 mU/ml total per well. Additional ROS exposures were conducted by adding 50 µl of a 2X stock of H_2O_2 or HOCl to 50 µl of a 2X suspension of bacterial cells. Exposures were allowed to proceed for 60 min at room temperature, then arrested by 10-fold dilution with ice-cold artificial sea water (ASW), followed by dilution-plating for CFUs. For HOCl treatments, arginine (1 mM) was incorporated in the 10-fold ASW diluent (Ferrante et al., 1987).

2.3. Viability assays

Viability of *P. marinus* was determined by differential staining with 3 µM fluorescein diacetate (FDA) and 0.01 mg/ml ethidium bromide (EtBr) (Rotman and Papermaster, 1966), and examination by fluorescence microscopy. Dead cells were indicated by red fluorescence, live cells by green fluorescence. FDA/EtBr staining was adequate for viability assessments of X/XO-and HOCl treated cells, but H_2O_2 treatments above 1 mM caused *P. marinus* cells to become transiently FDA negative for up to 12 h. The vast majority later regained FDA staining but not EtBr staining, indicating that they were not dead. Therefore, in H_2O_2 experiments, cells not staining with EtBr were counted as viable. As a confirmation that FDA negative, EtBr negative cells were viable, some experiments were also monitored with neutral red (0.01%) staining; 16 h following H_2O_2 treatments, parallel staining with FDA and neutral red showed that both measures of viability were in agreement (data not shown). Viability of *Vibrio*

strains was determined by serial dilution and plating on LB agar. Colonies were counted after 18 - 24 h incubation at 22 °C.

2.4. Peroxide destruction assays

The ability of live *P. marinus* and *V. splendidus* cells to remove hydrogen peroxide from solution was tested by suspending freshly harvested cells in PBS500, at OD_{600} of 2.0 and 0.025, respectively. Cells were challenged with 75 µM H_2O_2 in triplicate reactions in microtiter wells (200 µl final volume) at 22 °C. Samples were withdrawn at 20, 40, and 60 min, and assayed for remaining H_2O_2 using a microtiter adaptation of the FOX assay of Jiang et al. (1992). Standard curves were constructed using the same reagents as used for experiments. Viability of *P. marinus* and *V. splendidus* cells, after 1 h exposure to peroxides, was assessed by neutral red staining and dilution plating on LB, respectively.

For analysis of H_2O_2 destruction by cytosolic cell extracts, both *P. marinus* and *V. splendidus* were lysed in low osmotic buffer (25 mM $NaPO_4$, pH 7.2) containing 0.5% Triton X-100. Cell debris was removed by centrifugation (10,000g), and supernatants dialyzed overnight (4 °C) against 0.5 M NaCl buffered with 25 mM $NaPO_4$, pH 7.2, and containing 1 mM ascorbic acid (ASA). *P. marinus* and *V. splendidus* extracts were processed in parallel, and maintained no more than 24 h at 0 °C until assayed. Ten microliters of extract was assayed in reaction conditions identical to those described for live cells. FOX assays were conducted on 10 µl aliquots at 22 °C in a final volume of 200 µl.

Ascorbate peroxidase assays were conducted essentially as described by Amako et al. (1994). Ten microliter aliquots of dialyzed *P. marinus* or *V. splendidus* extract were added to a reaction mixture of 0.2 mM ASA in 25 mM $NaPO_4$, pH 7.2, mixed, and allowed to equilibrate for 15 s. Five microliters of a 10 mM H_2O_2 stock in 25 mM $NaPO_4$, pH 7.2, was then added, and the amount of reduced ASA in the reaction was monitored at 290 nm for 90 s. The slope of Abs_{290} decrease was calculated from 30 to 60 s. The decrease in Abs_{290} was converted to nM ascorbate using a molar absorbance coefficient of 2.8 $mM^{-1}cm^{-1}$. The spontaneous rate of ASA oxidation was subtracted from the H_2O_2-dependent rate, and for the data reported in Table 1, was less than 15% of the H_2O_2-dependent rate.

Table 1. Detection of ascorbate peroxidase activity in dialyzed extracts[a]

Source of extract	ΔA_{290} extract only [b]	ΔA_{290} extract with H_2O_2 [b]	Δ[ascorbate] (nmol/h/mg)
Perkinsus marinus	-0.008	-0.062	2430
Vibrio splendidus	+0.003	+0.001	0

[a]Ten microliters of P. marinus or V. splendidus extract (1 mg/ml protein) were assayed for APX activity as described in Section 2.
[b]Change in absorbance units/min from t = 30–60 s.

3. RESULTS AND DISCUSSION

3.1. Effects of X/XO system on viability of *P. marinus* and *V. splendidus*

Superoxide was generated using the xanthine/xanthine oxidase (X/XO) system with levels of XO up to 1000 mU/ml (Fig. 1A). At the highest level of XO tested (1000 mU/ml), the mean viability of *P. marinus* trophozoites decreased by 17%, which was not significant when analyzed by Student's *t*-test ($p = 0.06$). Although a 1000 mU/ml reaction has the potential to create 212 nM/ min superoxide, as explained in Section 2, our system produced only about 20% of the superoxide theoretically possible. Thus, we estimate that the 1000 mU reaction produced approximately 42 nM/min superoxide. Activated human

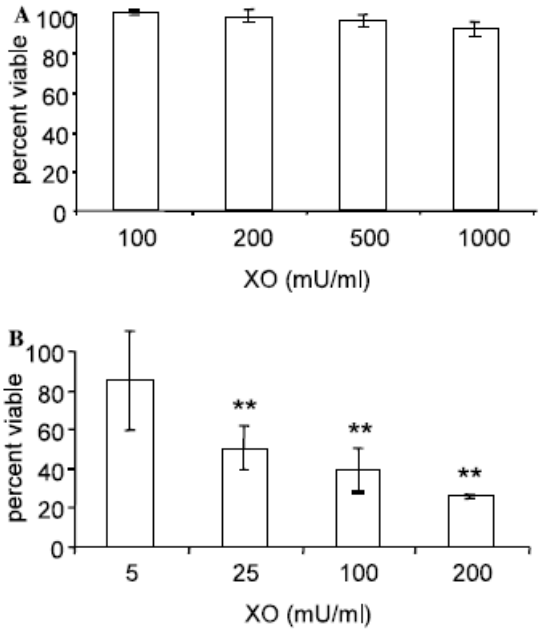

Figure 1. Percent viability after exposure to ROS generated by the X/XO system. (A) Percent viability of *Perkinsus marinus* trophozoites after a 1 h exposure to increasing amounts of XO. (B) Percent viability of *Vibrio splendidus* after a 1 h exposure to increasing amounts of XO. Viability measurements as in Section 2; error bars are SD. **p < 0:005 versus untreated controls.

neutrophils produce from 14.9 to 26 nM/ min superoxide per 10^6 cells (Bortolussi et al., 1987; Tan and Berridge, 2000). In a qualitative study, it is also reported that oyster hemocytes are relatively poor generators of reactive oxygen, as the 5 mU/ml treatment, all of these differences are statistically significant from the untreated control ($p < 0.005$). Published reports of the susceptibility of *Vibrio* spp. to X/XO or superoxide are scarce; however, stationary phase cultures of the gram negative pathogen *Listeria monocytogenes* are reported to be susceptible to as little as 64 mU/ml XO, representing 20 nM/min superoxide, while log phase cells are resistant to this level (Bortolussi et al., 1987). *V. splendidus* used in our study was grown overnight on LB agar, and likely consisted primarily of stationary phase cells.

3.2. Effects of H_2O_2 on viability of *P. marinus* and *V. splendidus*

We exposed *P. marinus* trophozoites to various levels of H_2O_2 for 1 h and assessed viability after a 1 h resting period. As shown in Fig. 2A, *P. marinus* mM, although the slight reduction of viability at 4 and 8 mM was statistically significant ($p < 0.005$). Only when the H_2O_2 concentration was raised above 100 mM did *P. marinus* viability decrease by more than 90%. Tolerance of *P. marinus* to H_2O_2 exposure appears high in comparison to the protistan parasites *Toxoplasma gondii* and *Entamoeba histolytica*, which are reported to be susceptible to exposure to 1 and 0.8 mM H_2O_2, respectively (Hughes et al., 1989; Murray and Cohn, 1979). Notable in the *E. histolytica* study was the observation that a virulent strain was slightly less susceptible to H_2O_2 than was an avirulent

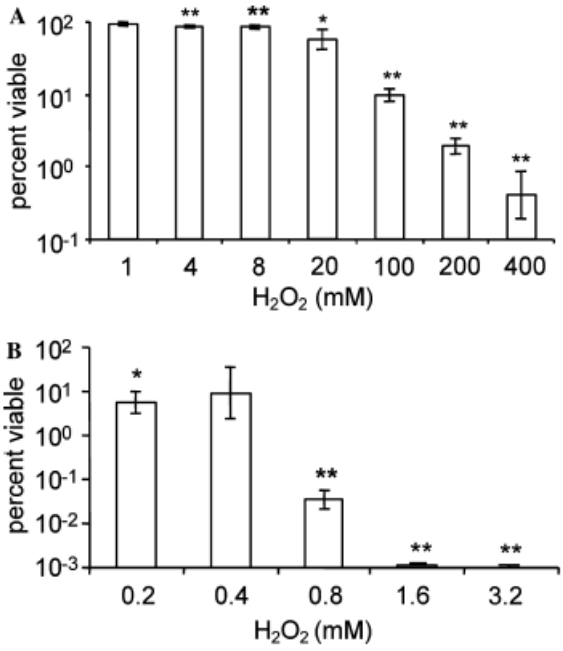

Figure 2. Percent viability after exposure to H_2O_2. (A) Viability of *Perkinsus marinus* trophozoites after a 1 h exposure to increasing amounts of H_2O_2. (B) Viability of *Vibrio splendidus* after a 1 h exposure to increasing amounts of H_2O_2. Viability measurements as in Section 2; error bars are SD. **p < 0:005, *p < 0:05 versus untreated controls.

strain (Ghadirian et al., 1986). It is interesting to note that *T. gondii* has catalase (CAT) activity, while *E. histolytica* lacks this activity, as do a number of other H_2O_2-sensitive parasites (Mehlotra, 1996; Weinbach and Diamond, 1974). There is no evidence of CAT activity in *P. marinus*, despite efforts by us and others to detect it (Pecher and Vasta, unpublished;Chu et al., 1998).

In contrast to the H_2O_2-tolerance of *P. marinus*, as little as 0.2 mM H_2O_2 decreased *V. splendidus* CFU by over 90%, and above 1 mM, CFU were lowered by over 99.99% (p < 0:005). Two other marine *Vibrio* species, *V. harveyi* and *V. angustum* (pathogenic and free living, respectively) are reported to be highly resistant to transient exposure to 2–100 mM H_2O_2, especially in stationary cultures (Ostrowski et al., 2001; Vattanaviboon and Mongkolsuk, 2001). The

dramatic difference between the H_2O_2 susceptibility of *V. splendidus* and these other *Vibrio* spp. may be explained by the fact that the *V. harveyi* and *V. angustum* studies were conducted with cell densities at least 10-fold higher than those used in this study. At high cell densities, bacterial CAT or other antioxidant enzymes have the potential to rapidly destroy H_2O_2. In separate studies on *V. cholera*, exposure of 10^6 cells/ml to 1 mM H_2O_2 resulted in greater than 99% reduction in CFU, whereas at a 10-fold higher cell density *V. cholera* removed the H_2O_2 from the assay medium and suffered less than 10% reduction in CFU (Schott et al., unpublished). Similar ‘‘threshold cell density’’ effects have been reported by others (Alcorn et al., 1994). In this study, *P. marinus* was tested at 2.5 x 10^6 cells/ml. Selected experiments conducted at twofold higher and twofold lower cell densities showed no discernable difference in susceptibility (data not shown).

3.3. Effects of hypochlorite on viability of *P. marinus* and *V. splendidus*

In phagocytic cells, the enzyme myeloperoxidase MPO produces HOCl using H_2O_2 and chloride. *In vitro*, *P. marinus* trophozoites were markedly more susceptible to hypochlorite than to H_2O_2 or the X/XO system (Fig. 3A). Exposure to only 25 μM HOCl reduced trophozoite viability by 42%, and exposure to 50, 100, and 200 μM reduced viability by 97, 98, and 99%, respectively. In this experiment, we also observed that inclusion of the radical scavenger arginine (10 mM) in the 50 lM HOCl treatment restored viability from 2 to 92% ($p < 0.005$). The susceptibility of *V. splendidus* to HOCl was comparable to that of *P. marinus*: although exposure to 25 μM HOCl resulted in no significant decrease in CFU,

exposure to 50, 100, and 200 µM treatments lowered CFUs by 63, 99.9, and 99.99%, respectively (Fig. 3B). Many bacterial and protistan species also show such differential sensitivity to HOCl over X/XO or H_2O_2 (Jepras and Fitzgeorge, 1986; Murray and Cohn, 1979), and are highly susceptible to killing by macrophage or neutrophils that produce HOCl (Ferrante et al., 1987; Vincendeau et al., 1989). The metazoan parasite *Schistosoma mansoni* is more susceptible to H_2O_2 than HOCl produced by hemocytes of the snail *Biomphalaria glabrata* (Hahn et al., 2001). There are reports of MPO-like activity in *C. virginica* hemocytes, though there are questions as to whether it is efficiently activated by exposure to live *P. marinus* (Anderson et al., 1997; Bramble and Anderson,

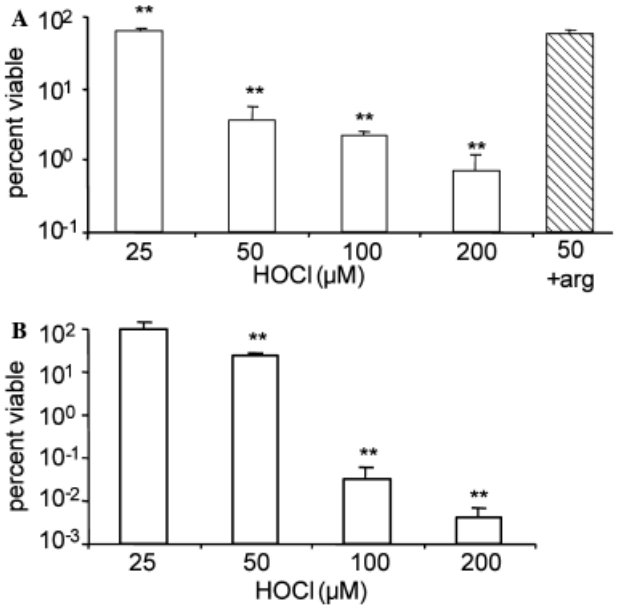

Figure 3. Percent viability after exposure to HOCl. (A) Viability of *Perkinsus marinus* trophozoites after 1 h exposure to increasing amounts of HOCl. Shaded column represents a parallel treatment with both 50 IM HOCl and 10mM arginine. (B) Viability of *Vibrio splendidus* after 1 h exposure to increasing amounts of HOCl as in Section 2. Viability measurements as in Section 2; error bars are SD. **p < 0:005 versus untreated controls.

1998). Our *in vitro* data show that the product of the MPO system, HOCl, is capable of killing *P. marinus*.

3.4. Ability of live *P. marinus* to degrade hydrogen peroxide

The low susceptibility of *P. marinus* to X/XO and H_2O_2 raised the question of how efficient the organism is at removing these ROS from solution. *P. marinus* is known to possess abundant SOD activity (Ahmed et al., 2003; Schott and Vasta, 2003; Wright et al., 2002), which likely converts superoxide into H_2O_2, but no mechanism for removing H_2O_2 has yet been described. In most organisms, catalase degrades H_2O_2 to water and molecular oxygen. Many organisms also possess glutathione peroxidase, which reduces H_2O_2 to water at the expense of reduced glutathione. In plants, ascorbate-dependent peroxidase (APX) is known to contribute to H_2O_2 removal (Amako et al., 1994). APX has also been identified in at least one protist (Boveris et al., 1980; Wilkinson et al., 2002).

We investigated the ability of suspensions of live *P. marinus* and *V. splendidus* to degrade H_2O_2 over a 1 h period, taking measurements at three intervals, and calculating the rate of H_2O_2 consumption per 10^8 cells for each interval. *P. marinus* removed 19.9, 21.9, and 21.3 nmol of H_2O_2 per 10^8 cells/h at incubation times of 25, 40, and 60 min (Fig. 4). In comparison, *V. splendidus* removed 35, 30, and 24 nmol per 108 cells/h at these three timepoints. We note that the rate of H_2O_2 removal by *P. marinus* remained constant over the 1 h assay period, while the H_2O_2 removal rate by *V. splendidus* diminished by more than 30%.

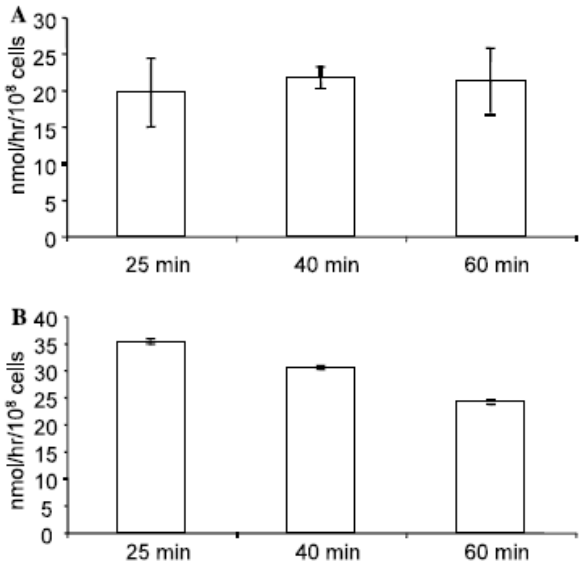

Figure 4. Hydrogen peroxide removal by live cells. The amount of H_2O_2 removed by live cells was measured at 25, 40, and 60 min, and the rate of H_2O_2 removal (nmol/h/108 cells) then calculated for each timepoint. Each timepoint is an average and SD of triplicate samples. (A) *Perkinsus marinus*; (B) *Vibrio splendidus*.

The relative abilities of *P. marinus* and *V. splendidus* to remove H_2O_2 appear incongruent with their susceptibilities to it. Indeed, during the assays to measure H_2O_2 removal, conducted at 75 µM H_2O_2, the viability of *V. splendidus* was decreased by 99%, while that of *P. marinus* dropped only 4%. Multiple factors will contribute to the ability of cells to degrade H_2O_2, including cell surface to volume ratio (s/v), cell wall/membrane permeability to H_2O_2, and the compartmentalization of catalytic or peroxidatic activities. Based on published data (Gauthier and Vasta, 1993; Jiang and Chai, 1996), we estimate that cultured *P. marinus* trophozoites are approximately 65 μm^3 spheres, with a s/v ratio of 1.2, while *Vibrio* spp. are approximately 1.0 μm^3 rods with a s/v ratio of 6.3. Thus, the modestly higher rate of H_2O_2 removal per cell by *V. splendidus* would be a relatively much higher rate if expressed in terms of cell volume. On the

other hand, slower diffusion of H_2O_2 into the much larger *P. marinus* cells could result in an underestimate of their internal peroxidatic potential. Although both organisms are surrounded by substantial cell walls in addition to cell membranes (Montes et al., 2002; Perkins and Menzel, 1967), the relative permeability of *P. marinus* to H_2O_2, as compared to *V. splendidus*, is not known.

In *Vibrio* spp., catalase is located in the cytosol, and may also be present in the periplasm (Yumoto et al., 2000). Niether the identity, nor the location, of potential H_2O_2-degrading activities of *P. marinus* is known. The two *P. marinus* SODs, however, are located in different compartments, consistent with their N-terminal leader sequences: PmSOD1 in the mitochondrion, and PmSOD2 possibly in unidentified structures/vesicles near the cell periphery (Schott and Vasta, 2003). Bacteria also have SODs targeted to both cytosol and periplasm (Korshunov and Imlay, 2002). We confirmed that like other *Vibrio* spp., crude extracts of *V. splendidus* possesses abundant catalase activity, using standard methods (Aebi, 1984; Ostrowski et al., 2001; Vattanaviboon and Mongkolsuk, 2001). In contrast, we have been unable to detect CAT activity or identify CAT gene sequences in *P. marinus*, despite extensive biochemical detection efforts using *P. marinus* extracts and extensive gene detection efforts using degenerate oligonucleotide-based amplifications from genomic *P. marinus* DNA (Pecher and Vasta, unpublished).

3.5. Peroxidase activity of *P. marinus* and *V. splendidus* extracts

The inability to detect catalase activity in *P. marinus* extracts motivated a search for alternative peroxidatic activities. Preliminary efforts to detect

glutathione peroxidase were unsuccessful; however, an uncommon activity, reported in another catalase-negative protistan parasite, *Trypanosoma cruzi*, is ascorbate-dependent peroxidase (APX) (Boveris et al., 1980; Wilkinson et al., 2002). APX activity may be easily overlooked, because the preservation of APX activity in cell extracts requires the constant presence of ascorbate. We dialyzed soluble cell extracts (1 mg/ml) of *P. marinus* and *V. splendidus* in buffer containing 1 mM ascorbate, and assessed their ability to remove H_2O_2 from solution with the FOX assay. As before, the rate of H_2O_2 removal was measured at 20, 40, and 60 min. The *P. marinus* extract removed 3.0, 2.9, and 3.5 nmol of H_2O_2/h/mg protein, while *V. splendidus* extract removed 16.4, 12.1, and 8.3 nmol/h/mg protein (Figs. 5A and B). It should be considered that as with live

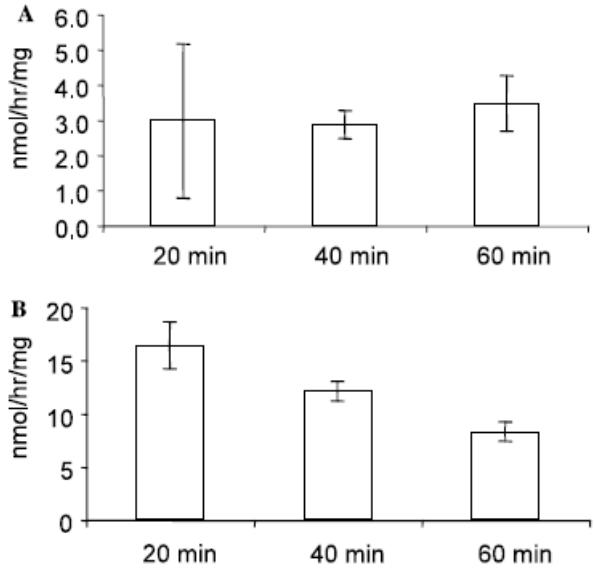

Figure 5. Hydrogen peroxide removal by dialyzed extracts. The amount of H_2O_2 removed by dialyzed cell extracts was measured at 25, 40, and 60 min, and the rate of H_2O_2 removal (nmol/h/mg protein) then calculated for each timepoint. Each timepoint represents the average and SD of triplicate samples. (A) *Perkinsus marinus*; (B) *Vibrio splendidus*.

cells, the dialyzed extract of *P. marinus* displays a constant level of peroxidatic activity, while the activity in the *V. splendidus* extract decreased by nearly half over the course of 1 h.

It is notable that the H_2O_2 degrading activity of *V. splendidus* decreased over time. There are reports that some CAT activities are susceptible to inactivation by high concentrations of H_2O_2 (e.g.,DeLuca et al., 1995), but whether this phenomenon is relevant to our observations has not been confirmed.

3.6. Evidence of ascorbate peroxidase in *P. marinus* extracts

If the removal of H_2O_2 from solution is partly or wholly due to the action of APX, then it should be directly reflected by an H_2O_2-dependent oxidization of ascorbate. To investigate this, we conducted standard APX assays using a spectrophotometric method that follows the oxidation of ascorbate (Abs at 290 nm). In a representative experiment (Table 1), there was significant APX activity in *P. marinus* extracts (ΔA290 of -0.062 min^{-1}), but not in *V. splendidus* extracts (ΔA290 of +0.001 min^{-1}). When expressed in terms of ascorbate oxidation per mg protein, the rate observed in *P. marinus* extracts is 2430 nmol/h/mg protein. This is nearly three orders of magnitude greater than the measured rate of H_2O_2 destruction in the assays depicted in Fig. 4, which were conducted using the same extracts as used for APX assays. There may be several factors contributing to this discrepancy. First, the APX assays were conducted for only 2 min, following a 30 s delay period for mixing reagents. When APX assays were extended for 20 min, the rate of ascorbate oxidation diminished by as much as

50% (not shown), suggesting that the contribution of APX to long-term H_2O_2 removal may be transient. Second, because it is typical for ascorbate to oxidize over time as a result of dissolved oxygen in the buffer and exposure to light, the ascorbate present in dialyzed extracts is partially oxidized, and oxidized ascorbate cannot serve as an electron donor for reduction of H_2O_2. It is possible that in addition to APX activity, *P. marinus* possesses another, less transient, activity that removes H_2O_2 more slowly.

In conclusion, the results presented here support the hypothesis that, like a number of other pathogens that are resistant to intra-phagocytic killing, *P. marinus* is relatively tolerant to selected ROS (Ghadirian et al., 1986). In spite of lacking CAT activity, *P. marinus* is over 100 times more tolerant than *V. splendidus* to the superoxide generating system of X/XO, and over 100 times more tolerant to H_2O_2. In contrast, the susceptibility of *P. marinus* to HOCl is equivalent to the sensitivity displayed by *V. splendidus*. To our knowledge, there are no reported enzymes that detoxify HOCl in any system, although there are other biologically relevant mechanisms for alleviating the toxic effects of HOCl, such as the radical scavengers arginine and ascorbate (Ferrante et al., 1987; Hu et al., 1993). Preliminary measurements show that *P. marinus* extracts contain at least 2 mM ascorbate (data not shown;Schaus et al., 1986). It remains an open question as to whether ascorbate serves as a HOCl scavenger as well as a cofactor for the H_2O_2-destroying enzyme, APX. In a broader context, the findings of this study do not exclude the possibility that other factors secreted by *P.*

marinus may contribute to directly suppress the generation of ROS by oyster hemocytes, as suggested by other authors (Anderson, 1999).

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REFERENCES

Aebi, H. 1984. Catalase *in vitro*. Methods Enzymol. 105, 121-126.

Ahmed, H., Schott, E. J., Gauthier, J. D. and Vasta, G. R. 2003. Superoxide dismutases from the oyster parasite *Perkinsus marinus:* purification, biochemical characterization, and development of a plate microassay for activity. Anal. Biochem. 318, 132-141.

Alcorn, T. M., Zheng, H. Y., Gunther, M. R., Hassett, D. J. and Cohen, M. S. 1994. Variation in hydrogen peroxide sensitivity between different strains of *Neisseria gonorrhoeae* is dependent on factors in addition to catalase activity. Infect. Immun. 62, 2136 - 2140.

Amako, K., Chen, G.-X. and Asada, K. 1994. Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant Cell Physiol. 35, 497-504.

Anderson, R. S. 1999. *Perkinsus marinus* secretory products modulate superoxide anion production by oyster (*Crassostrea virginica*) haemocytes. Fish Shellfish Immunol. 9, 51-60.

Anderson, R. S., Paynter, K. T. and Burreson, E. B. 1992. Increased reactive oxygen intermediate production by hemocytes withdrawn from *Crassostrea virginica* infected with *Perkinsus marinus*. Biol. Bull. 183, 476-481.

Anderson, R. S., Brubacher, L. L., Calvo, L. M., Burreson, E. M. and Unger, M. A. 1997. Effect of *in vitro* exposure to tributyltin on generation of oxygen metabolites by oyster hemocytes. Environ. Res. 74, 84-90.

Azevedo, C. 1989. Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsea) parasite of the clam *Ruditapes decussatus* from Portugal. J. Parasitol. 75, 627-635.

Birnboim, H. C. and Sandhu, J. 1997. Levels of DNA strand breaks and superoxide in phorbol ester-treated human granulocytes. J. Cell. Biochem. 66, 219-228.

Bortolussi, R., Vandenbroucke-Grauls, C. M., van Asbeck, B. S. and Verhoef, J. 1987. Relationship of bacterial growth phase to killing of *Listeria monocytogenes* by oxidative agents generated by neutrophils and enzyme systems. Infect. Immun. 55, 3197-3203.

Boveris, A., Sies, H., Martino, E. E., Docampo, R., Turrens, J. F. and Stoppani, A. O. 1980. Deficient metabolic utilization of hydrogen peroxide in *Trypanosoma cruzi*. Biochem. J. 188, 643-648.

Bramble, L. H. and Anderson, R. S. 1998. A comparison of the chemiluminescent responses of *Crassostrea virginica* and *Morone saxatilis* phagocytes to zymosan and viable *Listonella anguillarum*. Dev. Comp. Immunol. 22, 55-61.

Brawn, K. and Fridovich, I. 1981. DNA strand scission by enzymically generated oxygen radicals. Arch. Biochem. Biophys. 206, 414 - 419.

Brugerolle, G. 2002. *Cryptophagus subtilis*: a new parasite of cryptophytes affiliated with the Perkinsozoa lineage. Eur. J. Protistol. 37, 379-390.

Chu, F. L., Volety, A. K. and Armknecht, S. 1998. Antioxidant enzymes, potential virulent factors, in different strains of the oyster protozoan parasite, *Perkinsus marinus*. J. Shellfish Res. 17.

DeLuca, D. C., Dennis, R. and Smith, W. G. 1995. Inactivation of an animal and a fungal catalase by hydrogen peroxide. Arch. Biochem. Biophys. 320, 129-134.

Dukan, S., Belkin, S. and Touati, D. 1999. Reactive oxygen species are partially involved in the bacteriocidal action of hypochlorous acid. Arch. Biochem. Biophys. 367, 311-316.

Ferrante, A., Hill, N. L., Abell, T. J. and Pruul, H. 1987. Role of myeloperoxidase in the killing of *Naegleria fowleri* by lymphokine-altered human neutrophils. Infect. Immun. 55, 1047-1050.

Gardner, P. R. and Fridovich, I. 1991. Superoxide sensitivity of the *Escherichia coli* aconitase. J. Biol. Chem. 266, 19328-19333.

Gauthier, J. D. and Vasta, G. R. 1993. Continuous *in vitro* culture of the oyster parasite *Perkinsus marinus*. J. Invertebr. Pathol. 62, 321-323.

Ghadirian, E., Somerfield, S. D. and Kongshavn, P. A. 1986. Susceptibility of *Entamoeba histolytica* to oxidants. Infect. Immun. 51, 263-267.

Goggin, C. L. and Barker, S. C. 1993. Phylogenetic position of the genus *Perkinsus* (Protista, Apicomplexa) based on small subunit ribosomal RNA. Mol. Biochem. Parasitol. 60, 65-70.

Greger, E. A., Drum, A. S. and Elston, R. A. 1995. Measurement of oxidative activity in hemocytes of the pacific razor clam, *Siliqua patula*, and the oyster, *Crassostrea gigas*, using lucigenin- and luminol-dependent chemiluminescence. J. Invertebr. Pathol. 65, 48-60.

Hahn, U. K., Bender, R. C. and Bayne, C. J. 2001. Killing of *Schistosoma mansoni* sporocysts by hemocytes from resistant *Biomphalaria glabrata*: role of reactive oxygen species. J. Parasitol. 87, 292-299.

Hu, M. L., Louie, S., Cross, C. E., Motchnik, P. and Halliwell, B. 1993. Antioxidant protection against hypochlorous acid in human plasma. J. Lab. Clin. Med. 121, 257 -262.

Hughes, H. P., Boik, R. J., Gerhardt, S. A. and Speer, C. A. 1989. Susceptibility of *Eimeria bovis* and *Toxoplasma gondii* to oxygen intermediates and a new mathematical model for parasite killing. J. Parasitol. 75, 489-497.

Jepras, R. I. and Fitzgeorge, R. B. 1986. The effect of oxygen-dependent antimicrobial systems on strains of *Legionella pneumophila* of different virulence. J. Hyg. (Lond.) 97, 61-69.

Jiang, X. and Chai, T. J. 1996. Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable, nonculturable cells. Appl. Environ. Microbiol. 62, 1300-1305.

Korshunov, S. S. and Imlay, J. A. 2002. A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of Gram-negative bacteria. Mol. Microbiol. 43, 95-106.

La Peyre, J. F., Chu, F. L. and Vogelbein, W. K. 1995. *In vitro* interaction of *Perkinsus marinus* merozoites with eastern and Pacific oyster hemocytes. Dev. Comp. Immunol. 19, 291-304.

Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gelebart, F., Cordevant, C., Lange, M. and Poulet, S. A. 2001. A *Vibrio splendidus* strain is associated

with summer mortality of juvenile oysters *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). Dis. Aquat. Org. 46, 139-145.

Levine, N. D. 1978. *Perkinsus* gen. n. and other new taxa in the protozoan phylum *Apicomplexa*. J. Parasitol. 64, 549.

Mehlotra, R. K. 1996. Antioxidant defense mechanisms in parasitic protozoa. Crit. Rev. Microbiol. 22, 295-314.

Meyers, J. A., Burreson, E. M., Barber, B. J. and Mann, R. 1991. Susceptibility of diploid and triploid pacific oysters *Crassostrea gigas* (Thunberg, 1793) and eastern oysters, *Crassostrea virginica* (Gmelin, 1791), to *Perkinsus marinus*. J. Shellfish Res. 10, 433-437.

Montes, J. F., Durfort, M., Lladó, A. and García Valero, J. 2002. Characterization and immunolocalization of a main proteinaceous component of the cell wall of the protozoan parasite *Perkinsus atlanticus*. Parasitology 124, 477-484.

Murray, H. and Cohn, Z. 1979. Macrophage oxygen-dependent antimicrobial activity. I. Susceptibility of *Toxoplasma gondii* to oxygen intermediates. J. Exp. Med. 150, 938-949.

Nakamura, M., Mori, K., Inooka, S. and Nomura, T. 1985). In vitro production of hydrogen peroxide by the amoebocytes of the scallop, *Patinopecten yessoensis* (Jay). Dev. Comp. Immunol. 9, 407-417.

Norén, F., Moestrup, Ø. and Rehnstam-Holm, A.-S. 1999. *Parvilucifera infectans* Norén et Moestrup gen. et sp. nov. (Perkinsozoa phylum nov.): a parasitic flagellate capable of killing toxic microalgae. Eur. J. Protistol. 35, 233-254.

Ohno, Y. G. and Gallin, J. I. 1985. Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils. Studies utilizing the inactivation of myeloperoxidase by hydrogen peroxide and azide. J. Biol. Chem. 260.

Ostrowski, M., Cavicchioli, R., Blaauw, M. and Gottschal, J. C. 2001. Specific growth rate plays a critical role in hydrogen peroxide resistance of the marine oligotrophic ultramicrobacterium *Sphingomonas alaskensis* strain RB2256. Appl. Environ. Microbiol. 67, 1292-1299.

Perkins, F. O. 1996. The structure of *Perkinsus marinus* (Mackin, Owen, and Collier, 1950) Levine, 1978 with comments on taxonomy and phylogeny of *Perkinsus* spp. J. Shellfish Res. 15, 67-87.

Perkins, F. O. and Menzel, R. W. 1967. Ultrastructure of sporulation in the oyster pathogen *Dermocystidium marinum*. J. Invertebr. Pathol. 9, 205-229.

Rotman, B. and Papermaster, B. W. 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc. Natl. Acad. Sci. USA 55, 134 -141.

Saldarriaga, J. F., McEwan, M. L., Fast, N. M., Taylor, F. J. R. and Keeling, P. J. 2003. Multiple protein phylogenies show that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellate lineage. Int. J. Syst. Evol. Microbiol. 53, 355-365.

Schaus, E. E., Kutnink, M. A., O'Connor, D. K. and Omaye, S. 1986. A comparison of leukocyte ascorbate levels measured by the 2,4dinitrophenylhydrazine ethod with high-performance liquid chromatography. Biochem. Med. Metab. Biol. 36, 369-376.

Schott, E. J. and Vasta, G. R. 2003. The PmSOD1 gene of the protistan parasite *Perkinsus marinus* complements the sod2Δ mutant of *Saccharomyces cerevisiae*, and directs an iron superoxide dismutase to mitochondria. Mol. Biochem. Parasitol. 126, 81-92.

Siddall, M. E., Reece, K. S., Graves, J. E. and Burreson, E. M. 1997. 'Total evidence' refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa. Parasitology 115, 165-176.

Takahashi, K. and Mori, K. 2000. NADPH oxidase-like activity in hemocytes of the Pacific oyster *Crassostrea gigas*. Fish Pathol. 35, 15-19.

Tan, A. S. and Berridge, M. V. 2000. Superoxide produced by activated neutrophils efficiently reduces the tetrazolium salt, WST-1 to produce a soluble formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening antiinflammatory agents. J. Immunol. Methods 238, 59-68.

Vattanaviboon, P. and Mongkolsuk, S. 2001. Unusual adaptive, cross protection responses and growth phase resistance against peroxide killing in a bacterial shrimp pathogen, *Vibrio harveyi*. FEMS Microbiol. Lett. 200, 111-116.

Vincendeau, P., Daulouede, S. and Veyret, B. 1989. Role of hypochlorous acid in *Trypanosoma musculi* killing by phagocytes. Parasitology 98, 253 -257.

Vissers, M. C., Stern, A., Kuypers, F., van den Berg, J. and Winterbourn, C. C. 1994. Membrane changes associated with lysis of red blood cells by hypochlorous acid. Free Radic. Biol. Med. 16, 703-712.

Volety, A. K. and Chu, F. L. 1995. Suppression of chemiluminescence of eastern oyster (*Crassostrea virginica*) hemocytes by the protozoan parasite *Perkinsus marinus*. Dev. Comp. Immunol. 19, 135-142.

Weinbach, E. C. and Diamond, L. S. 1974. *Entamoeba histolytica*. I. Aerobic metabolism. Exp. Parasitol. 35, 232-243.

Wilkinson, S. R., Obado, S. O., Mauricio, I. L. and Kelly, J. M. 2002. *Trypanosoma cruzi* expresses a plant-like ascorbate-dependent hemoperoxidase localized to the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA 99, 13453-13458.

Wright, A. C., Ahmed, H., Gauthier, J. D., Silva, A. M. and Vasta, G. R. 2002. cDNA cloning and characterization of two iron superoxide dismutases from the oyster parasite *Perkinsus marinus*. Mol. Biochem. Parasitol. 123, 73-77.

Yumoto, I., Ichihashi, D., Iwata, H., Istokovics, A., Ichise, N., Matsuyama, H., Okuyama, H. and Kawasaki, K. 2000. Purification and characterization of a catalase from the facultatively psychrophilic bacterium *Vibrio rumoiensis* S-1(T) exhibiting high catalase activity. J. Bacteriol. 182, 1903-1909.

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IDENTIFICATION OF A SECOND RRNA GENE UNIT IN THE *PERKINSUS ANDREWSI* GENOME [6]

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ABSTRACT

Perkinsus species are parasitic protozoa of mollusks, currently classified within the Perkinsozoa, a recently established phylum that is basal to the Apicomplexa and Dinozoa. Ribosomal RNA (rRNA) genes and their intergenic spacers have been used to support the taxonomy of *Perkinsus* species, the description of new species, and to develop molecular probes for their detection and identification. We previously described ultrastructure, behavior in culture, and partial sequence of the rRNA locus of a *Perkinsus* species isolated from the baltic clam *Macoma balthica*. The rRNA genes and intergenic spacers of this *Perkinsus* isolate differed from those described in the currently accepted species to a degree that led to its designation as a new species, *Perkinsus andrewsi*. In this study, we identify an additional rRNA gene unit (rRNA-B) in the *P. andrewsi* holotype, and report the complete sequences of both rRNA gene units. Except for the 5.8S, all regions of the rRNA-B gene unit exhibited sequence differences from that initially described (rRNA-A). Each rRNA gene unit is arranged in a "head to tail" tandem repeat. This is the first report demonstrating two distinct rRNA units in a *Perkinsus* species.

Key Words. ICS, IGS, ITS, LSU, *Macoma balthica*, SSU.

INTRODUCTION

Perkinsus species (Perkinsozoa, Perkinsea) (Norén, Moestrup, and Rehnstam-Holm 1999) are protistan parasites of mollusks, often associated with disease. *Perkinsus marinus* was initially classified as a fungus within the genus *Dermocystidium* (*D. marinum*; Mackin, Owen, and Collier 1950). Ultrastructural studies on its zoospore stage, however, revealed morphological characteristics that resemble those of apicomplexan parasites, leading to the establishment of a new genus *Perkinsus* within the phylum Apicomplexa and renaming *D. marinum* as *P. marinus* (Levine 1978). Taxonomic affinities of *P. marinus* with *Parvilucifera infectans*, a protistan parasite of dinoflagellates, led Norén, Moestrup, and Rehnstam-Holm (1999) to establish the new phylum Perkinsozoa that now includes the genera *Perkinsus*, *Parvilucifera*, and *Cryptophagus* (Brugerolle 2002; Norén, Moestrup, and Rehnstam-Holm 1999). Based on sequence comparisons of actin and small subunit (SSU) ribosomal RNA (rRNA) genes, Siddal et al. (1997) suggested that *Perkinsus* species were closely related to the Dinozoa, and should be excluded from the Apicomplexa. Recent phylogenetic studies that included protein (actin and tubulins) and SSU rRNA gene phylogenies supported this view, and it has been proposed that *Perkinsus* is the earliest divergent group from the Dinozoa (Kuvardina et al. 2002; Saldarriaga et al. 2003).

Sequences of rRNA genes and intergenic regions have significantly contributed to the taxonomic characterization of *Perkinsus* species (Fong et al. 1993; Goggin 1994; Goggin and Baker 1993), to support new species

designations (Coss et al. 2001; Kotob et al. 1999), and to develop molecular probes for their detection and identification (Coss et al. 2001; de la Herrán et al. 2000; Marsh, Gauthier, and Vasta 1995; Penna, Khan, and French 2001; Robledo, Coss, and Vasta 2000; Robledo et al. 1998; 1999; 2002; Yarnall et al. 2000).

The organization of the rRNA gene cluster of *Perkinsus* species differs from that of most eukaryotes described so far. Unlike the typical eukaryotic rRNA gene cluster, in *Perkinsus* species the 5S gene is linked to the SSU rRNA gene (Coss et al. 2001; de la Herrán et al. 2000; Marsh, Gauthier, and Vasta 1995). This was corroborated by the characterization of the complete rRNA gene units of *P. atlanticus* and *P. marinus* (Robledo et al. 2002; JAFR and GRV, unpublished). In these species, the rRNA gene unit consists of the following regions: 5S, intergenic spacer (IGS), SSU, internal transcribed spacer (ITS) 1, 5.8S, ITS2, and large subunit (LSU). Within a rRNA gene cluster, the rRNA gene units are arranged as “head to tail” tandem repeats that are separated by intercluster spacers (ICS) (de la Herrán et al. 2000; Robledo et al. 2002). A similar rRNA gene organization was described for some fungi and algae (reviewed by Kawai et al. 1995), and the apicomplexan parasite *Toxoplasma* (Guay et al. 1992). However, in other Apicomplexa, such as *Plasmodium*, *Babesia*, *Theileria*, and *Cryptosporidium*, instead of the typical tandem organization of multiple rRNA gene units, these (from one to ten copies) are unlinked, and further, the 5S is unlinked to the other rRNA genes of each rRNA unit. In some species, a higher level of complexity of the rRNA genes has been

identified: the multiple rRNA gene units may not be identical, and up to seven distinct rRNA units have been described (Dame and McCutchan 1983; Gardner et al. 2002; Le Blancq et al. 1997; Reddy et al. 1991). In *P. falciparum* four rRNA gene units (A, B, C, and D) have been reported, which transcribe at least two life stage-specifically expressed rRNA types (A and S) (Gunderson et al. 1987; McCutchan et al. 1988). Recently, a fifth rRNA gene unit has been identified that is almost identical to the S-type rRNAs in the SSU and 5.8S gene sequences, but differs significantly in the LSU gene sequence from the LSU sequences of the A- and S-type rRNAs (Gardner et al. 2002). The presence of two distinct SSU and LSU gene sequences was also reported in dinoflagellate species within the genus *Alexandrium* (Scholin, Anderson, and Sogin 1993; Scholin et al. 1994; Yeung et al. 1996).

We previously described ultrastructure, behavior in culture, and partial sequence of the rRNA locus of a *Perkinsus* species isolated from the baltic clam *Macoma balthica* (Coss, Robledo, and Vasta 2001; Coss et al. 2001). The degree of difference of all rRNA genes and intergenic regions examined was comparable to or greater than differences between the currently accepted *Perkinsus* species, which led to the designation of the *M. balthica* isolate as *P. andrewsi* (Coss et al. 2001). Here, we describe an additional *P. andrewsi* rRNA gene unit (rRNA-B), distinct from the one initially identified (referred to as rRNA-A herein), and we report the complete characterization of the two rRNA gene units (rRNA-A and rRNA-B). This is the first report for the presence of two distinct rRNA gene units in a *Perkinsus* species.

MATERIALS AND METHODS

P. andrewsi cell culture

The *P. andrewsi* monoclonal holotype culture (PAND-A8-4a, ATCC 50807; Coss et al. 2001) was maintained in DMEM:HAM's (1:2) medium supplemented with 5% (v/v) fetal bovine serum (Gauthier and Vasta 1995). It was subjected to five rounds of subcloning by limiting dilution as described elsewhere (Gauthier and Vasta 1995; Coss, Robledo, and Vasta 2001). Briefly, for each round of subcloning a cell suspension at a density of 3 cells/ml was plated into 96-well plates with 100 µl/well, 50 µl of 0.2-µm filter-sterilized culture medium added, and incubated at 28 °C for 24 h. Wells containing single cells were identified using an inverted microscope. After an additional incubation of 5 d, the entire content of each selected well was transferred to single wells in a 24-well plate. Between each round of subcloning the cultures were incubated for 3-4 d.

PCR amplification, cloning, and sequencing

DNA from *in vitro* propagated *P. andrewsi* (PAND-A8-4a) was extracted using the DNeasy Tissue kit (QIAGEN, Valencia, CA). The IGS was PCR-amplified with the primers NTS1 and NTS2 under PCR conditions that have been optimized for the amplification of the IGS of *P. marinus* (Robledo et al. 1999). To obtain complete rRNA gene unit sequences, three different amplicons containing rRNA genes and intergenic regions were generated by PCR. Primers used are listed in Table 1, and their relative positions within the rRNA gene unit are shown in Fig. 1. Partial IGS and the complete SSU were amplified using the primer M8

Table 1. PCR primers for the amplification of rRNA genes and intergenic regions of *Perkinsus andrewsi* and probe design for Southern analysis.

Primer	Sequence (5 '- 3')	Specificity	Reference
M5	AAC CAT CCC GAC TAC CAT CTG G	IGS-B	Thispresent study
M6	TTC ATC ACT TGA GTT GCG	IGS-B	Thispresent study
M8	GCG AAA ATT GAC TTT CAG GTC G	IGS-B	Thispresent study
NTS1	AAG TCC TTA GGG TGC TGC	SSU	Robledo et al. 1999
NTS2	ATG AGC CAT TCG CAG TTT CGC C	SSU	Robledo et al. 1999
NTS6	ATT GTG TAA CCA CCC CAG GC	IGS-A	Coss et al. 2001
NTS7	AAG TCG AAT TGG AGG CGT GGT GAC	IGS-A	Coss et al. 2001
PER1	TAG TAC CCG CTC ATY GTG G	IGS	Robledo et al. 2002
PER2	TGC AAT GCT TGC GAG CT	IGS	Robledo et al. 2002
PMITS3	GGT AAT CTC ACA CAC ATC AGG C	LSU	Thispresent study
UPRA	AAC CTG GTT GAT CCT GCC AGT	SSU	Medlin et al. 1988
UPRB	TGA TCC TTC TGC AGG TTC ACC TAC	SSU	Medlin et al. 1988

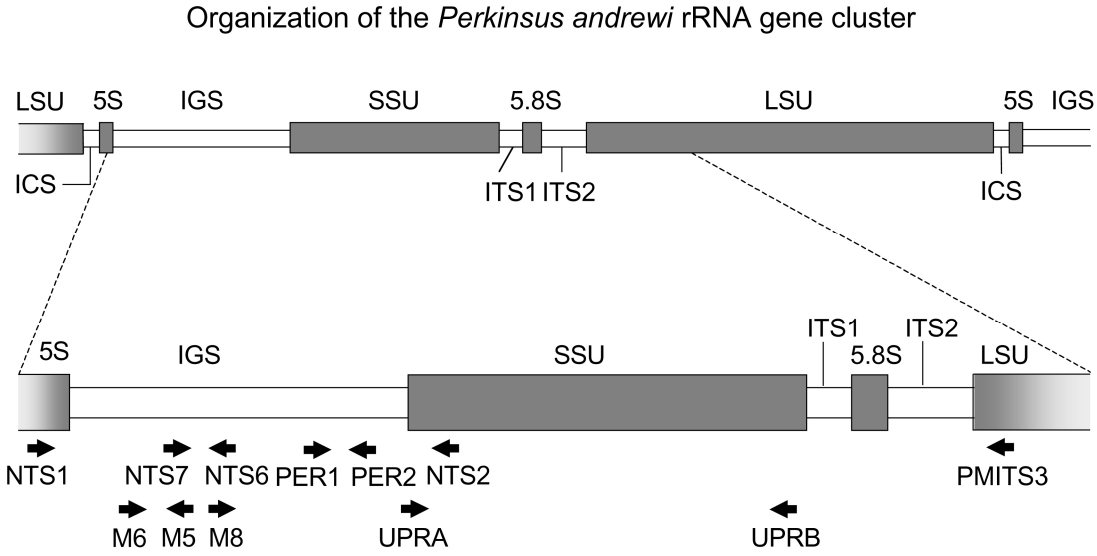

Fig. 1. Localization of primers (arrows) used for the amplification of rRNA genes and intergenic regions of *Perkinsus andrewsi.* ICS, intercluster spacer; IGS, intergenic spacer; ITS, internal transcribed spacer; LSU, large subunit; SSU, small subunit.

(this study), and UPRB from Medlin et al. (1988). Partial IGS, the complete SSU, and the complete ITS1-5.8S-ITS2 region were amplified using the IGS specific

primers NTS7 (Coss et al. 2001) and M8 (this study) and the primer PMITS3 (this study). Partial IGS, the complete SSU, the ITS1-5.8S-ITS2 region, and the LSU-ICS-5S region were amplified using the primers NTS7 and M8 (Coss et al. 2001; this study) and UPRA (Medlin et al. 1988).

PCR products were separated by electrophoresis using 1% agarose gels and visualized by staining with ethidium bromide (EtBr) (0.5 µg/ml). Amplicons were gel-purified using the QIAquick Gel Extraction kit (QIAGEN). Purified amplicons were cloned using the Promega pGEMII vector system (Promega Corporation, Madison, WI). At least three clones obtained from 10 PCR amplifications containing the IGS, eight clones obtained from at least two PCR amplifications containing partial sequence of the IGS and the complete SSU region, two clones from two PCR amplifications containing partial sequence of the IGS and the complete SSU-ITS1-5.8S-ITS2 region, and one clone from one PCR amplification containing partial sequence of the IGS and the complete SSU-ITS1-5.8S-ITS2-LSU-ICS-5S region were sequenced using additional internal primers.

To assess the potential intraclonal IGS variability, IGS amplicons were generated in five independent PCR amplifications using the primers NTS1 and NTS2 (Robledo et al. 1999) under the following optimized PCR conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 65 °C for 45 s, 72 °C for 1 min, with a final extension at 72 °C for 10 min. PCR products were size-separated, gel-purified, cloned, and sequenced as described above. For each amplicon, at least nine clones were sequenced.

rRNA sequences of *P. andrewsi* (accession number AF102171), *P. marinus* (AF497479), *P. atlanticus* (AF140295), *P. chesapeaki* (isolate G117) (AF042707 and AF091541), *Perkinsus* species isolated from *Mya arenaria* (ATCC 50864) (AF440464, AF440465, AF440466, and AF440467), and *Tagelus plebeius* (ATCC 50866, AF440468, AF440469, AF440470, and AF440471) were obtained from GenBank™. Sequences were aligned using ClustalW with default parameters (Thompson, Higgins, and Gibson 1994). Because of expected low sequence identities between intergenic regions, all IGS, ITS1, ITS2, and ICS sequences were anchored with *P. andrewsi* SSU sequence (AF102171). A similarity search for the *P. andrewsi* ICS sequences was performed using BLASTn (Altschul et al. 1990).

Assessment of IGS representation

To assess the relative representation of the two IGSs identified, serially diluted *P. andrewsi* genomic DNA was amplified with the primers NTS1 and NTS2 (Robledo et al. 1999) using the optimized PCR conditions. PCR products were separated and visualized as described above. Gel photographs (Polaroid black and white instant pack film 667, Polaroid, Waltham, MA) were scanned at high resolution (1,200 dpi). The intensity of amplicons on the digital images was analyzed using the software NIH Image 1.62 (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Because the intensity of EtBr-stained amplicons is proportional to their length (Sambrook and Russell 2001; Tanabe et al. 2002), intensity values for smaller amplicons were

normalized to the length of the largest amplicon by the ratio of the size of the largest amplicon to the size of the amplicon of interest.

Single-cell PCR.

A *P. andrewsi* cell suspension was incubated in fluid thioglycollate medium (FTM) for one week (Ray 1952). During the incubation in FTM, *Perkinsus* species trophozoites enlarge and arrest in the prezoosporagium stage. To obtain single cells, 5 µl of the FTM treated culture was diluted 10-fold in distilled water. This cell suspension was further serially diluted in 50 µl of distilled water in a 96-well plate until wells with single cells were identified under an inverted microscope. The entire content of wells containing single cells was transferred to a 0.5-ml thin walled microcentrifuge tube and frozen at -20 °C. The samples were thawed, dried in a speed vacuum concentrator, resuspended in distilled water to a final volume of 22 µl, boiled for 3 min, and the IGS amplified as described above. The PCR products were tested by PCR for the presence of *P. andrewsi* IGS sequences using primers specific for the IGS-A (NTS6 and NTS 7) (Coss et al. 2001) and IGS-B (M5; this study), and universal primers for amplification of the IGS (NTS1) (Robledo et al. 1999) (Table 1).

Southern analysis

High molecular weight *P. andrewsi* genomic DNA was extracted following Green (1997) with slight modifications. Briefly, approximately 3.0 x 10^6 cells of a log-phase culture were lysed over night at 55 °C in sucrose/proteinase K cell lysis buffer (150 mM NaCl, 15 mM Na citrate, 1 mM EDTA, 200 µg/ml proteinase

K, 1% (w/v), sodium dodecyl sulphate (SDS), 27% (w/v) sucrose). DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with isopropanol, washed with 70% ethanol and resuspended in TE buffer (10mM Tris, 1mM EDTA, pH 8.0). To remove RNA, the sample was treated with 1 mg/ml RNAse A, and the DNA precipitated with ice-cold ethanol (100%). Four µg of *P. andrewsi* DNA were digested with the restriction enzymes *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I (New England BioLabs Inc., Beverly, MA, Promega, Invitrogen Corp. Carlsbad, CA) following the manufacturers' recommendations. Controls consisted of plasmids containing the IGS-A and IGS-B (20 ng and 2 ng, respectively), and uncut *P. marinus* genomic DNA (4 µg). DNA digests and control samples were fractionated on a 0.8% agarose gel, stained with EtBr, and transferred onto a Nylon membrane (Hybond XL, Amersham Biosciences Corp., Piscataway, NJ) by capillary blotting (Sambrock and Russell, 2001). The membrane was hybridized in an aqueous prehybridization/hybridization solution (450mM NaCl, 45mM Na citrate, 2 mg/l Ficoll 400, 2 mg/l polyvinylpyrrolidone, 2 mg/l bovine serum albumin, 0.1% (w/v) SDS, 100 µg/ml salmon sperm DNA) at 68 °C overnight (Brown, 1993) with either an IGS-A specific probe (probe A, 290 bp), an IGS-B specific probe (probe B, 305 bp), or a probe specific to *Perkinus* species IGSs (probe PER, 315 bp). The membrane was stripped two to three times by boiling in 0.1% (w/v) SDS between hybridizations. Probes A and PER were generated by PCR from a plasmid containing the IGS-A using primers specific for the IGS-A (NTS6 and NTS7) and primers specific to *Perkinsus* species (PER1 and PER2, Robledo et al. 2002). Probe B was generated by

PCR from a plasmid containing the IGS-B using IGS-B specific primers (M5 and M6, this study). The probes were labeled with [α^{32}P] dCTP using *redi*prime™ II (Amersham Biosciences Corp.). Autoradiographic film (Kodak X-Omat Blue XB-1, Rochester, NY) was exposed to the nylon membrane for 30 min at room temperature after hybridization with probe A, 3 h at -80 °C after hybridization with probe B, and 1 h at room temperature after hybridization with probe PER. To demonstrate specificity of the probes, exposures were extended to 16 - 18 h at -80 °C.

RESULTS

Sequence of the rRNA locus

PCR amplification of *P. andrewsi* DNA using primers that flank the IGS region yielded a prominent amplicon of the expected size (1,670 bp) and a smaller additional product (1,540 bp) (Fig. 2). Sequence analysis of the smaller amplicon revealed sequences of the 5S and SSU rRNA in the 3' and 5' ends, respectively, suggesting that this sequence represented an IGS. Alignment analysis showed that this IGS sequence (hereafter IGS-B) differed in 419 positions (27.3%) from the previously described *P. andrewsi* IGS sequence (Coss et al. 2001; hereafter IGS-A).

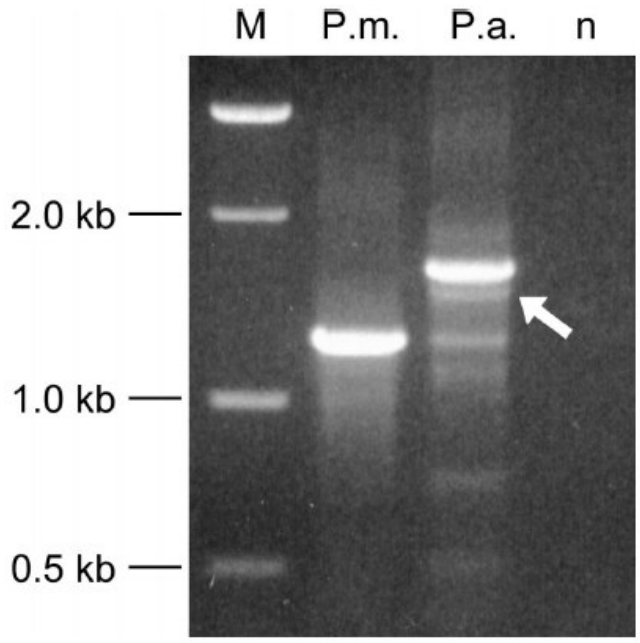

Fig. 2. Agarose gel electrophoresis of PCR-amplified products with IGS primers. P.a., *Perkinsus andrewsi* DNA; P.m., *P. marinus* DNA; n, negative control, PCR reaction contained the same mixture as the other PCR reactions except DNA was substituted with sterile distilled water; M, 1 kb DNA ladder. Note the additional 1.5-kb amplicon (arrow) in *P. andrewsi*, which corresponds to the later identified intergenic spacer of the rRNA-B gene unit.

PCR amplification using primers based on the IGS-B (primer M8) and primers based on conserved regions in the 3' end of the SSU (primer UPRB) and the 5' end of the LSU (primer PMITS3) resulted in amplicons of 2,522 bp and 3,274 bp, respectively. Sequence analysis showed that both amplicons contained part of the IGS-B, and a complete SSU rRNA sequence. In addition, the 3,274-bp amplicon contained an ITS1-5.8S-ITS2 sequence, and sequences present in the 5' end of the LSU. Sequence analysis of clones that contained the IGS-B sequence and the SSU-ITS-1-5.8S-ITS2 region showed that except for the 5.8S, all sequences of the rRNA genes and intergenic regions associated with the IGS-B differed from the initially described *P. andrewsi* rRNA genes and

intergenic regions. This demonstrated contiguity of the newly described sequences.

To isolate and characterize the LSU, the 5S, and the ICS of both rRNA gene units, amplicons were generated by PCR using primers specific for the IGS-A (primer NTS6) and IGS-B (primer M5), and a primer based in a conserved region of the 5' end of the SSU (primer UPRA) (Fig. 1). The obtained amplicons were 7,042 bp and 6,881 bp long, respectively. Both amplicons contained a partial IGS sequence, the complete SSU, the ITS1-5.8-ITS2 region, the complete LSU, and a region consisting of the ICS and the 5S that were contiguous with the LSU and the IGS, respectively. The LSU sequences of both gene units were 3,528 bp long and showed 99.1% identity. The 5S-A and B sequences were 121 bp long and to 99.2% identical. The ICSs of the rRNA-A and -B units differed both in size and sequence. The ICS-A (124 bp) and the ICS-B (109 bp) differed in 47 positions (37.9%, based on the length of ICS-A). A database search for both ICS using BLASTn (Altschul et al. 1990) failed to identify similar sequences. Consensus sequences of the LSU-ISC-5S region of the rRNA-A unit and the complete rRNA-B unit were deposited at GenBank™ (AY305327 and AY305326, respectively).

Sequence comparison of the *P. andrewsi* rRNA genes and intergenic spacer with *Perkinsus* species and isolates

Sequence comparisons of the rRNA genes and intergenic regions of the *P. andrewsi* rRNA-A and –B gene units, *P. marinus*, *P. atlanticus*, and *P.*

chesapeaki are summarized in Table 2. Sequence differences were most pronounced in the IGS and the ICS.

In *Perkinsus* species isolated from *M. arenaria* and *T. plebeius,* four distinct sequence types of the ITS1-5.8S-ITS2 region were reported (Dungan et al. 2002). One of the four reported variant types of ITS2-5.8S-ITS2 sequences (AF440465 and AF440469) of each *Perkinsus* species isolated from *M. arenaria* and *T. plebeius* showed 100% identity with the ITS1-5.8S-ITS2 region of *P. andrewsi* rRNA-A. The *P. andrewsi* unit B ITS1-5.8S-ITS2 sequence showed high sequence identity (99.6%) to the respective *P. chesapeaki* sequence, and to two of the four variant sequence types (AF440464, AF440467, AF440468, and AF440471) of each *Perkinsus* species isolated from *M. arenaria* and *T. plebeius* with 100%, 99.9%, 99.6% and 100% identity, respectively. One variant sequence form of the ITS1-5.8S-ITS2 sequences from the *Perkinsus* species from *T. plebeius* (AF440470) showed high sequence identity towards the *P. andrewsi* unit A sequence at the 3' end and high sequence identities towards *P. chesapeaki* and the *P. andrewsi* unit B sequence at the 5' end (Fig. 3).

Assessment of representation of the rRNA-A and rRNA-B units

The relative amounts of *P. andrewsi* IGS-A and IGS-B amplicons varied with the amplification conditions selected during the PCR optimization (Fig. 2, 4). A semi-quantitative assessment carried out by titration of genomic DNA template showed that the detection limit for IGS-A and IGS-B was between 0.5-pg and 5-pg of genomic DNA (Fig. 4). Analysis of the intensities of the amplicons obtained

Table 2. Comparison of the rRNA genes and intergenic spacers between *Perkinsus andrewsi* A and B rRNA units, *P. atlanticus, P. marinus,* and *P. chesapeaki* (isolate G117).

	Percent identity of the 5S			
	P. andrewsi rRNA-B	*P. atlanticus*	*P. marinus*	
P. andrewsi rRNA-A	99.2	95.2	97.5	
P. andrewsi rRNA-B		96.0	98.3	
P. atlanticus			97.6	
	Percent identity of the IGS			
	P. andrewsi rRNA-B	*P. atlanticus*	*P. marinus*	
P. andrewsi rRNA-A	72.7	49.1	50.8	
P. andrewsi rRNA-B		52.3	51.2	
P. atlanticus			72.2	
	Percent identity of the SSU			
	P. andrewsi rRNA-B	*P. atlanticus*	*P. marinus*	*P. chesapeaki*
P. andrewsi rRNA-A	99.8	99.1	98.5	99.6
P. andrewsi rRNA-B		99.3	98.7	99.7
P. atlanticus			99.3	99.0
P. marinus				98.5
	Percent identity of the ITS1			
	P. andrewsi rRNA-B	*P. atlanticus*	*P. marinus*	*P. chesapeaki*
P. andrewsi rRNA-A	94.7	85.9	79.6	94.7
P. andrewsi rRNA-B		88.3	79.6	100.0
P. atlanticus			80.1	88.3
P. marinus				86.2

Table 2 continued

	Percent identity of the 5.8S			
	P. andrewsi rRNA-B	*P. atlanticus*	*P. marinus*	*P. chesapeaki*
P. andrewsi rRNA-A	100.0	98.7	98.7	99.4
P. andrewsi rRNA-B		98.7	98.7	99.4
P. atlanticus			100.0	98.1
P. marinus				98.1
	Percent identity of the ITS2			
	P. andrewsi rRNA-B	*P. atlanticus*	*P. marinus*	*P. chesapeaki*
P. andrewsi rRNA-A	91.8	79.2	79.1	82.3
P. andrewsi rRNA-B		78.4	80.2	99.7
P. atlanticus			92.5	79.4
P. marinus				80.2
	Percent identity of the LSU			
	P. andrewsi rRNA-B	*P. atlanticus*	*P. marinus*	
P. andrewsi rRNA-A	99.1	97.9	98.0	
P. andrewsi rRNA-B		98.2	98.2	
P. atlanticus			98.8	
	Percent identity of the ICS			
	P. andrewsi rRNA-B	*P. atlanticus*	*P. marinus*	
P. andrewsi rRNA-A	62.1	31.5	31.8	
P. andrewsi rRNA-B		29.8	29.3	
P. atlanticus			61.1	

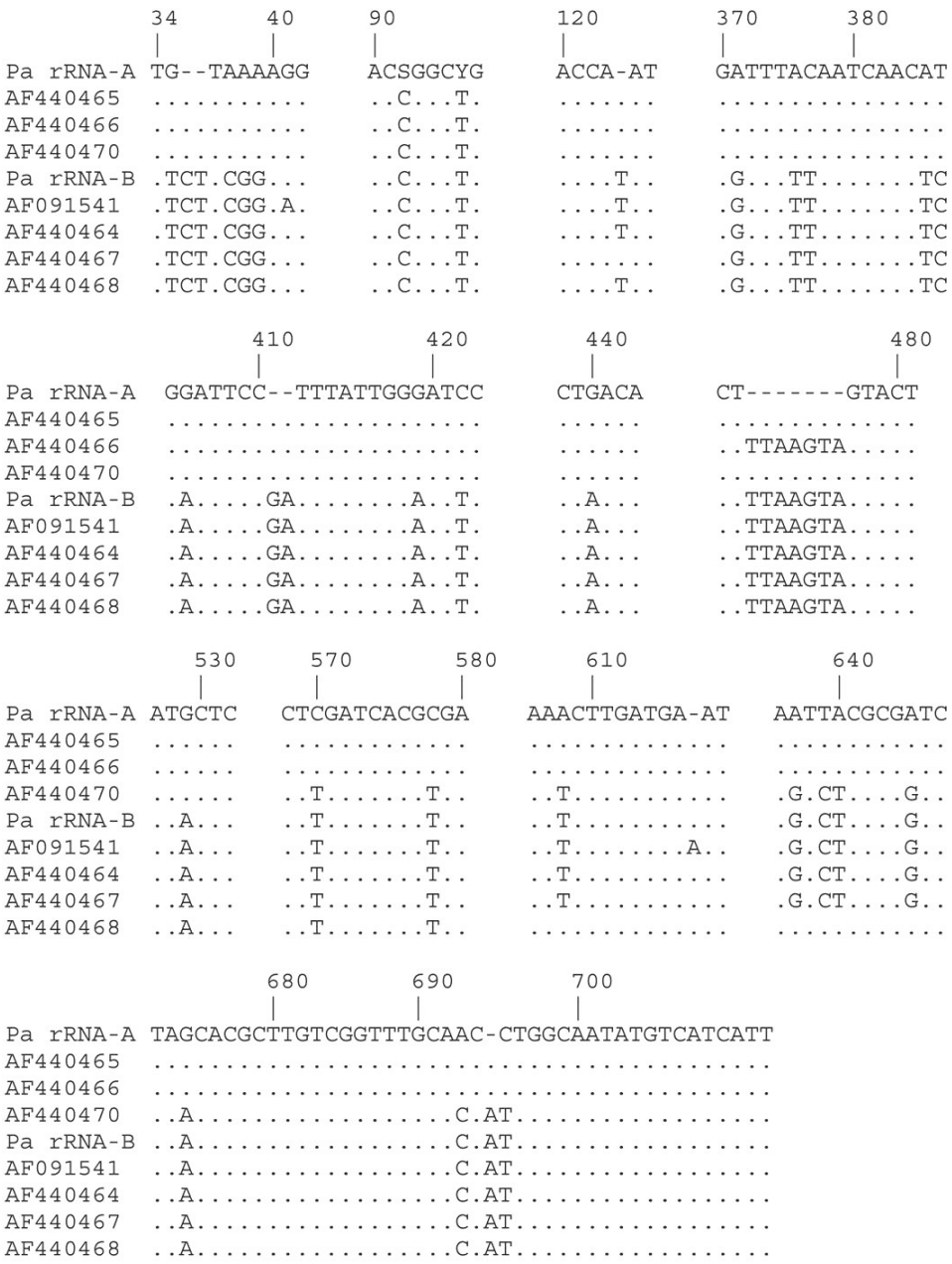

Fig. 3. Sequence alignment of the ITS1-5.8S-ITS2 regions of *Perkinsus andrewsi* (ATCC 50807), *P. chesapeaki* (isolate G117), a *Perkinsus* species isolated from *Mya arenaria* (ATCC 50864), and a *Perkinsus* species isolated from *Tagelus plebeius* (ATCC 50866) using ClustalW. Only the regions where differences occur are depicted. The numbers represent nucleotide positions in the ITS1-5.8S-ITS2 sequence of the *P. andrewsi* rRNA-A gene unit (AF102171). Pa rRNA-A, rRNA gene unit A of *P. andrewsi* (AF102171); Pa rRNA-B, rRNA gene unit B of *P. andrewsi* (AY305326); AF091541, *P. chesapeaki* (isolate G117); AF440464, AF440465, AF440466, AF440467, ITS1-5.8S-ITS2 type a, b, c, and d sequences from the *Perkinus* species isolated from *M. arenaria*; AF440468, AF440470, ITS1-5.8S-ITS2 type a and c sequences from the *Perkinus* species isolated from *T. plebeius*. The type b and d sequences of the *T. plebeius* isolate (AF440469 and AF440471) that are identical to the type b and a sequences of the *M. arenaria* isolate (AF440465 and AF440464), respectively, are not depicted.

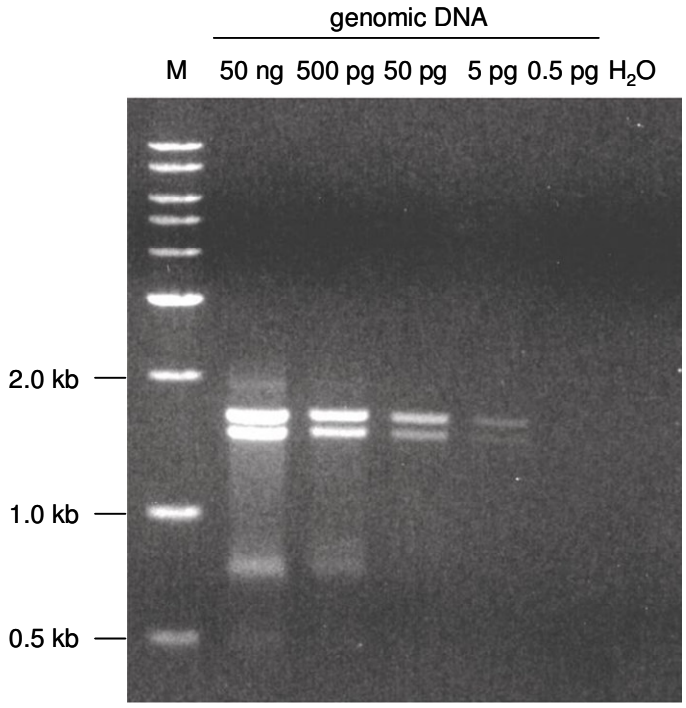

Fig. 4. Agarose gel of PCR-amplified products with IGS primers using optimized conditions. Decreasing amounts of *Perkinsus andrewsi* genomic DNA (50 ng to 0.5 pg) were used to assess the relative representation of the two intergenic spacers. n, negative control, PCR reaction contained the same mixture as the other PCR reactions except DNA was substituted with sterile distilled water; M, 1 kb DNA ladder.

under the optimized PCR conditions showed that the intensity of the IGS-B amplicon was approximately 90% of the intensity of the IGS-A.

Single-cell IGS-specific PCR

The *P. andrewsi* clonal culture characterized in this study had been subjected to three rounds of subcloning (Coss, Robledo and Vasta 2001). However, to rule out the possibility that rRNA-A and rRNA-B originated in different (non-clonal) *P. andrewsi* cells, we carried out PCR amplification of IGS-A and IGS-B from single *P. andrewsi* cells, using primers specific for IGS-A (Coss et al. 2001) and IGS-B (this study). In each single cell, both primer sets yielded

amplicons of the expected sizes for IGS-A and IGS-B (Fig. 5), confirming that each *P. andrewsi* cell contains both IGS sequences.

Intraclonal variability of IGS in *P. andrewsi*

A total of 12 clones containing IGS-A sequences and nine clones containing IGS-B sequences obtained from five independent PCR amplifications were sequenced. Within the clones that had the IGS-A sequence, variability occurred at three positions. Three clones had an insertion-deletion at position 573 that was preceded by a stretch of eight A, whereas the remaining clones had an additional A at this position. Transitions from T to C occurred in three clones

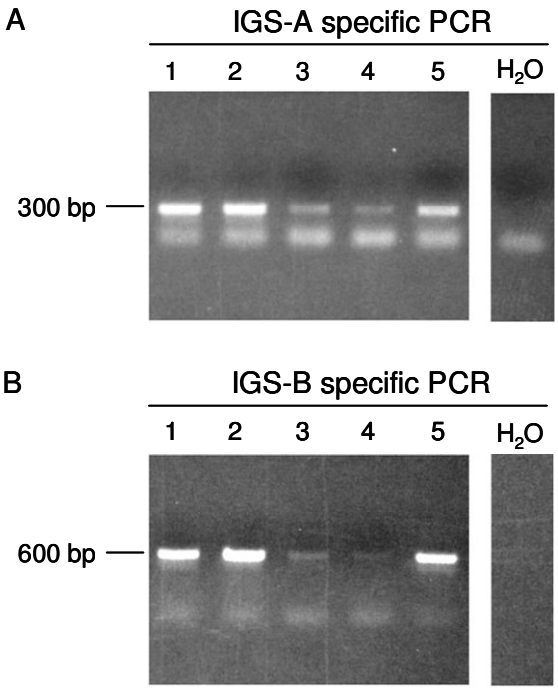

Fig 5. Agarose gels of amplified products of the single cell PCR experiment. IGS-A specific primers (A) and IGS-B specific primers (B) were used to verify the multiple IGS-amplicons obtained from single cells (1 - 5); n, negative control, PCR reaction contained the same mixture as the other PCR reactions except DNA was substituted with sterile distilled water.

at position 1,038, and from C to T in two clones at position 1,047. A consensus IGS-A sequence of the 12 clones was deposited in GenBank™ (AY305328). All 12 clones, however, differed in nine positions (0.7%) to the previously described IGS-A (AF102171). Changes were transitions from A to G (four positions) and T to C (five positions). Sequence alignment of the IGS-A and IGS-B showed that the IGS-A was identical to IGS-B in these positions (data not shown). The IGS-B sequences of all nine clones analyzed were identical.

Southern analysis

To confirm the presence of the additional rRNA gene unit in the *P. andrewsi* genome, *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I restriction digests of *P. andrewsi* genomic DNA, undigested *P. marinus* genomic DNA, and plasmids containing the IGS-A and IGS-B respectively, were blotted and hybridized with probes specific for the IGS-A, IGS-B, and a probe designed to detect presumably IGSs of all *Perkinsus* species (Fig. 6). The IGS-A specific probe hybridized strongly with fragments of about 3.3 kb, 8.0 kb, 5.5 kb, and 1.1 kb in the *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I digested *P. andrewsi* genomic DNA respectively (Fig. 6 A). Additional weak hybridization was observed with fragments of 4.5 kb and 3.8 kb as well as with 4.9 kb and 4.0 kb fragments in the *Bst*EII and *Eco*RI digested *P. andrewsi* DNA respectively (Fig. 6 A). The IGS-A-specific probe hybridized very weakly with plasmid containing the IGS-B (20 ng) but did not hybridize with *P. marinus* DNA, (4 µg; no signal was detected after an 18 h exposure at -80 °C) (Fig. 6 A). The IGS-B-specific probe strongly hybridized with fragments of approximately 8.0 kb, 8.0 kb, 3.0 kb, and 0.9 kb in the restriction-digested *P.*

andrewsi DNA, but only hybridized very weakly with 20 ng DNA from a with plasmid containing the IGS-A (20 ng) and did not hybridize with *P. marinus* DNA (4 µg) (Fig. 6 B). These results revealed the specificity of the probes to the IGS-A and IGS-B. The probe specific for *Perkinsus* species IGSs (probe PER) hybridized with two fragments of approximately 8.0 kb and 3.3 kb in the *Bam*HI digested *P. andrewsi* DNA, with one fragment of 8.0 kb in the *Bst*EII digested *P. andrewsi* DNA, three fragments of approximately 5.5 kb, 3.0 kb, and 1.8 kb in the *Eco*RI digested *P. andrewsi* DNA, and with two fragments of 6.5 kb and 2.9 kb in

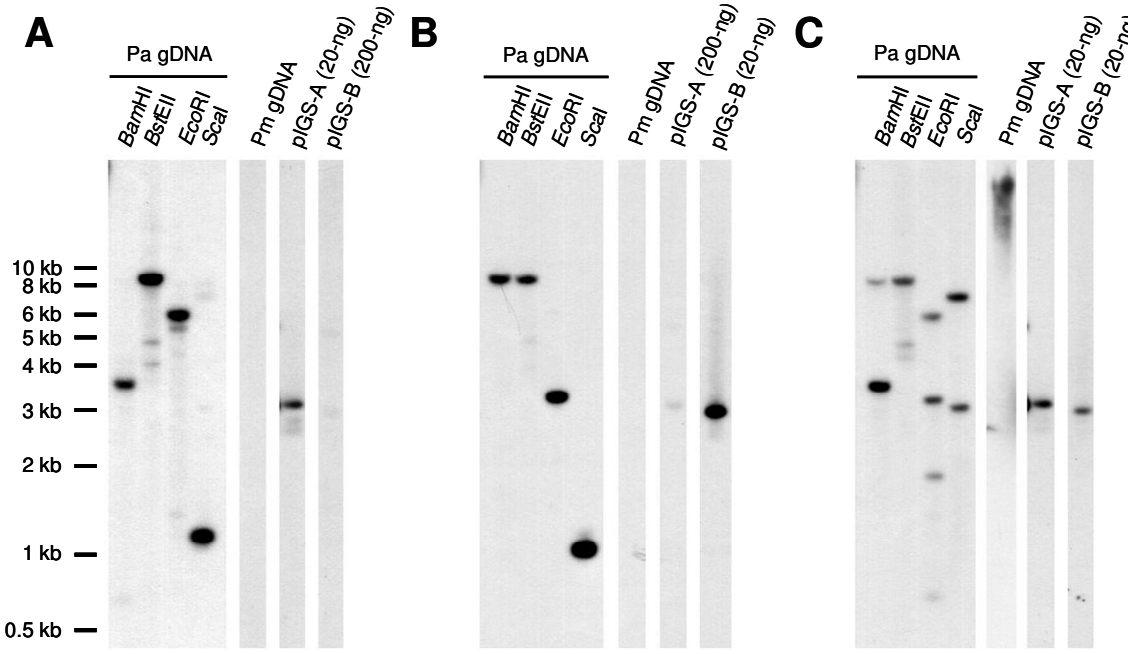

Fig. 6. Southern analysis of *Perkinsus andrewsi* genomic DNA. *Perkinsus andrewsi* genomic DNA, digested with *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I was size seperated on a 0.8% agarose gel, blotted onto a Hybond XL nylon membrane (Amersham Bioscience) and hybridized with an IGS-A specific probe (A), an IGS-B specific probe (B), and with a probe specific to *Perkinsus* species IGSs (C). Undigested *P. marinus* genomic DNA (4 µg) and 20 or 200 ng of plasmid DNA containing the IGS-A and IGS-B were used as controls. Autoradiographic films have been exposed to the nylon membrane for 30 min at room temperature (A), 3 h at at -80 °C (B), and 1 h at room temperature (C), except for the autoradiographic films showing the *P. marinus* genomic DNA that have been exposed for 18 h at -80 °C. IGS, intergenic spacer; Pa gDNA, *P. andrewsi* genomic DNA; Pm gDNA, *P. marinus* genomic DNA, uncut; pIGS-A, plasmid containing the IGS-A; pIGS-B, plasmid containing the IGS-B.

the *Sca*l digested *P. andrewsi* DNA (Fig. 6 C). Probe PER hybridized strongly with both plasmids, and somewhat weaker with *P. marinus* genomic DNA. A stronger signal with *P. marinus* genomic DNA was obtained by a longer exposure (18 h) of the autoradiograhic film (Fig. 6 C). The banding patterns observed with the three probes were consistent with fragments predicted from the rRNA-A and rRNA-B sequences (Fig. 7), with the exception of additional weak hybridizations of probe A and PER with the *Bst*EII and *Eco*RI digests of *P. andrewsi* DNA (Fig. 6 A, C).

DISCUSSION

PCR amplification of *P. andrewsi* genomic DNA using primers that anneal to conserved regions of the 5S and the SSU resulted in two distinct amplicons representing IGS sequences (IGS-A and IGS-B). These were confirmed by the presence of of the 5S and the SSU gene flanking sequences. The sequence of the IGS-A was identical to the previously described *P. andrewsi* IGS (Coss et al. 2001), except in nine positions. Because the IGS-A sequence described in this study represents a consensus sequence of 12 clones obtained from five independent PCR reactions, these differences most likely represent intraclonal IGS sequence variability (see below). The IGS-B, however, differed considerably from the IGS-A. Using primers specific to the IGS-B sequence and IGS-A specific primers from Coss et al. (2001), the presence of both IGS sequences in a single *P. andrewsi* cell was confirmed. Single cells were isolated by the "limiting dilution" method, routinely used for subcloning of *Perkinsus* cultures.

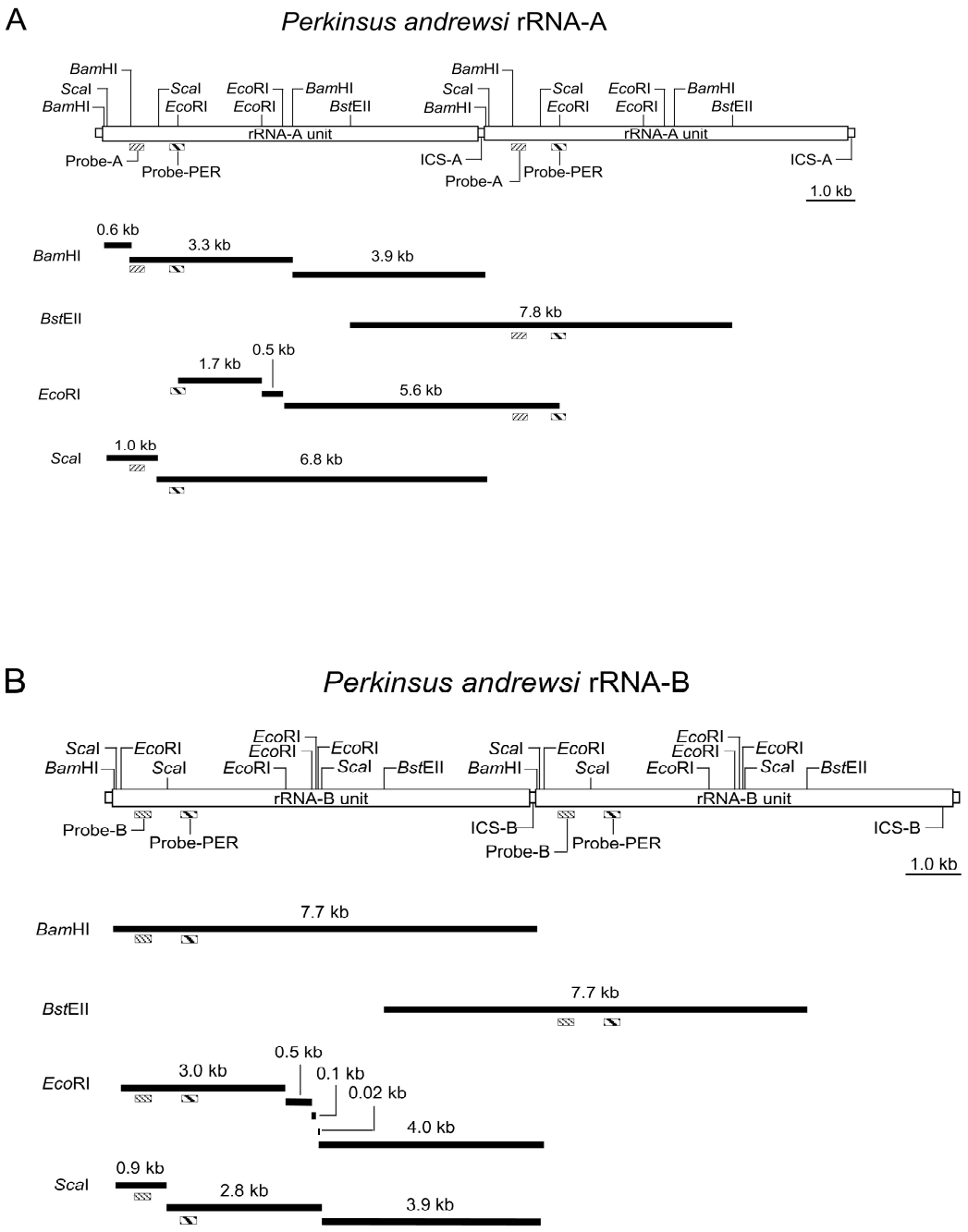

Fig. 7. Predicted restriction map of two *Perkinsus andrewsi* rRNA gene units digested with *Bam*HI, *Bst*EII, *Eco*RI, and *Sca*I and the fragments created by these endonucleases. A, *Perkinsus andrewsi* rRNA-A; B, *P. andrewsi* rRNA-B. The hybridization sites of probe A (▨), probe B (▧), and probe PER (◪) are indicated. ISC-A, intercluster spacer of the rRNA-A gene unit; ICS-B, intercluster spacer of the rRNA-A gene unit.

Although for the single cell PCR assay, serial dilutions were carried out in distilled water to facilitate concentration of the DNA at the following step, we have experimentally demonstrated by using neutral red and 4',6'-diamidino-2-phenylindole (DAPI) that during the time that takes to complete the procedure, the nucleus and nuclear DNA are retained (WTP and GRV unpublished). Therefore, since the IGS-A and -B specific primers target sequences in the non-transcribed spacer region of the IGS, potential cross contamination is highly unlikely. Variability of amplicon intensities may have been due to differences in DNA yields by the crude DNA extraction procedure used. Alternatively, the different cells could be in different stages of the cell cycle and thus might contain more than one genome equivalent.

We observed no intraclonal sequence variability within the IGS-B and only low sequence variability within the IGS-A, strongly suggesting that *P. andrewsi* possesses two distinct IGS sequences.

There are at least two possible organizations of the two IGS sequences within the *P. andrewsi* rRNA gene cluster(s). In one model, both IGS-A and IGS-B could be randomly dispersed and associated with identical rRNA genes, resulting in rRNA gene units that only differ in their IGSs. Alternatively, *P. andrewsi* could possess two distinct rRNA gene units, containing either IGS-A or IGS-B associated with different rRNA genes and intergenic regions. To address this question, amplicons obtained by PCR using an IGS-B-specific primer and conserved primers located in the 3' end of the SSU or in the 5' end of the LSU were cloned and sequenced. In all clones the IGS-B sequences were associated

with rRNA genes and intergenic regions that differed in their sequences from the previously described rRNA genes and intergenic regions associated with the IGS-A. Results from Southern analysis also corroborated the presence of two distinct rRNA units in the *P. andrewsi* genome. Hybridization patterns of restriction digests of *P. andrewsi* genomic DNA were consistent with restriction length fragment polymorphisms predicted on the basis of two distinct rRNA gene units being present in a single cell. Weak hybridizations observed in some digests tested with the IGS-A-specific probe and the probe specific to *Perkinsus* species IGS could represent intraclonal variability either in the rRNA-A gene unit, especially in the IGS-A, or alternatively, in the 5' or 3' end sequences of the rRNA gene clusters. Lack of sufficient sequence information upstream or downstream of the rRNA gene clusters prevents us from confirming either alternative.

Taken together, these observations provide strong evidence that *P. andrewsi* contains two distinct rRNA gene units, which we designated as rRNA-A and rRNA-B. Further, Southern analysis and PCR suggest that each rRNA gene unit is arranged as a "head to tail" tandem repeat. All three probes hybridized with only one fragment in genomic DNA samples that have been digested with *Bst*EII, an endonuclease that cuts once in each rRNA gene unit (based on restriction maps on the complete rRNA sequences). The observed fragments are slightly smaller than 8.0 kb, which corresponds approximately to the length of each rRNA gene unit (7.8 kb for the rRNA-A gene unit, and 7.7 kb for the rRNA-B gene unit). The presence of multiple distinct rRNA gene units has been

described in vertebrates (Cortadas and Pavon 1982), in *Trypanosoma cruzi* (Souto and Zingales 1993), in the dinoflagellate *Alexandrium* (Scholin, Anderson, and Sogin 1993; Scholin et al. 1994; Yeung et al. 1996), and in the apicomplexans *Theileria* (Bishop et al. 2000), *Babesia* (Dalrymple 1990; Reddy et al. 1991), *Cryptosporidium* (Le Blancq et al. 1997), and *Plasmodium* (reviewed by McCutchan et al. 1995). In most *Plasmodium* species examined so far, four rRNA gene units (A, B, C, and D), with the exception of *P. falciparum* that carries seven rRNA gene units (Dame and McCutchan 1984; Wellems et al. 1987; Waters 1994; Waters et al. 1997; Carlton et al. 2002; Gardner et al. 2002). The gene units transcribe at least two distinct ribosomal types (A and S) that are stage-specifically expressed (Dame and McCutchan 1984; Wellems et al. 1987; Waters 1994; Waters et al. 1997). Sequence comparison showed that high percent identities are found between rRNA gene units that transcribe for the same rRNA types (McCutchan et al. 1995). rRNA gene units that transcribe for the different ribosomal types show less percent identity. In *P. berghei* for example, sequence analysis of the SSU-A and SSU-C showed that both genes are 96.5% identical (Gunderson, McCutchan, and Sogin 1986; Gunderson et al. 1987).

Based on the structural differences postulated for the different life stage-specific expressed ribosome types in *Plasmodium* species, it has been proposed that these ribosome types are also functionally different (Gunderson et al. 1987; Rodgers et al. 1996; Thompson et al. 1999) and that the parasite relies on functional distinct ribosomes to complete its life cycle in a mammalian and insect

host (Gunderson et al. 1987). However, a recent study showed that in *P. berghei* the rRNA genes encoded by the respective gene units do not differ in the sequence of important core regions and thus lack structural differences, and both ribosome types, A and S, are expressed in life stages in the insect vector (van Spaendonk et al. 2001). The observations in *P. berghei* question the presence of functional different ribosomal types and their proposed requirement for completion of the life cycle. Instead, based on the atypical organization of the rRNA gene, van Spaendonk et al. (2001) proposed that each unit is controlled by different promoters, an arrangement that would enable the parasite to adjust the production of ribosomes depending on the need.

In *P. andrewsi,* genes of the rRNA-A and -B units show a high degree of identity: SSU-A and -B show 99.8% identity, LSU-A and -B 99.1% identity, the 5S-A and -B 99.2% identity, and the 5.8S 100.0% identity, suggesting that the two units do not transcribe structural and functional distinct ribosomal types. However, the IGSs of rRNA-A and -B units show only 72.7% identity. The IGS carries all elements required for transcription and termination of the RNA genes, and is critical for the regulation of the rDNA metabolism (De Lucchini, Andronico, and Nardi 1997; Reeder 1990; Sollner-Webb and Mougey 1991). In contrast to *P. atlanticus* and *P. marinus*, which possess a single rRNA gene unit type (Robledo et al. 2002; JAFR and GRV, unpublished), *P. andrewsi* has two distinct rRNA gene units. Whether these units are selectively regulated, as proposed for *P. berghei* (van Spandonk et al., 2001), remains to be demonstrated.

The two different rRNA gene units could be attributed to the presence of different alleles. Although the ploidy status of *Perkinsus* species has not yet been resolved, it has been suggested that *P. marinus* trophozoites are diploid (Reece, Bushek, and Graves 1997; Reece et al. 2001). Assuming that this is correct and that it can be extended to *P. andrewsi*, it is possible that: (a) the *P. andrewsi* monoclonal culture is heterozygous for the rRNA units; (b) both rRNA unit types are contiguously located on the same chromosome; or (c) the rRNA unit types are located on different chromosomes. Evidence suggests that *Perkinsus* species have multiple copies of rRNA gene units (de la Herrán et al. 2000). Hence, until further investigations are conducted to address the linkage of the two rRNA units, how these are localized and organized within the rRNA array(s) remains an open question.

The presence of multiple distinct rRNA gene units in a single *Perkinsus* species adds new complexity to the use of rRNA sequences for taxonomic analysis and species designations based on molecular characters. It also underscores the need of rigorous characterization of the complete rRNA gene locus sequences to determine whether polymorphisms observed in intergenic regions represent inter- or intraclonal variability or, as in the case for *P. andrewsi*, represent distinct rRNA gene units. This study shows low sequence variability within the IGS of each rRNA gene unit of *P. andrewsi*. Only 0.7% variability was observed in the IGS-A sequence, and none for the IGS-B. In *P. marinus*, two distinct sequence types (type I and II) differing in only seven nucleotide positions out of 307 bp within the non-transcibed spacer of the IGS were identified

(Robledo et al. 1999), suggesting that in this species the IGS also has a low degree of sequence variability.

In a recent study, Dungan et al. (2002) reported intraclonal variability of ITS sequences in clonal cultures of *Perkinsus* species isolated from *M. arenaria* and *T. plebeius*. Four variant ITS1-5.8S-ITS2 types ("a" to "d") were described. Sequence comparison of the eight sequences with *P. andrewsi* A and B unit ITS-5.8S-ITS2 sequences show that the type "a" of the *M. arenaria* isolate and type "d" of the *T. plebeius* isolate are identical to the ITS1-5.8S-ITS2-B sequences of *P. andrewsi*. Similarly, the type "b" of both isolates is identical to the rRNA-A ITS1-5.8S-ITS2 sequence of *P. andrewsi*. The remaining two types of each isolate appear to be intermediate, sharing sequences with the *P. andrewsi* A and B unit, respectively. Dungan et al. (2002) obtained the ITS1-5.8S-ITS2 sequences by PCR amplification using conserved primers, which has the potential to produce sequence artifacts by *in vitro* recombination, and thus, may mislead genetic analysis (Meyerhans, Vartanian, and Wain-Hobson 1990; Judo, Wedel, and Wilson 1998; Tanabe et al. 2002). Tanabe et al. (2002) analyzed PCR amplifications of a low copy gene (*Msp1*) from different *P. falciparum* strains, and observed up to 11.5% *in vitro* recombination when conserved primers were used. *In vitro* recombination sequence artifacts can be avoided by either sequencing the genes of interest directly from digested genomic DNA, or by the use of at least one allele-specific primer (Tanabe et al. 2002). We chose the second approach and used either rRNA-A- or rRNA-B-specific primers in combination with a conserved primer to amplify partial rRNA sequences that

contain the ITS1-5.8S-ITS2 region. No *in vitro* recombination was observed with this system, but if primers based on conserved regions were used to co-amplify partial A and B type rRNA sequences, sequence “chimeras” were identified (data not shown).

Sequence comparison of the two rRNA units of *P. andrewsi* and *P. chesapeaki*, a *Perkinsus* species isolated from *M. arenaria*, revealed that the SSU-B gene and the ITS1-5.8-ITS2-B sequence have high percent identity to the respective sequences of *P. chesapeaki*, suggesting that *P. chesapeaki* and *P. andrewsi* are closely related. However, in *P. chesapeaki* only one rRNA gene unit was described, and no intraclonal variability has been reported (Kotob et al. 1999). For the *Perkinsus* isolates from *M. arenaria* and *T. plebeius*, only partial rRNA sequences have been characterized. Characterization of the complete rRNA gene loci from these isolates and from the *P. chesapeaki* holotype (McLaughlin et al. 2000), currently unavailable, would reveal whether the observed sequence polymorphisms represent either the presence of distinct rRNA gene units or variability within a single unit. Furthermore, it would confirm the presence of the intermediate forms described, and contribute to resolve the relationship between *P. andrewsi, P. chesapeaki*, and the *Perkinsus* isolates from *M. arenaria* and *T. plebeius*.

A preliminary search in *P. marinus* and *P. atlanticus* by PCR, the two other *Perkinsus* species from which the complete rRNA locus has been characterized, failed to reveal the presence of additional rRNA gene units (Robledo et al. 2002; JAFR and GRV, unpublished). Thus, in this regard, the *P. andrewsi* holotype

appears to be unique among the *Perkinsus* species characterized at present time.

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LITERATURE CITED

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.*, **215**:403--410.

Bishop, R., Gobright, E., Spooner, P., Allsopp, B., Sohanpal, B. & Collins, N. 2000. Microsequence heterogeneity and expression of the LSU rRNA genes within the two single copy ribosomal transcription units of *Theileria parva*. *Gene*, **257**:299--305.

Brown, T. 1993. Hybridization analysis of DNA blots. *In*: Ausubel, F. A., Brent, R. Kingston, R. E., Moore, Seidman, J. G., Smith, J. A. & Struhl, K. (ed.), Current protocols in molecular biology, John Wiley & Sons, New York. p. 2.10.1--2.10.16.

Brugerolle, G. 2002. *Cryptophagus subtilis*: a new parasite of cryptophytes affiliated with the Perkinsozoa lineage. *Eur. J. Protistol.*, **37**:379--390.

Carlton, J. M., Angiuoli, S. V., Suh, B. B., Kooij, T. W., Pertea, M., Silva, J. C., Ermolaeva, M. D., Allen, J. E., Selengut, J. D., Koo, H. L., Peterson, J. D., Pop, M., Kosack, D. S., Shumway, M. F., Bidwell, S. L., Shallom, S. J., van Aken, S. E., Riedmuller, S. B., Feldblyum, T. V., Cho, J. K., Quackenbush, J., Sedegah, M., Shoaibi, A., Cummings, L. M., Florens, L., Yates, J. R., Raine, J. D., Sinden, R. E., Harris, M. A., Cunningham, D. A., Preiser, P. R., Bergman, L. W., Vaidya, A. B., van Lin, L. H., Janse, C. J., Waters, A. P., Smith, H. O., White, O. R., Salzberg, S. L., Venter, J. C., Fraser, C. M., Hoffman, S. L., Gardner, M. J. & Carucci, D. J. 2002. Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature*, **419**:512--519.

Cortadas, J. & Pavon, M. C. 1982. The organization of ribosomal genes in vertebrates. *EMBO J.*, **1**:1075--1080.

Coss, C. A., Robledo, J. A. F. & Vasta, G. R. 2001. Fine structure of clonally propagated *in vitro* life stages of a *Perkinsus* sp. isolated from the Baltic clam *Macoma balthica*. *J. Eukaryot. Microbiol.*, **48**:38--51.

Coss, C. A., Robledo, J. A. F, Ruiz, G. M. & Vasta, G. R. 2001. Description of *Perkinsus andrewsi* n. sp. isolated from the Baltic clam (*Macoma balthica*) by characterization of the ribosomal RNA locus, and development of a species-specific PCR-based diagnostic assay. *J. Eukaryot. Microbiol.*, **48**:52--61.

Dalrymple, B. P. 1990. Cloning and characterization of the rRNA genes and flanking regions from *Babesia bovis*: use of the genes as strain discriminating probes. *Mol. Biochem. Parasitol.*, **43**:117--124.

Dame, J. B. & McCutchan, T. F. 1983. The four ribosomal DNA units of the malaria parasite *Plasmodium berghei*. Identification, restriction map, and copy number analysis. *J. Biol. Chem.*, **258**:6984--6990.

Dame, J. B. & McCutchan, T. F. 1984. Identification of 5 S and 5.8 S ribosomal RNA molecules and their genes in *Plasmodium berghei*. *Mol. Biochem. Parasitol.*, **11**:301--307.

de la Herrán, R., Garrido-Ramos, M. A., Navas, J. I., Ruiz Rejón, C. & Ruiz Rejón, M. 2000. Molecular characterization of the ribosomal RNA gene region of *Perkinsus atlanticus*: its use in phylogenetic analysis and as a target for a molecular diagnosis. *Parasitology*, **120**:345--353.

De Lucchini, S., Andronico, F. & Nardi, I. 1997. Molecular structure of the rDNA intergenic spacer (IGS) in *Triturus*: implications for the hypervariability of rDNA loci. *Chromosoma*, **106**:315--326.

Dungan, C. F., Hamilton, R. M., Hudson, K. L., McCollough, C. B. & Reece, K. S. 2002. Two epizootic diseases in Chesapeake Bay commercial clams, *Mya arenaria* and *Tagelus plebeius*. *Dis. Aquat. Org.*, **50**:67--78.

Fong, D., Rodriguez, R., Koo, K., Sun, J., Sogin, M. L., Bushek, D., Littlewood, D. T. & Ford, S. E. 1993. Small subunit ribosomal RNA gene sequence of the oyster parasite *Perkinsus marinus*. *Mol. Mar. Biol. Biotechnol.*, **2**:346--350.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M. S., Nene, V., Shallom, S. J., Suh, B., Peterson, J., Angiuoli, S., Pertea. M., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M. & Barrell, B. 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature*, **419**:498--511.

Gauthier, J. D. & Vasta, G. R. 1995. *In vitro* culture of the eastern oyster parasite *Perkinsus marinus*: Optimization of the methodology. *J. Invertebr. Pathol.*, **66**:156--168.

Goggin, C. L. & Barker, S. C. 1993. Phylogenetic position of the genus *Perkinsus* (Protista, Apicomplexa) based on small subunit ribosomal RNA. *Mol. Biochem. Parasitol.*, **60**:65--70.

Goggin, C. L. 1994. Variation in the two internal transcribed spacers and 5.8S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). *Mol. Biochem. Parasitol.*, **65**:179--182.

Green, E. D. 1997. Genome analysis: a laboratory manual. Cold Spring Harbor Laboratory Press, Plainview, New York.

Guay, J. M., Huot, A., Gagnon, S., Tremblay, A. & Levesque, R. C. 1992. Physical and genetic mapping of cloned ribosomal DNA from *Toxoplasma gondii*: primary and secondary structure of the 5S gene. *Gene*, **114**:165--171.

Gunderson, J. H., McCutchan, T. F. & Sogin, M. L. 1986. Sequence of the small subunit ribosomal RNA gene expressed in the bloodstream stages of *Plasmodium berghei*: evolutionary implications. *J. Protozool.*, **33**:525--529.

Gunderson, J. H., Sogin, M. L., Wollett, G., Hollingdale, M., de la Cruz, V. F., Waters, A. P. & McCutchan, T. F. 1987. Structurally distinct, stage-specific ribosomes occur in *Plasmodium*. *Science*, **238**:933--937.

Judo, M. S., Wedel, A. B. & Wilson, C. 1998. Stimulation and suppression of PCR-mediated recombination. *Nucleic Acids Res.*, **26**:1819--1825.

Kawai, H., Muto, H., Fujii, T. & Kato, A. 1995. A linked 5S rRNA gene in *Scytosiphon lomentaria* (Scytosiphonales, Phaeophyceae). *J. Phycol.*, **31**:306--311.

Kotob, S. I., McLaughlin, S. M., van Berkum, P. & Faisal, M. 1999. Characterization of two *Perkinsus* spp. from the softshell clam, *Mya arenaria* using the small subunit ribosomal RNA gene. *J. Eukaryot. Microbiol.*, **46**:439--444.

Kuvardina, O. N., Leander, B. S., Aleshin, V. V., Myl'nikov, A. P., Keeling, P. J. & Simdyanov, T. G. 2002. The phylogeny of colpodellids (Alveolata) using small subunit rRNA gene sequences suggests they are the free-living sister group to apicomplexans. *J. Eukaryot. Microbiol.*, **49**:498--504.

Le Blancq, S. M., Khramtsov, N. V., Zamani, F., Upton, S. J. & Wu, T. W. 1997. Ribosomal RNA gene organization in *Cryptosporidium parvum*. *Mol. Biochem. Parasitol.*, **90**:463--478.

Levine, N. D. 1978. *Perkinsus* gen. n. and other new taxa in the protozoan phylum Apicomplexa. *J. Parasitol.*, **64**:549.

Mackin, J. G., Owen, H. M. & Collier, A. 1950. Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). *Science*, **111**:328--329.

Marsh, A. G., Gauthier, J. D. & Vasta, G. R. 1995. A semiquantitative PCR assay for assessing *Perkinsus marinus* infections in the eastern oyster, *Crassostrea virginica*. *J. Parasitol.*, **81**:577--583.

McCutchan, T. F., Li, J., McConkey, G. A., Rogers, M. J. & Waters, A. P. 1995. The cytoplasmic ribosomal RNAs of *Plasmodium* spp. *Parasitol. Today*, **11**:134--138.

McCutchan, T. F., de la Cruz, V. F., Lal, A. A., Gunderson, J. H., Elwood, H. J. & Sogin, M. L. 1988. Primary sequences of two small subunit ribosomal RNA genes from *Plasmodium falciparum*. *Mol. Biochem. Parasitol.*, **28**:63--68.

McLaughlin, S. M., Tall, B. D., Shaheen, A., Elsayed, E. E. & Faisal, M. 2000. Zoosporulation of a new *Perkinsus* species isolated from the gills of the softshell clam *Mya arenaria*. *Parasite*, **7**:115--122.

Medlin, L., Elwood, H. J., Stickel, S. & Sogin, M. L. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, **71**:491--499.

Meyerhans, A., Vartanian, J. P. & Wain-Hobson S. 1990. DNA recombination during PCR. *Nucleic Acids Res.*, **18**:1687--1691.

Norén, F., Moestrup, Ø. & Rehnstam-Holm, A.-S. 1999. *Parvilucifera infectans* Norén et Moestrup gen. et sp. nov. (Perkinsozoa phylum nov.): A parasitic flagellate capable of killing toxic microalgae. *Eur. J. Protistol.*, **35**:233--254.

Penna, M. S., Khan, M. & French, R. A. 2001. Development of a multiplex PCR for the detection of *Haplosporidium nelsoni, Haplosporidium costale* and *Perkinsus marinus* in the eastern oyster (*Crassostrea virginica*, Gmelin, 1971). *Mol. Cell Probes*, **15**: 385--390.

Ray, S. M. 1952. A culture technique for the diagnosis of infection with *Dermocystidium marinum* in oysters. *Science*, **116**:360--361.

Reddy, G. R., Chakrabarti, D., Yowell, C. A. & Dame, J. B. 1991. Sequence microheterogeneity of the three small subunit ribosomal RNA genes of *Babesia bigemina*: expression in erythrocyte culture. *Nucleic Acids Res.*, **19**:3641--3645.

Reece, K. S., Bushek, D. & Graves, J. E. 1997. Molecular markers for population genetic analysis of *Perkinsus marinus*. *Mol. Mar. Biol. Biotechnol.*, **6**:197--206.

Reece, K. S., Bushek, D., Hudson, K. L. & Graves, J. E. 2001. Geographic distribution of *Perkinsus marinus* genetic strains along the Atlantic and Gulf Coasts of the USA. *Mar. Biol.*, **139**:1047--1055.

Reeder, R. H. 1990. rRNA synthesis in the nucleolus. *Trends Genet.*, **6**:390--395.

Robledo, J. A. F., Coss, C. A. & Vasta, G. R. 2000. Characterization of the ribosomal RNA locus of *Perkinsus atlanticus* and development of a polymerase chain reaction-based diagnostic assay. *J. Parasitol.*, **86**:972--978.

Robledo, J. A. F., Nunes, P. A., Cancela, M. L. & Vasta, G. R. 2002. Development of an *in vitro* clonal culture and characterization of the rRNA gene cluster of *Perkinsus atlanticus*, a protistan parasite of the clam *Tapes decussatus*. *J. Eukaryot. Microbiol.*, **49**:414--422.

Robledo, J. A. F., Wright, A. C., Marsh, A. G. & Vasta, G. R. 1999. Nucleotide sequence variability in the nontranscribed spacer of the rRNA locus in the oyster parasite *Perkinsus marinus*. *J. Parasitol.*, **85**:650--656.

Robledo, J. A. F., Gauthier, J. D., Coss, C. A., Wright, A. C. & Vasta, G. R. 1998. Species-specificity and sensitivity of a PCR-based assay for *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica*: a comparison with the fluid thioglycollate assay. *J. Parasitol.*, **84**:1237--1244.

Rogers, M. J., Gutell, R. R., Damberger, S. H., Li, J., McConkey, G. A., Waters, A. P. & McCutchan, T. F. 1996. Structural features of the large subunit rRNA expressed in *Plasmodium falciparum* sporozoites that distinguish it from the asexually expressed subunit rRNA. *RNA*, **2**:134--145.

Saldarriaga, J. F., McEwan, M. L., Fast, N. M., Taylor, F. J. R. & Keeling P. J. 2003. Multiple protein phylogenies show that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellate lineage. *Int. J. Syst. Evol. Microbiol.*, **53**:355--365.

Sambrook, J. & Russell, D. W. 2001. Molecular Cloning. A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scholin, C. A., Anderson, D. M. & Sogin, M. L. 1993. Two distinct small-subunit ribosomal RNA genes in the North American toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae). *J. Phycol.*, **29**:209--216.

Scholin, C. A., Herzog, M., Sogin, M. L. & Anderson, D. M. 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). 2. Sequence analysis of a fragment of the LSU rRNA gene. *J. Phycol.*, **30**:999--1011.

Siddall, M. E., Reece, K. S., Graves, J. E. & Burreson, E. M. 1997. 'Total evidence' refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa. *Parasitology*, **115**:165--176.

Sollner-Webb, B. & Mougey, E. B. 1991. News from the nucleolus: rRNA gene expression. *Trends Biochem. Sci.*, **16**:58--62.

Souto, R. P. & Zingales, B. 1993. Sensitive detection and strain classification of *Trypanosoma cruzi* by amplification of a ribosomal RNA sequence. *Mol. Biochem. Parasitol.*, **62**:45--52.

Tanabe, K., Sakihama, N., Farnert, A., Rooth, I., Bjorkman, A., Walliker, D. & Ranford-Cartwright, L. 2002. *In vitro* recombination during PCR of *Plasmodium falciparum* DNA: a potential pitfall in molecular population genetic analysis. *Mol. Biochem. Parasitol.*, **122**:211--216.

Thompson, J. D., Higgins, D. G. & Gibson, T. J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**:4673--4680.

Thompson, J., van Spaendonk, R. M., Choudhuri, R., Sinden, R. E., Janse, C. J. & Waters, A. P. 1999. Heterogeneous ribosome populations are present in *Plasmodium berghei* during development in its vector. *Mol. Microbiol.*, **31**:253--260.

van Spaendonk, R. M., Ramesar, J., van Wigcheren, A., Eling, W., Beetsma, A. L., van Gemert, G. J., Hooghof, J., Janse, C. J. & Waters, A. P. 2001. Functional equivalence of structurally distinct ribosomes in the malaria parasite, *Plasmodium berghei*. *J. Biol. Chem.*, **276**:22638--22647.

Waters, A. P. 1994. The ribosomal RNA genes of *Plasmodium*. *Adv*. *Parasitol*., **34**:33--79.

Waters, A. P., van Spaendonk, R. M., Ramesar, J., Vervenne, R. A., Dirks, R. W., Thompson, J. & Janse, C. J. 1997. Species-specific regulation and switching of transcription between stage-specific ribosomal RNA genes in *Plasmodium berghei*. *J. Biol. Chem*., **272**:3583--3589.

Wellems, T. E., Walliker, D., Smith, C. L., do Rosario, V. E., Maloy, W. L., Howard, R. J., Carter, R. & McCutchan, T. F. 1987. A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of *Plasmodium falciparum*. *Cell*, **49**:633--642.

Yarnall, H. A., Reece, K. S., Stokes, N. A. & Burreson, E. M. 2000. A quantitative competitive polymerase chain reaction assay for the oyster pathogen *Perkinsus marinus*. *J. Parasitol*., **86**:827--37.

Yeung, P. K., Kong, K. F., Wong, F. T. & Wong, J. T. 1996. Sequence data for two large-subunit rRNA genes from an Asian strain of *Alexandrium catenella*. *Appl. Environ. Microbiol*., **62**:4199--4201.

L. Abstracts of contributions to scientific meetings

L. 1. Fish bioassay for Pfiesteria piscicida *toxicity: characterization of a culture flask assay format*

Michael S. Quesenberg *, Keiko Saito*, Danara N. Krupatkina, Nuala O'Leary, Wolf T. Pecher, Mohamed Alavi, Todd Miller, Rachel E. Schneider, Robert M. Belas, Jonathan R. Deeds, Alan R. Place, Yonathan Zohar, Gerardo R. Vasta.
CDC National meeting on *Pfiesteria.* Atlanta, GA, October 2000.

Abstract

The description of the heterotrophic dinoflagellate *Pfiesteria piscicida* or its toxin(s) as causative agent of fish lesions and fish deaths along the mid-Atlantic estuaries has led to the design and implementation of bioassays aimed at revealing its potential toxigenicity. We designed a bioassay in which clonal strains or environmental isolates of *P. piscicida* are exposed to fish in 750 ml culture flasks, and examined the relationship between proliferation profiles of *P. piscicida* and fish deaths. Experiments were carried out with environmental water and sediment samples collected by the Core Facility for Culture of Dinoflagellates at COMB or kindly provided by the Maryland Department of Natural Resources, and characterized dinoflagellate cultures (*P. piscicida, Gyrodinium galatheanum*, *Prorocentrum minimum*, CCMP1828, CCMP1829, and CCMP1834), co-incubated with sets of two small sheepshead minnows (*Cyprinodon variegatus*) at 23℃ under a 14 h/10h light/dark cycle. Parameters regularly monitored included numbers and compositions of dinoflagellate,

protozoa, and bacterial populations, “water quality” (pH; ammonia, nitrate, and nitrite levels), and time of fish death. The presence of fish in experimental flasks induced proliferation of *P. piscicida* and *Cryptoperidiniopsis* sp. (but not of *G. galatheanum* or “Shepherd’s crook”) with populations raising between 6 and 10 days, and declining 4 to 5 days thereafter. In some experimental flasks, fish deaths occurred during or soon after dinoflagellate cell numbers were maximal. Multiple bacterial strains (some recognized fish pathogens), protozoa and possibly dinoflagellates carried by the experimental fish, colonized experimental and control flasks. Levels of ammonia, nitrites and nitrates increased gradually and after three weeks reached levels beyond acceptable limits for fish survival. We conclude that (a) fish deaths within approximately 14 days may be due to the proliferation of *Pfiesteria piscicida* or *Pfiesteria*-like dinoflagellates; (b) deaths between 14 and 21 days may be attributed to either the proliferation of toxic dinoflagellates, pathogenic bacteria and/or deteriorating water quality; and (c) deaths beyond a period of approximately three weeks can be attributed to deteriorated water quality [Supported by grants NIEHS 1PO1 ES09563, and ECOHAB NA860P0492]. * Contributed equally to this study.

L. 2. Further molecular characterization of **Perkinsus andrewsi** *and related isolates*

Wolf T. Pecher, José A. F. Robledo, Cathleen A. Coss, Gerardo R. Vasta.

Annual Meeting of the National Shellfisheries Association. Mystic, CT, April 14-18, 2002.

Abstract

We previously described ultrastructure, behaviour in culture, and the rRNA locus of a *Perkinsus* species isolated from the baltic clam *Macoma balthica*. The morphological characterization did not reveal features remarkable enough to clearly indicate that the isolate was a distinct *Perkinsus* species (Coss et al. 2001a). However, the degree of difference of all rRNA genes and intergenic regions examined was comparable to or greater than differences between accepted *Perkinsus* species, which lead to its designation as *P. andrewsi* n. sp. (Coss et al 2001a, b). Here we report further molecular characterization of *P. andrewsi* and the characterization of an additional isolate from the hard clam *Mercenaria mercenaria*. Based on the molecular characterization both isolates are closely related. Their relationship with other recognized *Perkinsus* species will be discussed. [Supported by Grant No. NA06RG0101-5 from ODRP, NOAA, through the Maryland Sea Grant College, to GRV].

References:

Coss, C. A., Robledo, J. A., Vasta, G. R. 2001a. J. Eukaryot. Microbiol. 48: 38-51.

Coss, C. A., Robledo, J. A., Ruiz, G. M., Vasta, G. R. 2001b. J. Eukaryot. Microbiol. 48: 52-61.

L. 3. The antioxidant system of Perkinsus marinus

Eric J. Schott, José A. F. Robledo, Wolf T. Pecher, Florence Okafor, Gerardo R. Vasta. Annual Meeting of the National Shellfisheries Association. Mystic, CT, April 14-18, 2002.

Abstract

Spread of the protistan parasite *Perkinsus marinus* within the oyster *Crassostrea virginica* is believed to be via engulfment and migration by phagocytic hemocytes. Because phagocytosis of killed *P. marinus* trophozoites elicits production of reactive oxygen intermediates (ROIs) by oyster hemocytes, but phagocytosis of live trophozoites does not, *P. marinus* appears to have mechanisms to prevent the accumulation or production of ROIs. We previously described two Fe-type SOD genes (*PmSOD1, PmSOD2*) from *P. marinus* and demonstrated the ability of their products to convert $O_2^{-}\cdot$ to H_2O_2. *P. marinus* is resistant to moderate levels of H_2O_2, suggesting that it also possesses a mechanism for H_2O_2 removal. However, significant efforts to detect *P. marinus* catalase activity and gene sequences have been unsuccessful. Instead, we have found that *P. marinus* trophozoites possess abundant ascorbate dependent peroxidase (APX) activity. We have partially purified *P. marinus* APX, which co-migrates with a 35 kD band on non-denaturing gels. Continuing genetic, biochemical, and cellular studies of *P. marinus* FeSODs and APX will contribute to further characterize the *P. marinus* antioxidant defense system. [Supported by

Grant No NA06RG0101-5 from ODRP, NOAA, through the Maryland Sea Grant, to GRV].

L. 4. Identification of a second rRNA gene unit in the Perkinsus andrewsi *genome*

Wolf T. Pecher, José A. F. Robledo, Cathleen A. Coss, Gerardo R. Vasta. The 10th International Congress of Parasitology Associations. Vancouver, BC, August 4-9, 2002.

Abstract

Our laboratory previously described ultrastructure, behaviour in culture, and the rRNA locus of a *Perkinsus* species isolated from the baltic clam *Macoma balthica* which lead to its designation as *P. andrewsi* n. sp. (Coss et al 2001a, b). Here we report the presence of an additional rRNA gene unit (rRNA-II) in *P. andrewsi*, distinct from that initially described (rRNA-I). DNA from *in vitro* propagated *P. andrewsi* was extracted using the DNeasy Tissue kit (QIAGEN, Valencia, California). The intergenic spacer (IGS) was amplified, cloned, and sequenced following Robledo et al. (1999). Partial IGS and the complete small subunit (SSU) were amplified using primers designed on the IGS sequence of *P. andrewsi* and universal primers for the SSU from Medlin et al. (1988). Except for the 5.8S-II, all regions of the rRNA-II gene unit exhibited sequence differences with those in rRNA-I. The *P. andrewsi* IGS-II is noteworthy because it also differs in length from the IGS-I and from those in other *Perkinsus* species. This is the first report for the presence of two distinct rRNA loci in a *Perkinsus* species.

From the taxonomic standpoint and for species designations based on rRNA genes, it is critical to consider the possible presence and structure of additional rRNA gene units (Supported by Grant No. NA06RG0101-5 from ODRP, NOAA, through the Maryland Sea Grant College, to GRV).

References:

Coss, C. A., Robledo, J. A., Vasta, G. R. 2001a. *J. Eukaryot. Microbiol.* 48: 38-51.

Coss, C. A., Robledo, J. A., Ruiz, G. M., Vasta, G. R. 2001b. *J. Eukaryot. Microbiol.* 48: 52-61.

Medlin, L., Elwood H. J., Stickel, S., Sogin, M. L. 1988. *Gene*. 71:491-9.

Robledo, J. A., Wright, A. C., Marsh, A. G., Vasta, G. R. 1999. *J. Parasitol.* 85: 650-6.

L. 5. Flask bioassay for ichthyocidal activity of Pfiesteria piscicida

Keiko Saito, Michael S. Quesenberry, Danara N. Krupatkina, José A. F. Robledo, Tomás Drgon, Wolf T. Pecher, Nuala O'Leary, Mohamed Alavi, Todd Miller, Rachel E. Schneider, Robert Belas, Jonathan R. Deeds, Alan R. Place, Yonathan Zohar and Gerardo R. Vasta. Xth International Conference on Harmful Algae, St Pete Beach, FL, USA, October 21-25, 2002.

Abstract

The dinoflagellate *P. piscicida* has been reported as the causative agent of fish lesions and deaths, and deleterious effects on human health. We designed a bioassay in which fish are exposed to dinoflagellate strains or environmental consortia (e.g. environmental water or sediment samples) in 750-mL culture flasks, and examined the relationships among dinoflagellate growth profiles, *P. piscicida* presence, and fish deaths. Assay development was accomplished with clonal cultures (*P. piscicida*, *Karlodinium micrum*,

Prorocentrum minimum, CCMP1828, -1829, and -1834) co-incubated with fish (*Cyprinodon variegatus*). Variables characterized included water quality (pH, dissolved oxygen, ammonia, nitrate and nitrite concentrations), the effect of the presence of fish on the growth and compositions of protist including dinoflagellate and bacterial populations, and time of fish death. This assay enables the assessment of acute effects of ichthyocidal dinoflagellates on fish during the first 10 days (Stage A) of the experimental course. Fish deaths during the subsequent 10 to 20 days (Stage B) may be attributed to the proliferation of ichthyocidal dinoflagellates, pathogenic bacteria and/or deteriorating water quality**,** whereas those beyond a period of approximately three weeks (Stage C) can be most certainly attributed to deteriorated water quality. Application of the flask assay to environmental samples [n=53] yielded fish deaths in all three stages. The majority of samples that resulted in fish death in stage A tested positive for *P. piscicida* by PCR. If implemented with cautious interpretation, this assay should prove useful in monitoring blooms for the presence of *P. piscicida* and other dinoflagellate species potentially harmful to fish. [Support: grants NIEHS 1PO1 ES09563 and ECOHAB NA860P0492]

L. 6. Application of genus-, species-, and strain-specific molecular probes to the detection of **Perkinsus** *spp. in the Atlantic coast of USA*

Wolf T. Pecher, José A. F. Robledo, Eric J. Schott, Gerardo R. Vasta.

International Conference on Shellfish Restoration. Charleston, SC, November 20-24, 2002.

Abstract

"Dermo" disease, caused by *P. marinus*, represents a major cause of mortality of the eastern oyster (*Crassostrea virginica*) along the Gulf and Atlantic coasts of the USA. Based on the application of the fluid thioglycolate medium assay (FTM) to natural and farmed oyster populations, the parasite has been reported to be expanding its distribution range to the northeastern regions, now reaching oyster populations in Maine. Unlike Dermo epizootics in the Gulf and Atlantic coasts, in some regions within the northeastern states infection prevalences as assessed by FTM may be surprisingly high, but does not necessarily correlate with oyster mortality. Although environmental factors strongly influence the outcome of *P. marinus* infections, a likely explanation of this observation is the presence of other *Perkinsus* species/strains that may be highly prevalent in the northeastern region, but exhibit little or no pathogenicity for *C. virginica*. Using species-specific molecular probes developed in our laboratory we are currently investigating the epizootiology of *Perkinsus* species along the East Coast (ME, MA, RI, CT, NY, NJ, DE, MD, VA) in oysters and other commercial bivalves such as hard clams, soft shell clams, and scallops, and

putative reservoirs for *Perkinsus* species, such as ribbed mussels. Samples are tested for *P. marinus* (Types I and II), *P. andrewsi*, and *P. atlanticus*. In addition, we are applying a *Perkinsus* "genus-specific" PCR-based assay that complements the species-specific assays, and should facilitate the detection of yet undescribed *Perkinsus* species in bivalves and the environment.

L. 7. Further molecular characterization of **Perkinsus andrewsi** *and a related isolate*

Wolf T. Pecher, José A. F. Robledo, Cathleen A. Coss, Gerardo R. Vasta.
International Conference on Shellfish Restoration. Charleston, SC, November 20-24, 2002.

Abstract

We previously described ultrastructure, behavior in culture, and the rRNA locus of a *Perkinsus* species isolated from the baltic clam *Macoma balthica*. The morphological characterization did not reveal features remarkable enough to clearly indicate whether the isolate was a distinct *Perkinsus* species. However, the degree of difference of rRNA genes and intergenic regions examined was comparable to or greater than differences between accepted *Perkinsus* species, which led to its designation as *P. andrewsi* n. sp. Here we report further molecular characterization of the holotype of *P. andrewsi* (ATCC 50807) and the characterization of an additional isolate from the hard clam *Mercenaria mercenaria*. In *P. andrewsi* an additional rRNA gene unit was found (rRNA-B) that is distinct from that initially described (rRNA-A). Except for the 5.8S-B, all

regions of the rRNA-B gene unit exhibited sequence differences from those in the rRNA-A gene unit. This is the first report of the presence of two distinct rRNA gene units in a *Perkinsus* species. In the *Perkinsus* isolate of *Mercenaria mercenaria* only one rRNA gene unit was found which is almost identical to the rRNA-B gene unit from *P. andrewsi.* The relationships of *P. andrewsi* and the *M. mercenaria* isolate to other recognized *Perkinsus* species will be discussed.

L. 8. The antioxidant pathway of Perkinsus marinus*: functional analysis and localization of two iron superoxide dismutases*

Eric J. Schott, José A. F. Robledo, Wolf T. Pecher, Florence Okafor, Gerardo R. Vasta. International Conference on Shellfish Restoration. Charleston, SC, November 20-24, 2002.

Abstract

The ecomomic and environmental impacts of *Perkinsus marinus* epizootics make imperative the understanding of this parasite's virulence mechanisms. It has been proposed that viable *P. marinus* trophozoites rapidly suppress or detoxify the reactive oxygen burst characteristic of oyster hemocytes. We now report that *in vitro* cultured *P. marinus* trophozoites are remarkably insensitive to transient exposure to superoxide and hydrogen peroxide (H_2O_2), but not hypochlorite. We cloned cDNAs of two *P. marinus* Fe-type superoxide dismutases (FeSODs) that have the potential to convert superoxide to H_2O_2 in vivo. Both cDNAs are able to complement SOD mutations in yeast or bacteria. Genomic sequences of PmSOD1 and PmSOD2 provide

insights into their relationship to each other and to FeSODs of other protistan parasites, and suggest differences in thier regulation. Recombinant FeSODs have been crystalized for structural determinations. Specific antisera raised against recombinant PmSOD1 and PmSOD2 show differential immunolocalization. The product of SOD activity is H_2O_2, which is cytotoxic and may react with iron to create the very reactive hydroxyl radical. Significant efforts to detect catalase in *P. marinus* by biochemical and molecular approaches were unsucessful; instead, we identified ascorbate-dependent peroxidase (APX) activity. The activity was purified over 1000-fold, and by electrophoretic analysis to be a 35 kDa protein. The unique characteristics of the *P. marinus* anitoxidant system may provide the basis for disease prevention or therapy strategies.

L. 9. Assessment of the epizootiology of Perkinsus spp. *at the Atlantic Coast of the USA using genus-, species-, and strain-specific molecular probes*

Wolf T. Pecher, Eric J. Schott, José A. F. Robledo, Gerardo R. Vasta. Annual Meeting of the National Shellfisheries Association, New Orleans, LA, April 13-17, 2003.

Abstract

P. marinus represents a major cause of mortality of the eastern oyster (*Crassostrea virginica*) along the Gulf of Mexico and Atlantic coasts of the USA. Based the fluid thioglycolate medium (FTM) assay, *Perkinsus* infections attributed to *P. marinus* have been reported as far north as Maine but although

infection prevalence in Northeast regions may be high, it may not correlate with oyster mortality. In addition to the influence of environmental factors, the presence of other *Perkinsus* species/strains that exhibit reduced pathogenicity for *C. virginica* may explain these observations. Two recently described species, *P. chesapeaki* and *P. andrewsi* that test positive by the FTM assay, can also be present in clams and oysters, but their virulence remains unknown. Thus, the accurate prevalence assessment of *Perkinsus* spp. is needed for the detailed understanding of epizootic events. To discriminate between *P. marinus*, *P. andrewsi* and other *Perkinsus* species our laboratory has developed species-specific PCR-based assays. We are applying these molecular probes to investigate the epizootiology of *Perkinsus* species and strains in oysters, hard clams, and other shellfish along the East Coast (from ME to VA). [Supported by ODRP, NOAA award NA06RG0101-5, through the MD Sea Grant College, to GRV].

L. 10. The antioxidant pathway of **Perkinsus marinus***: functional analysis and localization of two iron superoxide dismutases*

Eric J. Schott, José A. F. Robledo, Wolf T. Pecher, Florence Okafor, Gerardo R. Vasta. Annual Meeting of the National Shellfisheries Association, New Orleans, LA, April 13-17, 2003.

Abstract

The economic and environmental impacts of *Perkinsus marinus* epizootics make imperative the understanding of this parasite's virulence mechanisms. It

has been proposed that viable *P. marinus* trophozoites rapidly suppress or detoxify reactive the oxygen burst characteristic of oyster hemocytes. We now report that cultured *P. marinus* trophozoites are remarkably insensitive to transient exposure to superoxide and hydrogen peroxide (H_2O_2), but not hypochlorite. These findings are consistent with two observations: (1) Viable trophozoites are able to destroy hydrogen peroxide *in vitro*; (2) extracts of *P. marinus* contain abundant iron-type superoxide dismutase (FeSOD) activity, as well as ascorbate dependent peroxidase (APX) activity. We previously described the cloning and characterization of two *P. marinus* FeSODs that have the potential to convert superoxide to H_2O_2 in vivo. Recombinant PmSOD1 and PmSOD2 proteins have been crystallized for structural analyses, and used to raise specific antisera for immunolocalizations. The APX activity appears to be a 35 kD protein. Continuing analysis of *P. marinus* SOD and APX functions will be presented. The unique characteristics of the *P. marinus* antioxidant system may provide the basis for disease prevention or therapy strategies [Supported by ODRP, NOAA award NA06RG0101-5, through the MD Sea Grant College, to GRV].

L. 11. **Perkinsus andrewsi** ***has a second rRNA gene unit***

Wolf T. Pecher, José A. F. Robledo, Gerardo R. Vasta. 11th East Coast Protozoology Conference. University of Maryland, Baltimore County, Catonsville, MD, June 6-8, 2003.

Abstract

Perkinsus species are parasitic protozoa of mollusks, found worldwide. Our laboratory previously described ultrastructure, behavior in culture, and partial sequence of the rRNA locus of a *Perkinsus* species isolated from the baltic clam *Macoma balthica*, which led to its designation as a new species, *P. andrewsi* (Coss et al. 2001a, b). In this study, we identify an additional rRNA gene unit (rRNA-B) in *P. andrewsi*, and report the complete sequences of both rRNA gene units. DNA from *in vitro* propagated *P. andrewsi* was extracted using the DNeasy Tissue kit (QIAGEN). rRNA genes and intergenic regions were amplified by PCR, cloned, and sequenced. To assess the relative representation of the rRNA units, their intergenic spacers (IGSs) were PCR-amplified from serially diluted *P. andrewsi* genomic DNA and the intensity of the amplicons analyzed using the software NIH Image 1.62 (http://rsb.info.nih.gov/nih-image/). Both IGSs appeared to be equally amplified, suggesting similar representation of the rRNA units. Except for the 5.8S, all regions of the rRNA-B gene unit exhibited sequence differences with that initially described (rRNA-A). This is the first report for the presence of two distinct rRNA units in a *Perkinsus* species [Supported by ODRP, NOAA award NA06RG0101-5, through the MD Sea Grant

College].

References:

Coss, C. A., Robledo, J. A., Vasta, G. R. 2001a. *J. Eukaryot. Microbiol.* 48: 38-51.

Coss, C. A., Robledo, J. A., Ruiz, G. M., Vasta, G. R. 2001b. *J. Eukaryot. Microbiol.* 48: 52-61.

L. 12. The antioxidant system of **Perkinsus marinus**

Eric J. Schott, José A. F. Robledo, Wolf T. Pecher, Florence Okafor, Adelbar M. Silva, Gerardo R. Vasta. 11th East Coast Protozoology Conference. University of Maryland, Baltimore County, Catonsville, MD, June 6-8, 2003.

Abstract

Perkinsus marinus is a protistan parasite of the eastern oyster, *Crassostrea virginica*. Its taxonomic placement basal to the dinoflagellate lineage, and its ease of experimental manipulation, make it an attractive organism in which to study the mechanisms and evolution of intracellular survival. Phagocytosis of killed *P. marinus* trophozoites elicits production of reactive oxygen species (ROS) by oyster hemocytes, while phagocytosis of live trophozoites does not. Thus, *P. marinus* appears to prevent the production or accumulation of ROS. Exposure of cultured *P. marinus* trophozoites to various ROS reveals that the parasite is highly resistant to superoxide and H_2O_2, but susceptible to hypochlorite. We have characterized genes and activities for two *P. marinus* Fe-type superoxide dismutases that may convert superoxide to H_2O_2 *in vivo*. H_2O_2 is also deleterious to cell components, and typically removed by catalase activity. Efforts to detect catalase in *P. marinus* were unsuccessful; instead, we identified ascorbate-dependent peroxidase (APX) activity. APX

activity was enriched over 1000-fold by ion exchange and hydrophobic chromatography, and correlates with a 35 kD protein. Sequestration of iron from cultures of *P. marinus* resulted in a significant reduction of FeSOD and APX activities. [Supported by NOAA award NA06RG0101-5, through MD Sea Grant].

***L. 13. Antioxidant activities of the protistan parasite* Perkinsus marinus.**

Eric J. Schott, Wolf T. Pecher, Florence Okafor, José F. Robledo, Gerardo R. Vasta. Molecular Parasitology Meeting XIV. Marine Biological Laboratory, Woods Hole, MA, September 14-18, 2003.

Abstract

Perkinsus marinus, a facultative intracellular parasite of the eastern oyster, *Crassostrea virginica*, is responsible for devastation of oyster populations along the Atlantic and Gulf coasts of N. America. Both its taxonomic placement and ease of experimental manipulation make it an attractive organism in which to study mechanisms and evolution of intracellular survival. Although phagocytosis of killed *P. marinus* trophozoites elicits production of reactive oxygen species (ROS) by hemocytes, phagocytosis of live trophozoites does not. Thus, *P. marinus* appears to prevent the production or accumulation of ROS. We are investigating the role of parasite antioxidant enzymes in ROS suppression and intracellular survival. We previously characterized two *P. marinus* Fe-type superoxide dismutases that may convert superoxide to H_2O_2 in vivo. Molecular and immunofluorescence studies show that PmSOD1 is mitochondrially targeted; PmSOD2 may be targeted to another subcellular compartment (Schott and Vasta

2003, MBP 126:81). While H_2O_2 is membrane permeable and thus potentially deleterious to cell components, exposure of *P. marinus* trophozoites to various ROS reveals that the parasite is highly resistant to both superoxide and H_2O_2, but susceptible to hypochlorite. Live trophozoites and *P. marinus* extracts are able to remove H_2O_2 from solution. Although efforts to detect *P. marinus* catalase activity or gene sequences were unsuccessful, we identified abundant ascorbate-dependent peroxidase (APX) activity. APX activity was enriched over 1000-fold, and correlates with a 35 kDa protein by SDS-PAGE. Recently, we identified ESTs for thioredoxin and peroxiredoxin, which may also participate in ROS resistance. Efforts to define the roles of these antioxidants in ROS removal and intracellular survival are underway (Supported by Grant NA06RG0101-5 from ODRP, NOAA, through Maryland Sea Grant).

L. 14. Assessment of the distribution of **Perkinsus** *spp. along the Mid and North Atlanic Coast using molecular probes*

Wolf T. Pecher, Mohammad Alavi, Eric J. Schott, José A. F. Robledo, Laura Roth, Sean T. Berg, Harmony A. Hancock, Gerardo R. Vasta. 79th Annual meeting of the American Society of Parasitologists. Philadelphia, PA, July 24-28, 2004.

Abstract

Perkinsus spp. are facultative intracellular protistan parasite of molluscs. Three species (*P. marinus*, *P. chesapeaki* and *P. andrewsi*) are sympatric in Chesapeake Bay, infecting both oysters and clams. *P. marinus* is a major cause

of mortality of oysters (*Crassostrea virginica*) along the Gulf and Atlantic coasts of the USA. However, the pathogenicity of *P. andrewsi* and *P. chesapeaki* for oysters and their host types (*Macoma balthica* and *Mya arenaria*, respectively) is unknown. Based on the application of a diagnostic assay for *Perkinsus* spp. that lacks species-specificity, infections attributed to *P. marinus* have been reported in *C. virginica* as far north as Maine. But high infection prevalences observed have not correlated with expected oyster mortalities which may be explained by the presence of other *Perkinsus* spp./strains with little or no pathogenicity for *C. virginica*. Species-specific PCR-based diagnostic assays for *P. marinus*, *P. andrewsi* and other *Perkinsus* spp. developed in our laboratory were used to examine the distribution of *Perkinsus* spp. along the coast from Virginia to Nova Scotia in *C. virginica*, *Mercenaria mercenaria*, *Argopecten irradians* and *Geukensia* spp. collected monthly from June to November 2002. DNA was extracted from selected tissues and tested for the presence of *Perkinsus* spp. Preliminary results revealed that *P. marinus* and *P. andrewsi* are present in *C. virginica* from Virginia to Massachusetts. Furthermore, *P. marinus* and a yet unidentified *Perkinsus* sp./strain are found in oysters as far north as Maine. *P. marinus* is the most prevalent *Perkinsus* spp. in oysters, suggesting that either environmental conditions in the region surveyed favor *P. marinus* infections, or that the oyster is a less suitable host for other sympatric *Perkinsus* spp./strains. (Supported by Grant NA90A06RG0101-5 from NOAA through the MD Sea Grant College).

L. 15. Ascorbate-dependent peroxidase activity in the alveolate parasite, **Perkinsus marinus**

Eric J. Schott, Wolf T. Pecher, José A. F. Robledo, Gerardo R. Vasta. 79th Annual meeting of the American Society of Parasitologists. Philadelphia, PA, July 24-28, 2004.

Abstract

Perkinsus marinus, a protistan parasite of the eastern oyster, *Crassostrea virginica*, has become and attractive organism for the study of intracellular survival. Advantages include the propagation of clonal lines in fully defined medium, lack of pathogenicity to man, and ongoing gemonic initiatives. The early divergence of the Perkinsozoa from the dinoflagellate/apicomplexan branch of the Alveolata has added to interest in this recently established phylum. Viable *P. marinus* trophozoites are engulfed by oyster hemocytes, but suppress chemiluminescent responses normally associated with phagocytic activity, leading to the hypothesis that the parasite prevents reactive oxygen species (ROS) accumulation by enzymatic means. A manifestation of efficient ROS removal should be resistance to exogenous ROS. *In vitro*, cultured trophozoites are highly resistant to superoxide and hydrogen peroxide, but susceptible to hypochlorite. *P. marinus* trophozoites express at least two superoxide dismutases, but lack detectable catalase activity. Instead, they possess an ascorbate dependent peroxidase activity (APX) that may contribute to hydrogen peroxide removal *in vitro* and *in vivo*. Partial purification of *P. marinus* APX revealed a 35 kDa protein on SDS-PAGE, which, upon sequence analysis of

tryptic peptides, confirmed similarities to plant APX. Degenerate oligonucleotides designed on peptide sequences amplified two putative APX genes (*PmAPX1* and *PmAPX2*) from *P. marinus* cDNA. PmAPX1 and PmAPX2 differ greatly in their N-terminal regions, and appear to be encoded by single, unlinked genes. Rabbit antiserum raised against a synthetic peptide corresponding to PmAPX1 detects a protein of approximately 35 kDa on Western blots of soluble *P. marinus* extracts, and binds to recombinant PmAPX1. The molecular and serological tools developed for PmAPX1 and PmAPX2 will be used to study the expression of PmAPX during the host-phagocyte interaction (Supported by NOAA/Maryland Sea Grant award SA7528068-H; PI: Vasta, http://www.umbi.umd.edu/%7Ecomb/faculty/vasta/vasta.html.)

L. 16. Isolation and partial characterization of selected phosphatases of the protistan parasite Perkinsus marinus

Wolf T. Pecher, Gerardo R. Vasta. Molecular Parasitology Meeting XV. Marine Biological Laboratory, Woods Hole, MA, September 19-23, 2004.

Abstract

Perkinsus marinus is a facultative intracellular protistan parasite of the eastern oyster *Crassostrea virginica*, currently placed within the Perkinsozoa, a newly established phylum basal to the Dinozoa. *P. marinus* trophozoites are readily phagocytosed by oyster hemocytes, where they survive intracellular killing and proliferate. Upon phagocytosis, *P. marinus* suppresses the oxidative burst normally observed in oyster hemocytes, suggesting that it either scavenges

reactive oxygen intermediates (ROIs) or inhibits their production. Among other enzymes, phosphatases have been proposed to inhibit ROI production. Phosphatases are a heterologous group of enzymes involved in a variety of biological processes, including the modulation of host/pathogen interactions. *P. marinus* releases an acid phosphatase (ACP) activity that has been considered as a potential virulence factor. Here we report its partial biochemical characterization. At its optimum pH of 4.5, it is sensitive to Na-fluoride but resistant to okadaic acid and EDTA, suggesting that it may have a protein phosphatase 2B-like activity. In addition to the ACP activity, we identified several putative phosphatase sequences from *P. marinus* ESTs obtained from an ongoing functional genomics project. Based on these EST sequences, we have isolated and initiated the characterization of five putative phosphatase genes that in other systems have been proposed to be involved in host/pathogen interactions: a phosphotyrosine phosphatase, a protein phosphatase 2B, two protein phosphatase 2C, and a dual specificity phosphatase-like gene. Availability of the gene and mRNA sequences, and expression of recombinant proteins will enable the functional analysis of these phosphatases, including their potential involvement in intracellular survival of *P. marinus*. (Supported by Grant SA7528068-H from ODRP, NOAA through the MD Sea Grant College to GRV).

L. 17. *Ascorbate peroxidase and the antioxidant repertoir of* Perkinsus marinus

Eric J. Schott, Wolf T. Pecher, José A. F. Robledo, Gerardo R. Vasta. Molecular Parasitology Meeting XV. Marine Biological Laboratory, Woods Hole, MA, September 19-23, 2004.

Abstract

Perkinsus marinus, a parasite of the eastern oyster, *Crassostrea virginica*, is an increasingly useful organism for the study of mechanisms of intracellular survival. Major advantages include the ability to propagate clonal lines in defined medium, and rapidly developing genomic resources. Placement of the Perkinsozoa within the dinoflagellate/apicomplexan branch of the Alveolata adds to interest in this recently established phylum. Viable *P. marinus* trophozoites are engulfed by oyster hemocytes, but suppress chemiluminescence normally associated with phagocytic activity, leading to the hypothesis that the parasite prevents reactive oxygen species (ROS) accumulation by enzymatic means. A manifestation of efficient ROS removal should be resistance to exogenous ROS. *In vitro*, cultured trophozoites are resistant to superoxide and hydrogen peroxide, but susceptible to hypochlorite. *P. marinus* trophozoites express two superoxide dismutases, but lack detectable catalase activity. Instead, they possess an ascorbate dependent peroxidase activity (APX) that may contribute to hydrogen peroxide removal *in vitro* and in vivo. Partial purification of *P. marinus* APX revealed a 35 kDa protein on SDS-PAGE, which, upon partial sequence analysis, confirmed similarity to plant APX. Oligonucleotides designed on peptide

sequences amplified two putative APX genes (PmAPX1 and PmAPX1) from *P. marinus* cDNA. PmAPX1 and PmAPX2 differ in their predicted N-terminal regions, and appear to be encoded by single, unlinked genes. Rabbit antiserum raised against a peptide corresponding to PmAPX1 reacts with a ~35 kDa band on Western blots of soluble *P. marinus* extracts, and with recombinant PmAPX1 and PmAPX2. The molecular and serological tools developed for PmAPX1 and PmAPX2 will be used to study the expression of PmAPX during the host-phagocyte interaction (Supported by NOAA/Maryland Sea Grant).

L. 18. Perkinsus *spp. and* Bonamia *spp. infections in the asian oyster (*Crassostrea ariakensis*) maintained in a fully contained aquaculture setting*

Gerardo R. Vasta, Mohammad Alavi, Eric J. Schott, Keiko Saito, Satoshi Tasumi, Wolf T. Pecher. Annual Meeting of the National Shellfisheries Association. Philadelphia, PA, April 11-14, 2005.

Abstract

To assess the potential for the Asian oyster *Crassostrea ariakensis* to harbor oyster parasites, we maintained oysters in a fully contained aquaculture facility and monitored prevalence of *Perkinsus marinus, P. andrewsi*, *P. atlanticus*, *Haplosporidium nelsoni* (MSX) and *Bonamia ostrae* for 15 weeks. Detection of each parasite was by specific PCR. Two-year old triploid *C. ariakensis* originated in hatcheries employing Chesapeake Bay waters endemic for *P. marinus* and potentially other oyster pathogens. At the beginning of the

experiment, *Perkinsus* spp., *B. ostrae*, and MSX prevalences were 66%, 3%, and 0% respectively. After 11 weeks, *C. ariakensis* displayed *Perkinsus* spp. at greater than 80% prevalence. *B. ostrae* prevalence remained below 10%. Cumulative oyster mortality was 18%. Semi-quantitative PCR indicated that 100% of the dead animals were infected, and on average contained higher levels of *Perkinsus* spp. than did infected live animals. Water from tanks in which animals were maintained was also PCR-positive for the presence of *Perkinsus* spp. To examine the capacity of *C. ariakensis* hemocytes or plasma to kill or inhibit proliferation of *Perkinsus* spp., *P. marinus* trophozoites were exposed *in vitro* to oyster hemocytes or plasma for 24 and 72 hours, and parasite survival was assessed by measuring proliferation in nutrient medium for four weeks. Results suggest that although longer exposure time increases killing of *P. marinus* by *C. ariakensis* hemocytes, a fraction of *P. marinus* trophozoites survive, and proliferate when transferred to culture medium, even at 16:1 hemocyte to parasite ratios.

L. 19. Perkinsus *spp. and* Bonamia *spp. infections in* Crassostrea ariakensis *maintained in a fully contained aquaculture setting*

Eric J. Schott, José A. F. Robledo, Mohammad R. Alavi, Keiko Saito, Satoshi Tasumi, Wolf T. Pecher**,** Gerardo R. Vasta. Annual Meeting of the National Shellfisheries Association. Philadelphia, PA, April 11-14, 2005.

Abstract

In the Chesapeake Bay, diseases caused by *Perkinsus marinus* and *Haplosporidium nelsonii* (Dermo and MSX, respectively) have contributed to drastic declines of populations the native oyster, *Crassostrea virginica*. The Asian oyster, *C. ariakensis*, which grows readily to market size in Dermo-endemic Chesapeake Bay waters, is being considered for introduction to restore oyster populations. While apparently tolerant to *P. marinus*, *C. ariakensis* exposed to Bay waters may reach prevalences of up to 80%, raising the possibility that it could serve as a reservoir in which the parasite could increase its virulence. A crucial question to address is whether *P. marinus* can be transmitted from *C. ariakensis* to naïve *C. virginica*. We conducted cohabitation experiments with *Perkinsus*-infected *C. ariakensis* and *Perkinsus*-free *C. virginica*. The prevalence of *Perkinsus* infection in both 'donor' and potential 'recipient' populations was assessed at 2 and 4 weeks by PCR-based methods. After four weeks, *Perkinsus* was present in *C. virginica*. A potential protozoal disease of *C. ariakensis*, *Bonamia ostraea*, has been associated with mortalities of experimental populations of *C. ariakensis* in Pamlico Sound of NC. The

potential exists for *Bonamia* sp. to be present in Chesapeake Bay. We previously detected PCR amplicons indicative of *Bonamia* spp. in *C. ariakensis* reared in the Chesapeake Bay.We are conducting cohabitation studies in which *B. ostreae*-infected *Ostraea edulis* are co-cultured with *C. ariakensis* for four weeks. We are currently in the process of analyzing possible transmission using PCR-based methodologies.

L. 20. Application of molecular probes to assess the distribution of Perkinsus *spp. in the eastern oyster* Crassostrea virginica *along the Mid- and North Atlanic Coast of North America*

Wolf T. Pecher, Mohammad Alavi, Eric J. Schott, José A. F. Robledo, Gerardo R. Vasta. International Marine Biotechnology Conference. St John's, Newfoundland & Labrador, Canada, June 7-12, 2005.

Abstract

Perkinsus spp. are facultative intracellular protistan parasites of mollusks. In Chesapeake Bay, USA, three species, *P. marinus*, *P. chesapeaki* and *P. andrewsi*, are sympatric, infecting oysters and clams. In addition, unidentified *Perkinsus* spp. may be present. Although *P. marinus* is a recognized pathogen for the oyster *Crassostrea virginica*, it remains unknown whether *P. andrewsi* or *P. chesapeaki* are pathogenic. Our laboratory has developed specific PCR-based diagnostic assays for *P. marinus*, and *P. andrewsi,* and a “generic” PCR test designed to detect all *Perkinsus* spp. Infections with *Perkinsus* spp. have been observed in *C. virginica* as far north as Maine, USA, sometimes with high

prevalences but low mortalities. Thus, we hypothesized that in addition to *P. marinus*, *Perkinsus* spp./strains with little or no pathogenicity for *C. virginica* may be present in this region. Accordingly, we investigated the distribution of *Perkinsus* spp. infections in *C. virginica* along the Mid- and North Atlantic Coast of North America (from Nova Scotia to Virginia) applying the PCR-based assays. *P. marinus* and *P. andrewsi* were detected as far north as Maine. *P. marinus* appears to be most abundant, suggesting that it is either the dominant species in the study area, or, alternatively, that oysters are not suitable hosts for *P. andrewsi*. However, a considerable number of oysters tested positive with the generic PCR assay, but negative for *P. marinus* and *P. andrewsi*, suggesting that a yet undescribed *Perkinsus* sp. or strain is present in this area. (Supported by Grant SA7528068-H from ODRP, NOAA to GRV).

L. 21. Characterization of two putative protein phosphatase genes of the protistan oyster parasite Perkinsus marinus

Wolf T. Pecher, Gerardo R. Vasta. Molecular Parasitology Meeting XVI. Marine Biological Laboratory, Woods Hole, MA, September 11-15, 2005.

Abstract

Perkinsus marinus is a protistan parasite of the eastern oyster *Crassostrea virginica*. Infections are initiated by phagocytosis of *P. marinus* by oyster hemocytes. Upon phagocytosis *P. marinus* actively suppresses the oxidative burst normally observed during phagocytosis of yeast or heat-killed *P. marinus*, possibly by interfering with the production of reactive oxygen intermediates through parasite-

derived enzymes, such as phosphatases. Previously, we reported on the partial purification and biochemical characterization of an extracellular phosphatase activity and on the identification and partial characterization of several phosphatase-like genes from *P. marinus*, including a protein phosphatase (PP) 2B- and two PP2C-like genes. Since then, we further characterized the PP2B- and one of the PP2C-like genes that were designated as *PmPP2B* and *PmPP2C2*, respectively. *PmPP2B* is organized in 18 exons. Based on computational translation, the 1,560 bp long orf of *PmPP2B* encodes a 520 amino acid long protein with a predicted pI of 6.39 and a calculated molecular weight of 59 kDA. *PmPP2C2* has 11 exons. The orf of *PmPP2C2* is 1,344 bp long and encodes a putative 448 amino acid long protein with a predicted pI of 7.99 and a calculated molecular weight of 49 kDa. Interestingly, computational analysis of PmPP2C2 suggests the presence of a 19 amino acid long signal peptide. PmPP2C2 shows moderate and PmPP2B high percent similarities to putative protein phosphatases and hypothetical proteins of Apicomplexans. Recombinant expression of both putative phosphatase gene products is underway, which, together with the availability of the gene and mRNA sequences, will enable their functional analysis, including their potential involvement in intracellular survival of *P. marinus*. (Supported by Grant SA7528068-I from MD Sea Grant/NOAA to GRV).

L. 22. Characterization of a protein phosphatase 2C-like gene of Perkinsus marinus*, a protistan parasite of the eastern oyster,* Crassostrea virginica

Wolf T. Pecher, Gerardo R. Vasta. Molecular Parasitology Meeting XVII. Marine Biological Laboratory, Woods Hole, MA, September 10-14, 2006.

Abstract

Perkinsus marinus is a protistan parasite of the eastern oyster *Crassostrea virginica*. Infections are initiated by phagocytosis of *P. marinus* trophozoites by oyster hemocytes. Upon phagocytosis *P. marinus* actively suppresses the oxidative burst normally observed during phagocytosis of yeast or heat-killed *P. marinus*, possibly by interfering with the production of reactive oxygen species through parasite-derived enzymes, such as phosphatases. Previously, we reported on the partial purification and biochemical characterization of an extracellular phosphatase activity, and on the identification and partial characterization of several phosphatase-like genes from *P. marinus*, including two PP2C-like genes. Further characterization of the PP2C-like gene designated as *PmPP2C2* showed that it comprises 11 exons. The 1,344 bp long ORF encodes a putative 448 amino acid (aa) long protein of a calculated molecular weight of 49 kDa and predicted pI of 7.99, that has sequence similarities to hypothetical proteins of apicomplexans. Computational analysis suggests the presence of a 19 aa signal sequence. Preliminary Western blot analysis of cell extracts of the *P. marinus* strains CB5D4 and TxSc with polyclonal antibodies raised against recombinant PmPP2C2 revealed three major

bands (approximately 57 kDa, 49 kDa, and 43 kDa). Our data suggest differences in the band intensities between the strains. In *P. marinus* CB5D4, a recently isolated strain, the 49 kDa band has highest intensity, whereas in *P. marinus* TxSc, a strain that has been in culture for more than one decade, the intensity of the 57 kDa band is strongest. Functional analysis of the PmPP2C2-like gene including its potential involvement in intracellular survival of *P. marinus* is underway (Supported by Grants from MD Sea Grant/NOAA and NSF to GRV).

L. 23. Assessment of the distribution of Perkinsus *species in the eastern oyster* Crassostrea virginica *and hard clam* Mercenaria mercenaria *along the Mid- and North Atlantic-coast of North America using molecular probes*

Wolf T. Pecher, Mohammad R. Alavi, José A. F. Robledo, Eric J. Schott, Laura Roth, Sean T. Berg, Harmony A. Hancock, Gerardo R. Vasta. Aquaculture 2007. San Antonio, TX, February 26 - March 2, 2007.

Abstract

Perkinsus species are protistan endoparasites of mollusks. In Chesapeake Bay, *P. marinus*, *P. chesapeaki* and *P. andrewsi* are sympatric, infecting oysters and clams. In addition, unidentified *Perkinsus* species may be present. Although *P. marinus* is a recognized pathogen for the oyster *Crassostrea virginica*, it remains unknown whether *P. andrewsi* and *P. chesapeaki* are pathogenic. Infections with *Perkinsus* species have been observed in *C. virginica* as far north as Maine, USA, sometimes with high

prevalences but low mortalities. Thus, we hypothesized that in addition to *P. marinus*, *Perkinsus* species and/or strains with little or no pathogenicity for *C. virginica* may be present in this region. Our laboratory has developed specific PCR-based diagnostic assays for *P. marinus*, *P. andrewsi*, a *Perkinsus* species isolated from the hard clam *M. mercenaria*, and a "generic" PCR test designed to detect all *Perkinsus* species. Applying the PCR-based assays, we investigated the distribution of *Perkinsus* species infections in *C. virginica* collected monthly from June to September 2002 from seven sites along the Mid- and North-Atlantic Coast of North America (from Nova Scotia to Virginia). A total of 733 specimen were tested. *P. marinus* and *P. andrewsi* were detected as far north as Maine. *P. marinus* appeared to be the most abundant species. Furthermore, we evaluated the presence of the *Perkinsus* species in 225 *M. mercenaria*, collected over the same time period at three sites (from Maine to Virginia). However, we observed inhibition of PCR amplification in clam DNA samples. The addition of bovine serum albumin (at a final concentration of 1mg/ml) and 5% (v/v) dimethyl sulfoxide to the PCR reaction mixture and the use of a specialty DNA polymerase (*TaKaRa Ex Taq*TM, TaKaRa Bio Inc., Otsu, Shiga, Japan) removed the inhibitory effects of most DNA extracts. Using the modified PCR protocol *Perkinsus* infections were detected in *M. mercenaria* as far north as Maine. Compared to *C. virginica*, prevalences of *P. marinus* were significantly lower, suggesting that *M. mercenaria* is not an optimal host for *P. marinus*. Interestingly, in both hosts a number of individuals tested positive with the "generic" assay but negative with any of the species-specific assays used,

possibly suggesting the presence of additional *Perkinsus* species or strains not yet identified.

The study was supported by grants from ODRP, NOAA through the MD Sea Grant College to G. R. Vasta.

BIBLIOGRAPHY

Abollo, E., Casas, S. M., Ceschia, G. and Villalba, A. (2006). Differential diagnosis of *Perkinsus* species by polymerase chain reaction-restriction fragment length polymorphism assay. *Mol Cell Probes* **20**: 323-9.

Adak, S. and Datta, A. K. (2005). *Leishmania major* encodes an unusual peroxidase that is a close homologue of plant ascorbate peroxidase: a novel role of the transmembrane domain. *Biochem J* **390**: 465-74.

Adema, C. M., van der Knapp, M. P. W. and Sminia, T. (1991). Molluscan hemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates. *Rev Acquat Sci* **4**: 201-23.

Aebi, H. (1984). Catalase *in vitro*. *Methods Enzymol* **105**: 121-6.

Aguirre-García, M. M., Cerbon, J. and Talamas-Rohana, P. (2000). Purification and properties of an acid phosphatase from *Entamoeba histolytica* HM-1:IMSS. *Int J Parasitol* **30**: 585-91.

Ahmed, H., Schott, E. J., Gauthier, J. D. and Vasta, G. R. (2003). Superoxide dismutases from the oyster parasite *Perkinsus marinus:* purification, biochemical characterization, and development of a plate microassay for activity. *Anal Biochem* **318**: 132-41.

Alcorn, T. M., Zheng, H. Y., Gunther, M. R., Hassett, D. J. and Cohen, M. S. (1994). Variation in hydrogen peroxide sensitivity between different strains of *Neisseria gonorrhoeae* is dependent on factors in addition to catalase activity. *Infect Immun* **62**: 2136 - 40.

Allam, B. and Ford, S. E. (2002). Transport of particles across epithelia from oyster mantle cavity: a model for *Perkinsus marinus* invasion. *Abstracts of Natl Shellfish Assoc 13 Mystic, CT April 14-18, 2002*.

Allen, S. L., Misch, M. S. and Morrison, B. M. (1963). Variations in the electrophoretically separated acid phosphatases of *Tetrahymena*. *J Histochem Cytochem* **11**: 706-19.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**: 403-10.

Amako, K., Chen, G.-X. and Asada, K. (1994). Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. *Plant Cell Physiol* **35**: 497-504.

Andersen, M. R. and Omiecinski, C. J. (1992). Direct extraction of bacterial plasmids from food for polymerase chain reaction amplification. *Appl Environ Microbiol* **58**: 4080-2.

Anderson, R. A. (2001). A comparative study of anti-*Perkinsus marinus* activity in bivalve sera. *J Shellfish Res* **20**: 1011-7.

Anderson, R. S. (1996). Interaction of *Perkinsus marinus* with humoral factors and hemocytes of *Crassostrea virginica*. *J Shellfish Res* **15**: 127-34.

Anderson, R. S. (1999). *Perkinsus marinus* secretory products modulate superoxide anion production by oyster (*Crassostrea virginica*) haemocytes. *Fish Shellfish Immunol* **9**: 51-60.

Anderson, R. S., Paynter, K. T. and Burreson, E. B. (1992). Increased reactive oxygen intermediate production by hemocytes withdrawn from *Crassostrea virginica* infected with *Perkinsus marinus*. *Biol Bull* **183**: 476-81.

Anderson, R. S., Burreson, E. M. and Paynter, K. T. (1995). Defense responses of hemocytes withdrawn from *Crassostrea virginica* infected with *Perkinsus marinus*. *Biol Bull* **183**: 476-81.

Anderson, R. S., Brubacher, L. L., Calvo, L. M., Burreson, E. M. and Unger, M. A. (1997). Effect of *in vitro* exposure to tributyltin on generation of oxygen metabolites by oyster hemocytes. *Environ Res* **74**: 84-90.

Andrews, J. D. (1955). Notes on fungus parasites of bivalve mollusks in Chesapeak Bay. *Proc Natl Shellfish Assoc* **45**: 157-63.

Andrews, J. D. (1988). Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effect on the oyster industry. *Am Fish Soc Spec Publ* **18**: 47-63.

Andrews, J. D. (1996). History of *Perkinsus marinus*, a pathogen of oysters in Chesapeake Bay 1950 -1984. *J Shellfish Res* **15**: 13-6.

Andrews, J. D. and Hewatt, W. G. (1957). Oyster mortality studies in Virginia II. The fungus disease caused by *Dermocystidium marinum* in oysters of Chesapeake Bay. *Ecol Monographs* **27**: 1-25.

Andrews, J. D. and Ray, S. M. (1988). Management strategies to control the disease caused by *Perkinsus marinus*. *Am Fish Soc Spec Publ* **18**: 257-64.

Archambaud, C., Gouin, E., Pizarro-Cerda, J., Cossart, P. and Dussurget, O. (2005). Translation elongation factor EF-Tu is a target for Stp, a serine-threonine phosphatase involved in virulence of *Listeria monocytogenes*. *Mol Microbiol* **56**: 383-96.

Asojo, O. A., Schott, E. J., Vasta, G. R. and Silva, A. M. (2006). Structures of PmSOD1 and PmSOD2, two superoxide dismutases from the protozoan parasite *Perkinsus marinus*. *Acta Crystallograph Sect F Struct Biol Cryst Commun* **62**: 1072-5.

Atmar, R. L., Metcalf, T. G., Neill, F. H. and Estes, M. K. (1993). Detection of enteric viruses in oysters by using the polymerase chain reaction. *Appl Environ Microbiol* **59**: 631-5.

Azevedo, C. (1989). Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsea) parasite of the clam *Ruditapes decussatus* from Portugal. *J Parasitol* **75**: 627-35.

Barford, D., Das, A. K. and Egloff, M.-P. (1998). The structure and mechanism of protein phosphatases: insights into catalysis and regulation. *Annu Rev Biophys Biomol Struct* **27**: 133-64.

Beaven, A. E. and Paynter, K. T. (1999). Acidification of the phagosome in *Crassostrea virginica* hemocytes following engulfment of zymosan. *Biol Bull* **196**: 26-33.

Bendtsen, J. D., Nielsen, H., von Heijne, G. and Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* **340**: 783-95.

Birnboim, H. C. and Sandhu, J. (1997). Levels of DNA strand breaks and superoxide in phorbol ester-treated human granulocytes. *J Cell Biochem* **66**: 219-28.

Bishop, R., Gobright, E., Spooner, P., Allsopp, B., Sohanpal, B. and Collins, N. (2000). Microsequencence heterogeneity and expression of the LSU rRNA genes within the two copy ribosomal transcription units of *Theileria parva*. *Gene* **257**: 299-305.

Blackbourn, J., Bower, S. M. and Meyer, G. R. (1998). *Perkinsus qugwadi* sp. nov. (insertae sedis), a pathogenic protozoan parasite of the Japanese scallops, *Patinopecten yessoensis*, cultured in British Columbia, Canada. *Can J Zool* **76**: 942-53.

Bortolussi, R., Vandenbroucke-Grauls, C. M., van Asbeck, B. S. and Verhoef, J. (1987). Relationship of bacterial growth phase to killing of *Listeria monocytogenes* by oxidative agents generated by neutrophils and enzyme systems. *Infect Immun* **55**: 3197-203.

Boveris, A., Sies, H., Martino, E. E., Docampo, R., Turrens, J. F. and Stoppani, A. O. (1980). Deficient metabolic utilization of hydrogen peroxide in *Trypanosoma cruzi*. *Biochem J* **188**: 643-8.

Bramble, L. H. and Anderson, R. S. (1998). A comparison of the chemiluminescent responses of *Crassostrea virginica* and *Morone saxatilis* phagocytes to zymosan and viable *Listonella anguillarum*. *Dev Comp Immunol* **22**: 55-61.

Brawn, K. and Fridovich, I. (1981). DNA strand scission by enzymically generated oxygen radicals. *Arch Biochem Biophys* **206**: 414 - 9.

Brown, G. D. and Reece, K. S. (2003). Isolation and characterization of serine protease gene(s) from Perkinsus marinus. *Dis Aquat Org* **57**: 117-26.

Brown, T. (1993). Hybridization analysis of DNA blots. In *Current protocols in molecular biology*, pp. 2.10.1-2..6. Edited by F. A. Ausubel, R. Brent, R. E. Kingston, Moore, J. G. Seidman, J. A. Smith and K. Struhl. New York: John Wiley, Sons.

Brugerolle, G. (2002). *Cryptophagus subtilis*: a new parasite of cryptophytes affiliated with the Perkinsozoa lineage. *Eur J Protistol* **37**: 379-90.

Burreson, E. M. and Ragone Calvo, L. M. (1996). Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. *J Shellfish Res* **15**: 17-34.

Burreson, E. M., Reece, K. S. and Dungan, C. F. (2005). Molecular, morphological, and experimental evidence support the synonymy of *Perkinsus chesapeaki* and *Perkinsus andrewsi*. *J Eukaryot Microbiol* **52**: 258-70.

Burreson, E. M., Alvarez, R. S., Martinez, V. V. and Macedo, L. A. (1994). *Perkinsus marinus* (Apicomplexa) as a potential source of oyster *Crassostrea virginica* mortality in coastal lagoons of Tabasco, Mexico. *Dis Aquat Org* **20**: 77-82.

Bushek, D., Dungan, C. F. and Lewitus, A. J. (2002). Serological affinities of the oyster pathogen *Perkinsus marinus* (Apicomplexa) with some dinoflagellates (Dinophyceae). *J Eukaryot Microbiol* **49**: 11-6.

Carlton, J. M., Angiuoli, S. V., Suh, B. B., Kooij, T. W., Pertea, M., Silva, J. C., Ermolaeva, M. D., Allen, J. E., Selengut, J. D., Koo, H. L., Peterson, J. D., Pop, M., Kosack, D. S., Shumway, M. F., Bidwell, S. L., Shallom, S. J., van Aken, S. E., Riedmuller, S. B., Feldblyum, T. V., Cho, J. K., Quackenbush, J., Sedegah, M., Shoaibi, A., Cummings, L. M., Florens, L., Yates, J. R., Raine, J. D., Sinden, R. E., Harris, M. A., Cunningham, D. A., Preiser, P. R., Bergman, L. W., Vaidya, A. B., van Lin, L. H., Janse, C. J., Waters, A. P., Smith, H. O., White, O. R., Salzberg, S. L., Venter, J. C., Fraser, C. M., Hoffman, S. L., Gardner, M. J. and Carucci, D. J. (2002). Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* **419**: 512-9.

Casas, S. M., Grau, A., Reece, K. S., Apakupakul, K., Azevedo, C. and Villalba, A. (2004). *Perkinsus mediterraneus* n. sp., a protistan parasite of the European flat oyster *Ostrea edulis* from the Balearic Islands, Mediterranean Sea. *Dis Aquat Org* **58**: 231-44.

Charlet, M., Chernysh, S., Phillipe, H., Hetru, C., Hoffmann, J. A. and Bulet, P. (1996). Innate immunity: isolation of several cystein-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. *J Biol Chem* **271**: 21808-13.

Cheng, T. C. (1975). Functional morphology and biochemistry of molluscan phagocytes. *Ann N Y Acad Sci* **266**: 343-79.

Choi, K. S., Lewis, D. H., Powell, E. N., Frelier, P. F. and Ray, S. M. (1991). A polyclonal antibody developed from *Perkinsus marinus* hypnospores fails to cross react with other life stages of *P. marinus* in oyster (*Crassostrea virginica*) tissues. *J Shellfish Res* **10**: 411-5.

Chu, F. L. and La Peyre, J. F. (1993a). *Perkinsus marinus* susceptibility and defense-related activities in eastern oysters *Crassostrea virginica*: temperature effects. *Dis Aquat Org* **16**: 223-34.

Chu, F. L., La Peyre, J. F. and Burreson, C. S. (1993). *Perkinsus marinus* infection and potential defense-related activities in eastern oysters, *Crassostrea virginica*: salinity effects. *J Invertebr Pathol* **62**: 226-32.

Chu, F. L., Volety, A. K. and Armknecht, S. (1998a). Antioxidant enzymes, potential virulent factors, in different strains of the oyster protozoan parasite, *Perkinsus marinus*. *J Shellfish Res* **17**: 1315.

Chu, F.-L. and La Peyre, J. F. (1993b). Development of disease caused by the parasite, *Perkinsus marinus* and defense-related hemolymph factors in three populations of oysters from the Chesapeake Bay, USA. *J Shellfish Res* **12**: 21-7.

Chu, F.-L. E. (1996). Laboratory investigations of susceptibility, infectivity, and transmission of *Perkinsus marinus* in oysters. *J Shellfish Res* **15**: 57-66.

Chu, F.-L. E., Armknecht, S. and Volety, A. K. (1998b). Acid phosphatase and superoxide dismutase activities in different strains of the oyster protozoan parasite, *Perkinsus marinus*. In *Proc 3rd Int Symp Aquatic Animal Health*, pp. 112. Edited by A. S. Kane and S. L. Poynton. Baltimore, MD: Acquatic Pathology Center Press.

Cilia, V., Lafay, B. and Christen, R. (1996). Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. *Mol Biol Evol* **13**: 451-61.

Cortadas, J. and Pavon, M. C. (1982). The organization of ribosomal genes in vertebrates. *EMBO J* **1**: 1075-80.

Coss, C. A. (2000). Investigation of *Perkinsus* species from clams sympatric to oysters with emphasis on infections in the baltic clam *Macoma balthica* of Chesapeake bay. *Ph. D. Thesis*, George Washington University, Washington, DC. pp. 247.

Coss, C. A., Robledo, J. A. F. and Vasta, G. R. (2001a). Fine structure of clonally propagated in vitro life stages of a *Perkinsus* sp. isolated from the Baltic clam *Macoma balthica*. *J Eukaryot Microbiol* **48**: 38-51.

Coss, C. A., Robledo, J. A., Ruiz, G. M. and Vasta, G. R. (2001b). Description of *Perkinsus andrewsi* n. sp. isolated from the Baltic clam (*Macoma balthica*) by characterization of the ribosomal RNA locus, and development of a species-specific PCR-based diagnostic assay. *J Eukaryot Microbiol* **48**: 52-61.

Dalrymple, B. P., Peters, J. M., Bose, R. and Wright, I. G. (1996). A polymerase chain reaction method for the identification of genes encoding members of the Bv60/p58 family of rhoptry protein homologues in the genus *Babesia*. *Exp Parasitol* **84**: 96-100.

Dame, J. B. and McCutchan, T. F. (1983). The four ribosomal DNA units of the malaria parasite *Plasmodium berghei*. Identification, restriction map, and copy number analysis. *J Biol Chem* **258**: 6984-90.

Dame, J. B. and McCutchan, T. F. (1984). Identification of 5 S and 5.8 S ribosomal RNA molecules and their genes in *Plasmodium berghei*. *Mol Biochem Parasitol* **11**: 301-7.

de la Herrán, R., Garrido-Ramos, M. A., Navas, J. I., Ruiz Rejón, C. and Ruiz Rejón, M. (2000). Molecular characterization of the ribosomal RNA gene region of *Perkinsus atlanticus*: its use in phylogenetic analysis and as a target for a molecular diagnosis. *Parasitology* **120**: 345-53.

De Leo, F. R., Ulman, K. V., Davis, A. R., Jutila, K. L. and Quinn, M. T. (1996). Assembly of the human neutrophil NADPH oxidase involves binding of $p67^{phox}$ and flavocytochrome *b* to a common functional domain in $p47^{phox}$. *J Biol Chem* **271**: 17013-20.

De Lucchini, S., Andronico, F. and Nardi, I. (1997). Molecular structure of the rDNA intergenic spacer (IGS) in *Triturus*: implications for the hypervariability of rDNA loci. *Chromosoma* **106**: 315-26.

de Nadal, E., Fadden, R. P., Ruiz, A., Haystead, T. and Arino, J. (2001). A role for the Ppz Ser/Thr protein phosphatases in the regulation of translation elongation factor 1Balpha. *J Biol Chem* **276**: 14829-34.

DeLuca, D. C., Dennis, R. and Smith, W. G. (1995). Inactivation of an animal and a fungal catalase by hydrogen peroxide. *Arch Biochem Biophys* **320**: 129-34.

Dissing, J., Dahl, O. and Svensmark, O. (1979). Phosphonic and arsonic acids as inhibitors of human red cell acid phosphatase and their use in affinity chromatography. *Biochim Biophys Acta* **569**: 159-76.

Dover, G. (1982). Molecular drive: a cohesive mode of species evolution. *Nature* **299**: 111-7.

Dowling, J. D., Saha, A. K. and Glew, R. H. (1992). Virulence factors of the family *Legionellaceae*. *Microbiol Rev* **56**: 32-60.

Dukan, S., Belkin, S. and Touati, D. (1999). Reactive oxygen species are partially involved in the bacteriocidal action of hypochlorous acid. *Arch Biochem Biophys* **367**: 311-6.

Dungan, C. F. and Robertson, B. S. (1993). Binding specificities of mono and polyclonal antibodies to the protozoan oyster pathogen *Perkinsus marinus*. *Dis Aquat Org* **15**: 9-22.

Dungan, C. F. and Reece, K. S. (2006). In vitro propagation of two *Perkinsus* spp. parasites from Japanese Manila Clams *Venerupis philippinarum* and description of *Perkinsus honshuensis* n. sp. *J Eukaryot Microbiol* **53**: 1-11.

Dungan, C. F., Hamilton, R. M., Hudson, K. L., McCollough, C. B. and Reece, K. S. (2002). Two epizootic diseases in Chesapeake Bay commercial clams, *Mya arenaria* and *Tagelus plebeius*. *Dis Aquat Org* **50**: 67-78.

Elandalloussi, L. M., Leite, R. M., Afonso, R., Nunes, P. A., Robledo, J. A., Vasta, G. R. and Cancela, M. L. (2004). Development of a PCR-ELISA assay for diagnosis of *Perkinsus marinus* and *Perkinsus atlanticus* infections in bivalve molluscs. *Mol Cell Probes* **18**: 89-96.

Emanuelsson, O., Nielsen, H., Brunak, S. and von Heijne, G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* **300**: 1005-16.

Ferrante, A., Hill, N. L., Abell, T. J. and Pruul, H. (1987). Role of myeloperoxidase in the killing of *Naegleria fowleri* by lymphokine-altered human neutrophils. *Infect Immun* **55**: 1047-50.

Fong, D., Rodriguez, R., Koo, K., Sun, J., Sogin, M. L., Bushek, D., Littlewood, D. T. and Ford, S. E. (1993). Small subunit ribosomal RNA gene sequence of the oyster parasite *Perkinsus marinus*. *Mol Mar Biol Biotechnol* **2**: 346-50.

Ford, S. E. (1996). Range extension by the oyster parasite *Perkinsus marinus* into the northeastern United States: response to climate change? *J Shellfish Res* **15**: 45-56.

Fox, D. S., Cruz, M. C., Sia, R. A., Ke, H., Cox, G. M., Cardenas, M. E. and Heitman, J. (2001). Calcineurin regulatory subunit is essential for virulence and mediates interactions with FKBP12-FK506 in *Cryptococcus neoformans*. *Mol Microbiol* **39**: 835-49.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M. S., Nene, V., Shallom, S. J., Suh, B., Peterson, J., Angiuoli, S., M., P., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M. and Barrell, B. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**: 498-511.

Gardner, P. R. and Fridovich, I. (1991). Superoxide sensitivity of the *Escherichia coli* aconitase. *J Biol Chem* **266**: 19328-33.

Garreis, K. A., La Peyre, J. F. and Faisal, M. (1996). The effects of *Perkinsus marinus* extracellular products and purified proteases on oyster defence parameters in vitro. *Fish Shellfish Immunol* **6**: 581-97.

Gauthier, J. D. (1998). Development of an *in vitro* culture system for the characterization of gene expression in the oyster parasite *Perkinsus marinus*. *Ph. D. Thesis*, University of Maryland at College Park, College Park. pp.

Gauthier, J. D. and Vasta, G. R. (1993). Continuous *in vitro* culture of the oyster parasite *Perkinsus marinus*. *J Invertebr Pathol* **62**: 321-3.

Gauthier, J. D. and Vasta, G. R. (1995). *In vitro* culture of the eastern oyster parasite *Perkinsus marinus*: optimization of the methodology. *J Invertebr Pathol* **66**: 156-68.

Gauthier, J. D. and Vasta, G. R. (2002). Effects of plasma from bivalve mollusk species on the in vitro proliferation of the protistan parasite *Perkinsus marinus*. *J Exp Zool* **292**: 221-30.

Gauthier, J. D., Miller, C. R. and Wilbur, A. E. (2006). TAQMAN_MCG real-time PCR approach to quantification of *Perkinsus marinus* and *Perkinsus* spp. in oysters. *J Shellfish Res* **25**: 619-24.

Ghadirian, E., Somerfield, S. D. and Kongshavn, P. A. (1986). Susceptibility of *Entamoeba histolytica* to oxidants. *Infect Immun* **51**: 263-7.

Gilbert, L. A., Ravindran, S., Turetzky, J. M., Boothroyd, J. C. and Bradley, P. J. (2007). *Toxoplasma gondii* targets a protein phosphatase 2C to the nuclei of infected host cells. *Eukaryot Cell* **6**: 73-83.

Goggin, C. L. (1994). Variation in the two internal transcribed spacers and 5.8S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). *Mol Biochem Parasitol* **65**: 179-82.

Goggin, C. L. and Barker, S. C. (1993). Phylogenetic position of the genus *Perkinsus* (Protista, Apicomplexa) based on small subunit ribosomal RNA. *Mol Biochem Parasitol* **60**: 65-70.

Green, E. D. (1997). *Genome analysis: a laboratory manual*. Plainview, New York: Cold Spring Harbor Laboratory Press.

Greger, E. A., Drum, A. S. and Elston, R. A. (1995). Measurement of oxidative activity in hemocytes of the pacific razor clam, *Siliqua patula*, and the oyster, *Crassostrea gigas*, using lucigenin- and luminol-dependent chemiluminescence. *J Invertebr Pathol* **65**: 48-60.

Guay, J. M., Huot, A., Gagnon, S., Tremblay, A. and Levesque, R. C. (1992). Physical and genetic mapping of cloned ribosomal DNA from *Toxoplasma gondii*: primary and secondary structure of the 5S gene. *Gene* **114**: 165-71.

Gunderson, J. H., McCutchan, T. F. and Sogin, M. L. (1986). Sequence of the small subunit ribosomal RNA gene expressed in the bloodstream stages of *Plasmodium berghei*: evolutionary implications. *J Protozool* **33**: 525-9.

Gunderson, J. H., Sogin, M. L., Wollett, G., Hollingdale, M., de la Cruz, V. F., Waters, A. P. and McCutchan, T. F. (1987). Structurally distinct, stage-specific ribosomes occur in *Plasmodium*. *Science* **238**: 933-7.

Hahn, U. K., Bender, R. C. and Bayne, C. J. (2001). Killing of *Schistosoma mansoni* sporocysts by hemocytes from resistant *Biomphalaria glabrata*: role of reactive oxygen species. *J Parasitol* **87**: 292-9.

Hardy, S. W., Grant, P. T. and Fletcher, T. C. (1977). A haemagglutinin in the tissue fluid of the Pacific oyster, *Crassostrea gigas*, with specificity for sialic acid residues in glycoproteins. *Experientia* **33**: 767-9.

Harvell, C. D., Kim, K., Burkholder, J. M., Colwell, R. R., Epstein, P. R., Grimes, D. J., Hofmann, E. E., Lipp, E. K., Osterhaus, A. D., Overstreet, R. M., Porter, J. W., Smith, G. W. and Vasta, G. R. (1999). Emerging marine diseases--climate links and anthropogenic factors. *Science* **285**: 1505-10.

Hewatt, W. G. and Andrews, J. D. (1956). Temperature control experiments on the fungus disease, *Dermocystidium marinum*, of oysters. *Proc Natl Shellfish Assoc* **46**: 129-33.

Hill, W. E., Keasler, S. P., Trucksess, M. W., Feng, P., Kaysner, C. A. and Lampel, K. A. (1991). Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Appl Environ Microbiol* **57**: 707-11.

Hoffman, S. H. (1997). Preparation of yeast DNA. In *Current protocols in molecular biology*, pp. 13.1.1-.1.4. Edited by F. A. Ausubel, R. Brent, R. E. Kingston, Moore, J. G. Seidman, J. A. Smith and K. Struhl. New York: John Wiley, Sons.

Horton, P., Park, K.-J., Obayashi, T. and Nakai, K. (2006). Protein subcellular localization prediction with WoLF PSORT. *Proc APBc06* **39-48**.

Hu, M. L., Louie, S., Cross, C. E., Motchnik, P. and Halliwell, B. (1993). Antioxidant protection against hypochlorous acid in human plasma. *J Lab Clin Med* **121**: 257 -62.

Hubert, F., Noël, T. and Roch, P. (1996). A member of the arthropod defensin family from edible Mediterranean mussels (*Mytilus galloprovincialis*). *Eur J Biochem* **240**: 302-6.

Hughes, H. P., Boik, R. J., Gerhardt, S. A. and Speer, C. A. (1989). Susceptibility of *Eimeria bovis* and *Toxoplasma gondii* to oxygen intermediates and a new mathematical model for parasite killing. *J Parasitol* **75**: 489-97.

Jepras, R. I. and Fitzgeorge, R. B. (1986). The effect of oxygen-dependent antimicrobial systems on strains of *Legionella pneumophila* of different virulence. *J Hyg (Lond)* **97**: 61-9.

Jiang, X. and Chai, T. J. (1996). Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable, nonculturable cells. *Appl Environ Microbiol* **62**: 1300-5.

Jiang, Z. Y., Hunt, J. V. and Wolff, S. P. (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal Biochem* **202**: 384-9.

Judo, M. S., Wedel, A. B. and Wilson, C. (1998). Stimulation and suppression of PCR-mediated recombination. *Nucleic Acids Res* **26**: 1819-25.

Karolus, J., Sunila, I., Spear, S. and Volk, J. (2000). Prevalence of *Perkinsus marinus* (Dermo) in *Crassostrea virginica* along the Connecticut shoreline. *Aquaculture* **183**: 215-21.

Kawai, H., Muto, H., Fujii, T. and Kato, A. (1995). A linked 5S rRNA gene in *Scytosiphon lomentaria* (Scytosiphonales, Phaeophyceae). *J Phycol* **31**: 306-11.

Kennedy, V. S. (1991). Eastern oyster *Crassostrea virginica*. In *Habitat requirements for Chesapeake Bay living resources*. Edited by S. L. Funderburk, J. A. Mihursky, S. J. Jordan and D. Riley. Solomons, MD: Chesapeake Research Consortium, Inc.

Kennedy, V. S. (1996). The ecological role of the eastern oyster, *Crassostrea viriginica*, with remarks on disease. *J Shellfish Res* **15**: 177-83.

Kleinschuster, S. J. and Swink, S. L. (1993). A simple method for the in vitro culture of *Perkinsus marinus*. *The Nautilus* **107**: 76-8.

Klotz, M. G. and Loewen, P. C. (2003). The molecular evolution of catalatic hydroperoxidases: evidence for multiple lateral transfer of genes between prokaryota and from bacteria into eukaryota. *Mol Biol Evol* **20**: 1098-112.

Korshunov, S. S. and Imlay, J. A. (2002). A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of Gram-negative bacteria. *Mol Microbiol* **43**: 95-106.

Kotob, S. I., McLaughlin, S. M., Van Berkum, P. and Faisal, M. (1999a). Discrimination between two *Perkinus* spp. isolated from the softshell clam, *Mya arenaria*, by sequence analysis of two internal transcribed spacer regions and the 5.8S ribosomal RNA gene. *Parasitology* **199**: 363-8.

Kotob, S. I., McLaughlin, S. M., van Berkum, P. and Faisal, M. (1999b). Characterization of two *Perkinsus* spp. from the softshell clam, *Mya arenaria* using the small subunit ribosomal RNA gene. *J Eukaryot Microbiol* **46**: 439-44.

Krantz, G. E. and Jordan, S. J. (1996). Management alternatives for protecting *Crassostrea virginica* fisheries in *Perkinsus marinus* enzootic and epizootic areas. *J Shellfish Res* **15**: 167-76.

Kuvardina, O. N., Leander, B. S., Aleshin, V. V., Myl'nikov, A. P., Keeling, P. J. and Simdyanov, T. G. (2002). The phylogeny of Colpodellids (Alveolata) using small subunit rRNA gene sequences suggests they are the free-living sister group to Apicomplexans. *J Eukaryot Microbiol* **49**: 498-504.

La Peyre, J. F., Chu, F.-L. E. and Ragone, L. M. (1989). The effect of salinity and *Perkinsus marinus* infection on some immunological parameters of the oyster *Crassostrea virginica*. *J Shellfish Res* **8**: 469.

La Peyre, J. F., Faisal, M. and Burreson, E. M. (1993). *In vitro* propagation of the protozoan *Perkinsus marinus*, a pathogen of the eastern oyster, *Crassostrea virginica*. *J Eukaryot Microbiol* **40**: 304-10.

La Peyre, J. F., Chu, F. L. and Vogelbein, W. K. (1995a). *In vitro* interaction of *Perkinsus marinus* merozoites with eastern and Pacific oyster hemocytes. *Dev Comp Immunol* **19**: 291-304.

La Peyre, J. F., Yarnall, H. A. and Faisal, M. (1996). Contribution of *Perkinsus marinus* extracellular products in the infection of eastern oysters (*Crassostrea virginica*). *J Invertebr Pathol* **68**: 312-3.

La Peyre, J. F., Schafhauser, D. Y., Rizkhalla, E. H. and Faisal, M. (1995b). Production of serine proteases by the eastern oyster pathogen *Perkinsus marinus* (Apicomplexa) *in vitro*. *J Eukaryot Microbiol* **42**: 544-51.

Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gelebart, F., Cordevant, C., Lange, M. and Poulet, S. A. (2001). A *Vibrio splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). *Dis Aquat Org* **46**: 139-45.

LaCount, M. W., Handy, G. and Lebioda, L. (1998). Structural origins of L(+)-tartrate inhibition of human prostatic acid phosphatase. *J Biol Chem* **273**: 30406-9.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-5.

Le Blancq, S. M., Khramtsov, N. V., Zamani, F., Upton, S. J. and Wu, T. W. (1997). Ribosomal RNA gene organization in *Cryptosporidium parvum*. *Mol Biochem Parasitol* **90**: 463-78.

Leander, B. S. and Keeling, P. J. (2004). Early evolutionary history of dinoflagellates and apicomplexans (Alveolata) as inferred from Hsp90 and actin phylogenies. *J Phycol* **40**: 341-50.

Lester, R. J. G. and Davis, G. H. G. (1981). A new *Perkinsus* species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber*. *J Invertebr Pathol* **37**.

Levine, N. D. (1978). *Perkinsus* gen. n. and other new taxa in the protozoan phylum *Apicomplexa*. *J Parasitol* **64**: 549.

Lovelace, J. K., Dwyer, D. M. and Gottlieb, M. (1986). Purification and characterization of the extracellular acid phosphatase of *Leishmania donovani*. *Mol Biochem Parasitol* **20**: 243-51.

Luan, S. (2003). Protein phosphatases in plants. *Annu Rev Plant Biol* **54**: 63-92.

Lymbery, A. J. (1989). Host specificity, host range and host preference. *Parasitol Today* **5**: 298.

Mackin, J. G. (1951). Histopathology of infection of *Crassostrea virginica* (Gmelin) by *Dermocystidium marinum* Mackin, Owen and Collier. *Bull Mar Sci Gulf Carib* **1**: 72-87.

Mackin, J. G. (1962). Oyster diseases caused by *Dermosystidium marinum* and other microorganisms in Louisana. *Publ Inst Mar Sci Univ Texas* **7**: 132-229.

Mackin, J. G., Owen, H. M. and Collier, A. (1950). Preliminary note on the occurence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). *Science* **111**: 328-9.

Maeda, T., Wurgler-Murphy, S. M. and Saito, H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369**: 242-5.

Mamoun, C. B. and Goldberg, D. E. (2001). *Plasmodium* protein phosphatase 2C dephosphorylates translation elongation factor 1beta and inhibits its PKC-mediated nucleotide exchange activity in vitro. *Mol Microbiol* **39**: 973-81.

Marchler-Bauer, A., Anderson, J. B., Cherukuri, P. F., DeWeese-Scott, C., Geer, L. Y., Gwadz, M., He, S., Hurwitz, D. I., Jackson, J. D., Ke, Z., Lanczycki, C. J., Liebert, C. A., Liu, C., Lu, F., Marchler, G. H., Mullokandov, M., Shoemaker, B. A., Simonyan, V., Song, J. S., Thiessen, P. A., Yamashita, R. A., Yin, J. J., Zhang, D. and Bryant, S. H. (2005). CDD: a Conserved Domain Database for protein classification. *Nucleic Acids Res* **33**: D192-6.

Marsh, A. G., Gauthier, J. D. and Vasta, G. R. (1995). A semiquantitative PCR assay for assessing *Perkinsus marinus* infections in the eastern oyster, *Crassostrea virginica*. *J Parasitol* **81**: 577-83.

Martinéz-Murcia, A. J., Anton, A. I. and Rodriguez-Valera, F. (1999). Patterns of sequence variation in two regions of the 16S rRNA multigene family of *Escherichia coli*. *Int J Syst Bacteriol* **49 Pt 2**: 601-10.

McCutchan, T. F., Li, J., McConkey, G. A., Rogers, M. J. and Waters, A. P. (1995). The cytoplasmic ribosomal RNAs of *Plasmodium* spp. *Parasitol Today* **11**: 134-8.

McLaughlin, S. M. and Faisal, M. (1999). A comparison of the diagnostic assays for detection of *Perkinsus* spp. in the softshell clam *Mya arenaria*. *Aquaculture* **172**: 197-204.

McLaughlin, S. M., Tall, B. D., Shaheen, A., Elsayed, E. E. and Faisal, M. (2000). Zoosporulation of a new *Perkinus* species isolated from the gills of the softshell clam *Mya arenaria*. *Parasite* **7**: 115-22.

Medlin, L., Elwood, H. J., Stickel, S. and Sogin, M. L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* **71**: 491-9.

Mehlotra, R. K. (1996). Antioxidant defense mechanisms in parasitic protozoa. *Crit Rev Microbiol* **22**: 295-314.

Meyerhans, A., Vartanian, J. P. and Wain-Hobson, S. (1990). DNA recombination during PCR. *Nucleic Acids Res* **18**: 1687-91.

Meyers, J. A., Burreson, E. M., Barber, B. J. and Mann, R. (1991). Susceptibility of diploid and triploid pacific oysters *Crassostrea gigas* (Thunberg, 1793) and eastern oysters, *Crassostrea virginica* (Gmelin, 1791), to *Perkinsus marinus*. *J Shellfish Res* **10**: 433-7.

Mitta, G., Hubert, F., Noël, T. and Roch, P. (1999). Myticin, a novel cysteine-rich antimicrobial peptide isolated from haemocytes and plasma of the mussel *Mytilus galloprovincialis*. *Eur J Biochem* **265**: 71-8.

Montes, J. F., Durfort, M., Lladó, A. and García Valero, J. (2002). Characterization and immunolocalization of a main proteinaceous component of the cell wall of the protozoan parasite *Perkinsus atlanticus*. *Parasitology* **124**: 477-84.

Murray, H. and Cohn, Z. (1979). Macrophage oxygen-dependent antimicrobial activity. I. Susceptibility of *Toxoplasma gondii* to oxygen intermediates. *J Exp Med* **150**: 938-49.

Murrell, A., Kleeman, S. N., Barker, S. C. and Lester, R. J. G. (2002). Synonymy of *Perkinsus olseni* Lester & Davis, 1981 and *P. atlanticus* Azevedo, 1989 and an update on the phylogenetic position of the genus *Perkinsus*. *Bull Eur Assoc Fish Pathol* **22**: 258-65.

Nakamura, M., Mori, K., Inooka, S. and Nomura, T. (1985). In vitro production of hydrogen peroxide by the amoebocytes of the scallop, *Patinopecten yessoensis* (Jay). *Dev Comp Immunol* **9**: 407-17.

Newell, R. I. E. (1988). Ecological changes in Chesapeake Bay: are they the result of overharvesting the American oyster, *Crassostrea virginica*? In *Understanding the estuary: advances in Chesapeake Bay research*, pp. 539-46. Edited by M. P. Luynch and E. C. Krome. Solomons, MD: Chesapeake Bay Research Publication.

Noctor, G. and Foyer, C. H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* **49**: 249-79.

Norén, F., Moestrup, Ø. and Rehnstam-Holm, A.-S. (1999). *Parvilucifera infectans* Norén et Moestrup gen. et sp. nov. (Perkinsozoa phylum nov.): a parasitic flagellate capable of killing toxic microalgae. *Eur J Protistol* **35**: 233-54.

Ohno, Y. G. and Gallin, J. I. (1985). Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils. Studies utilizing the inactivation of myeloperoxidase by hydrogen peroxide and azide. *J Biol Chem* **260**.

Ohta, T. and Dover, G. A. (1984). The cohesive population genetics of molecular drive. *Genetics* **108**: 501-21.

Ostrowski, M., Cavicchioli, R., Blaauw, M. and Gottschal, J. C. (2001). Specific growth rate plays a critical role in hydrogen peroxide resistance of the marine oligotrophic ultramicrobacterium *Sphingomonas alaskensis* strain RB2256. *Appl Environ Microbiol* **67**: 1292-9.

Ottinger, C. A., Lewis, T. D., Shapiro, D. A., Faisal, M. and Kaattari, S. L. (2001). Detection of *Perkinsus marinus* extracellular proteins in tissues of the eastern oyster *Crassostrea virginica*: potential use in diagnostic assays. *J Aquat Anim Health* **13**: 133-41.

Pecher, W. T., Robledo, J. A. F. and Vasta, G. R. (2004). Identification of a second rRNA gene unit in the *Perkinsus andrewsi* genome. *J Eukaryot Microbiol* **51**: 234-45.

Penna, M. S., Khan, M. and French, R. A. (2001). Development of a multiplex PCR for the detection of *Haplosporidium nelsoni, Haplosporidium costale* and *Perkinsus marinus* in the eastern oyster (*Crassostrea virginica*, Gmelin, 1971). *Mol Cell Probes* **15**: 385-90.

Perkins, F. O. (1976). Zoospores of the oyster pathogen *Dermocystidium marinum*. I. Fine structure of the conoid and other sporozoan-like organelles. *J Parasitol* **62**: 959-74.

Perkins, F. O. (1988). Parasite morphology, strategy, and evolution. *Am Fish Soc Spec Publ* **18**: 93-111.

Perkins, F. O. (1996). The structure of *Perkinsus marinus* (Mackin, Owen, and Collier, 1950) Levine, 1978 with comments on taxonomy and phylogeny of *Perkinsus* spp. *J Shellfish Res* **15**: 67-87.

Perkins, F. O. and Menzel, R. W. (1967). Ultrastructure of sporulation in the oyster pathogen *Dermocystidium marinum*. *J Invertebr Pathol* **9**: 205-29.

Prajapati, S., Verma, U., Yamamoto, Y., Kwak, Y. T. and Gaynor, R. B. (2004). Protein phosphatase 2Cβ association with the IκB kinase complex is involved in regulating NF-κB activity. *J Biol Chem* **279**: 1739-46.

Qari, S. H., Goldman, I. F., Pieniazek, N. J., Collins, W. E. and Lal, A. A. (1994). Blood and sporozoite stage-specific small subunit ribosomal RNA-encoding genes of the human malaria parasite *Plasmodium vivax*. *Gene* **150**: 43-9.

Qiu, L., Song, L., Xu, W., Ni, D. and Yu, Y. (2007). Molecular cloning and expression of a Toll receptor gene homologue from Zhikong Scallop, *Chlamys farreri*. *Fish Shellfish Immunol* **22**: 451-66.

R Developer Core Team (2006). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Ray, S. M. (1952). A culture technique for the diagnosis of infection with *Dermocystidium marinum* in oysters. *Science* **116**: 360-1.

Ray, S. M. (1966). A review of the culture method for detecting *Dermocystidium marinum*, with suggested modifications and precautions. *Proc Natl Shellfish Assoc* **54**: 55-69.

Reddy, G. R., Chakrabarti, D., Yowell, C. A. and Dame, J. B. (1991). Sequence microheterogeneity of the three small subunit ribosomal RNA genes of *Babesia bigemina*: expression in erythrocyte culture. *Nucleic Acids Res* **19**: 3641-5.

Reece, K. S., Bushek, D. and Graves, J. E. (1997). Molecular markers for population genetic analysis of *Perkinsus marinus*. *Mol Mar Biol Biotechnol* **6**: 197-206.

Reece, K. S., Bushek, D., Hudson, K. L. and Graves, J. E. (2001). Geographic distribution of *Perkinsus marinus* genetic strains along the Atlantic and Gulf Coasts of the USA. *Mar Biol* **139**: 1047-55.

Reeder, R. H. (1990). rRNA synthesis in the nucleolus. *Trends Genet* **6**.

Reilly, T. J., Baron, G. S., Nano, F. E. and Kuhlenschmidt, M. S. (1996). Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from *Francisella tularensis*. *J Biol Chem* **271**: 10973-83.

Remaley, A. T., Kuhns, D. B., Basford, R. E., Glew, R. H. and Kaplan, S. S. (1984). Leishmanial phosphatase blocks neutrophil O_2 production. *J Biol Chem* **259**: 11173-5.

Remaley, A. T., Das, S., Campbell, P. I., LaRocca, G. M., Pope, M. T. and Glew, R. H. (1985a). Characterization of *Leishmania donovani* acid phosphatases. *J Biol Chem* **260**: 880-6.

Remaley, A. T., Glew, R. H., Kuhns, D. B., Basford, R. E., Waggoner, A. S., Ernst, L. A. and Pope, M. (1985b). *Leishmania donovani*: surface membrane acid phosphatase blocks neutrophil oxidative metabolite production. *Exp Parasitol* **60**: 331-41.

Rice, P., Longden, I. and Bleasby, A. (2000). EMBOSS: The European Molecular Biology Open Software Suite. *Trends Genet* **16**: 276-7.

Robledo, J. A., Wright, A. C., Marsh, A. G. and Vasta, G. R. (1999). Nucleotide sequence variability in the nontranscribed spacer of the rRNA locus in the oyster parasite *Perkinsus marinus*. *J Parasitol* **85**: 650-6.

Robledo, J. A. F., Coss, C. A. and Vasta, G. R. (2000). Characterization of the ribosomal RNA locus of *Perkinsus atlanticus* and development of a polymerase chain reaction-based diagnostic assay. *J Parasitol* **86**: 972-8.

Robledo, J. A. F., Nunes, P. A., Cancela, M. L. and Vasta, G. R. (2002). Development of an *in vitro* clonal culture and characterization of the rRNA gene cluster of *Perkinsus atlanticus*, a protistan parasite of the clam *Tapes decussatus*. *J Eukaryot Microbiol* **49**: 414-22.

Robledo, J. A. F., Gauthier, J. D., Coss, C. A., Wright, A. C. and Vasta, G. R. (1998). Species-specificity and sensitivity of a PCR-based assay for *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica*: a comparison with the fluid thioglycollate assay. *J Parasitol* **84**: 1237-44.

Rogers, M. J., Gutell, R. R., Damberger, S. H., Li, J., McConkey, G. A., Waters, A. P. and McCutchan, T. F. (1996). Structural features of the large subunit rRNA expressed in *Plasmodium falciparum* sporozoites that distinguish it from the asexually expressed subunit rRNA. *RNA* **2**: 134-45.

Rohde, K. (1980). Host specificity indices of parasites and their application. *Experientia* **36**: 1369-71.

Rotman, B. and Papermaster, B. W. (1966). Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc Natl Acad Sci USA* **55**: 134 -41.

Russell, S., Frasca, S., Jr., Sunila, I. and French, R. A. (2004). Application of a multiplex PCR for the detection of protozoan pathogens of the eastern oyster *Crassostrea virginica* in field samples. *Dis Aquat Org* **59**: 85-91.

Saha, A. K., Dowling, J. N., LaMarco, K. L., Das, S., Remaley, A. T., Olomu, N., Pope, M. and Glew, R. H. (1985). Properties of an acid phosphatase from *Legionella micdadei* which blocks superoxide anion production by human neutrophils. *Arch Biochem Biophys* **243**: 150-60.

Saldarriaga, J. F., McEwan, M. L., Fast, N. M., Taylor, F. J. R. and Keeling, P. J. (2003). Multiple protein phylogenies show that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellate lineage. *Int J Syst Evol Microbiol* **53**: 355-65.

Sambrook, J. and Russell, D. W. (2001). *Molecular Cloning. A Laboratory Manual*, 3rd ed. edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schaus, E. E., Kutnink, M. A., O'Connor, D. K. and Omaye, S. (1986). A comparison of leukocyte ascorbate levels measured by the 2,4dinitrophenylhydrazine ethod with high-performance liquid chromatography. *Biochem Med Metab Biol* **36**: 369-76.

Scholin, C. A., Anderson, D. M. and Sogin, M. L. (1993). Two distinct small-subunit ribosomal RNA genes in the North American toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae). *J Phycol* **29**: 209-16.

Scholin, C. A., Herzog, M., Sogin, M. L. and Anderson, D. M. (1994). Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). 2. Sequence analysis of a fragment of the LSU rRNA gene. *J Phycol* **30**: 999-1011.

Schott, E. J. and Vasta, G. R. (2003). The PmSOD1 gene of the protistan parasite *Perkinsus marinus* complements the sod2Δ mutant of *Saccharomyces cerevisiae*, and directs an iron superoxide dismutase to mitochondria. *Mol Biochem Parasitol* **126**: 81-92.

Schott, E. J., Pecher, W. T., Okafor, F. and Vasta, G. R. (2003a). The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species. *Exp Parasitol* **105**: 232-40.

Schott, E. J., Robledo, J.-A. F., Wright, A. C., Silva, A. M. and Vasta, G. R. (2003b). Gene organization and homology modeling of two iron superoxide dismutases of the early branching protist *Perkinsus marinus*. *Gene* **309**: 1-9.

Seo, J. K., Crawford, J. M., Stone, K. L. and Noga, E. J. (2005). Purification of a novel arthropod defensin from the American oyster, *Crassostrea virginica*. *Biochem Biophys Res Commun* **338**: 1998-2004.

Shakarian, A. M., Joshi, M. B., Yamage, M., Ellis, L. S., Debrabant, A. and Dwyer, D. M. (2003). Members of a unique histidine acid phosphatase family are conserved amongst a group of primitive eukaryotic human pathogens. *Mol Cell Biochem* **245**: 31-41.

Siddall, M. E., Reece, K. S., Graves, J. E. and Burreson, E. M. (1997). 'Total evidence' refutes the inclusion of *Perkinsus* species in the phylum Apicomplexa. *Parasitology* **115**: 165-76.

Smith, W. H. F. and Wessel, P. (1990). Gridding with continuous curvature splines in tension. *Geophysics* **55**: 293-305.

Sollner-Webb, B. and Mougey, E. B. (1991). News from the nucleolus: rRNA gene expression. *TIBS* **16**: 58-62.

Soniat, T. M. (1996). Epizootiology of *Perkinsus marinus* disease of eastern oysters in the Gulf of Mexico. *J Shellfish Res* **15**: 35-43.

Souto, R. P. and Zingales, B. (1993). Sensitive detection and strain classification of *Trypanosoma cruzi* by amplification of a ribosomal RNA sequence. *Mol Biochem Parasitol* **62**: 45-52.

Souto, R. P., Fernandes, O., Macedo, A. M., Campbell, D. A. and Zingales, B. (1996). DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi*. *Mol Biochem Parasitol* **83**: 141-52.

Splettstoesser, W. D. and Schuff-Werner, P. (2002). Oxidative stress in phagocytes - The enemy within? *Microsc Res Tech* **57**: 441-55.

Steinbeck, M. J., Khan, A. U. and Karnovsky, M. J. (1992). Intracellular singlet oxygen generation by phagocytosing neutrophils in response to particles coated with a chemical trap. *J Biol Chem* **267**: 13425-33.

Stelter, K., El-Sayed, N. M. and Seeber, F. (2007). The expression of a plant-type ferredoxin redox system provides molecular evidence for a plastid in the early dinoflagellate *Perkinsus marinus*. *Protist* **158**: 119-30.

Stoebe, B. and Maier, U. G. (2002). One, two, three: nature's tool box for building plastids. *Protoplasma* **219**: 123-30.

Stolf, B. S., Souto, R. P., Pedroso, A. and Zingales, B. (2003). Two types of ribosomal RNA genes in hybrid *Trypanosoma cruzi* strains. *Mol Biochem Parasitol* **126**: 73-80.

Sue-A-Quan, A. K., Fialkow, L., Vlahos, C. J., Schelm, J. A., Greinstein, S., Butler, J. and Downey, G. P. (1995). Inhibition of neutrophil oxidative burst and granule secretion by Wortmannin: potential role of MAP kinase and renaturable kinases. *J Cell Physiol* **172**: 94-108.

Takahashi, K. and Mori, K. (2000). NADPH oxidase-like activity in hemocytes of the Pacific oyster *Crassostrea gigas*. *Fish Pathol* **35**: 15-9.

Takekawa, M., Maeda, T. and Saito, H. (1998). Protein phosphatase 2Cα inhibits the human stress-responsive p38 and JNK MAPK pathways. *EMBO J* **17**: 4744-52.

Tan, A. S. and Berridge, M. V. (2000). Superoxide produced by activated neutrophils efficiently reduces the tetrazolium salt, WST-1 to produce a soluble formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening antiinflammatory agents. *J Immunol Methods* **238**: 59-68.

Tanabe, K., Sakihama, N., Farnert, A., Rooth, I., Bjorkman, A., Walliker, D. and Ranford-Cartwright, L. (2002). *In vitro* recombination during PCR of *Plasmodium falciparum* DNA: a potential pitfall in molecular population genetic analysis. *Mol Biochem Parasitol* **122**: 211-6.

Thompson, J., van Spaendonk, R. M., Choudhuri, R., Sinden, R. E., Janse, C. J. and Waters, A. P. (1999). Heterogeneous ribosome populations are present in *Plasmodium berghei* during development in its vector. *Mol Microbiol* **31**: 253-60.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673-80.

Ursitti, J. A., Mozdzanowski, J. and Speicher, D. W. (1995). Electroblotting from polyacrylamide gels. In *Current Protocols in Protein Science*, pp. 10.7.1-.7.4. Edited by J. E. Coligan, B. M. Dunn, D. W. Speicher and P. T. Wingfield. Hoboken, NJ: John Wiley & Sons Inc.

Van Etten, R. L. and Saini, M. S. (1978). Selective purification of tartrate-inhibitable acid phosphatases: rapid and efficient purification (to homogeneity) of human and canine prostatic acid phosphatases. *Clin Chem* **24**: 1525-30.

van Spaendonk, R. M., Ramesar, J., van Wigcheren, A., Eling, W., Beetsma, A. L., van Gemert, G. J., Hooghof, J., Janse, C. J. and Waters, A. P. (2001). Functional equivalence of structurally distinct ribosomes in the malaria parasite, *Plasmodium berghei*. *J Biol Chem* **276**: 22638-47.

Vannier-Santos, M. A., Martiny, A., Meyer-Fernandes, J. R. and de Souza, W. (1995). Leishmanial protein kinase C modulates host cell infection via secreted acid phosphatase. *Eur J Cell Biol* **67**: 112-9.

Vasta, G. R., Cheng, T. C. and Marchalonis, J. J. (1984). A lectin on the hemocyte membrane of the oyster (*Crassostrea virginica*). *Cell Immunol* **88**: 475-88.

Vasta, G. R., Sullivan, J. T., Cheng, J. J., Marchalonis, J. J. and Warr, G. W. (1982). A cell membrane-associated lectin of the oyster hemocyte. *J Invertebr Pathol* **40**: 367-77.

Vattanaviboon, P. and Mongkolsuk, S. (2001). Unusual adaptive, cross protection responses and growth phase resistance against peroxide killing in a bacterial shrimp pathogen, *Vibrio harveyi*. *FEMS Microbiol Lett* **200**: 111-6.

Villalba, A., Reece, K. S., Ordás, M. C., Casas, S. M. and Figueras, A. (2004). Perkinsosis in molluscs: a review. *Aquat Living Resour* **17**: 411-32.

Vincendeau, P., Daulouede, S. and Veyret, B. (1989). Role of hypochlorous acid in *Trypanosoma musculi* killing by phagocytes. *Parasitology* **98**: 253 -7.

Vissers, M. C., Stern, A., Kuypers, F., van den Berg, J. and Winterbourn, C. C. (1994). Membrane changes associated with lysis of red blood cells by hypochlorous acid. *Free Radic Biol Med* **16**: 703-12.

Volety, A. K. and Chu, F. L. (1995). Suppression of chemiluminescence of eastern oyster (*Crassostrea virginica*) hemocytes by the protozoan parasite *Perkinsus marinus*. *Dev Comp Immunol* **19**: 135-42.

Volety, A. K. and Chu, F. L. (1997). Acid phosphatase activity in *Perkinsus marinus*, the protistan parasite of the American oyster, *Crassostrea virginica*. *J Parasitol* **83**: 1093-8.

Warmka, J., Hanneman, J., Lee, J., Amin, D. and Ota, I. (2001). Ptc1, a type 2C Ser/Thr phosphatase, inactivates the HOG pathway by dephosphorylating the mitogen-activated protein kinase Hog1. *Mol Cell Biol* **21**: 51-60.

Waters, A. P. (1994). The ribosomal RNA genes of *Plasmodium*. *Adv Parasitol* **34**: 33-79.

Waters, A. P., van Spaendonk, R. M., Ramesar, J., Vervenne, R. A., Dirks, R. W., Thompson, J. and Janse, C. J. (1997). Species-specific regulation and switching of transcription between stage-specific ribosomal RNA genes in Plasmodium berghei. *J Biol Chem* **272**: 3583-9.

Wayne, L. G. and Diaz, G. A. (1986). A double staining method for differentiating between two classes of mycobacterial catalase in polyacrylamide electrophoresis gels. *Anal Biochem* **157**: 89-92.

Weinbach, E. C. and Diamond, L. S. (1974). *Entamoeba histolytica*. I. Aerobic metabolism. *Exp Parasitol* **35**: 232-43.

Wellems, T. E., Walliker, D., Smith, C. L., do Rosario, V. E., Maloy, W. L., Howard, R. J., Carter, R. and McCutchan, T. F. (1987). A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of *Plasmodium falciparum*. *Cell* **49**: 633-42.

Wentworth, P., Jr., McDunn, J. E., Wentworth, A. D., Takeuchi, C., Nieva, J., Jones, T., Bautista, C., Ruedi, J. M., Gutierrez, A., Janda, K. D., Babior, B. M., Eschenmoser, A. and Lerner, R. A. (2002). Evidence for antibody-catalyzed ozone formation in bacterial killing and inflammation. *Science* **298**: 2195-9.

Wessel, P. and Smith, W. H. F. (1998). New, improved versions of the Generic Mapping Tools released. *EOS Trans AGU* **79**: 579.

Wilkinson, S. R., Obado, S. O., Mauricio, I. L. and Kelly, J. M. (2002). *Trypanosoma cruzi* expresses a plant-like ascorbate-dependent hemoperoxidase localized to the endoplasmic reticulum. *PNAS* **99**: 13453-8.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol* **63**: 3741-51.

Wright, A. C., Ahmed, H., Gauthier, J. D., Silva, A. M. and Vasta, G. R. (2002). cDNA cloning and characterization of two iron superoxide dismutases from the oyster parasite *Perkinsus marinus*. *Mol Biochem Parasitol* **123**: 73-7.

Xia, Y., Clarkson, J. M. and Charnley, A. K. (2001). Acid phosphatases of *Metarhizium anisopliae* during infection of the tobacco hornworm *Manduca sexta*. *Arch Microbiol* **76**: 427-34.

Yamamori, T., Inanami, O., Nagahata, H., Cui, Y. D. and Kuwabra, M. (2000). Roles of p38 MAPK, PKC and PI-K in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes. *FEBS Lett* **467**: 253-8.

Yarnall, H. A., Reece, K. S., Stokes, N. A. and Burreson, E. M. (2000). A quantitative competitive polymerase chain reaction assay for the oyster pathogen *Perkinsus marinus*. *J Parasitol* **86**: 827-37.

Yeung, P. K., Kong, K. F., Wong, F. T. and Wong, J. T. (1996). Sequence data for two large-subunit rRNA genes from an Asian strain of *Alexandrium catenella*. *Appl Environ Microbiol* **62**: 4199-201.

Yumoto, I., Ichihashi, D., Iwata, H., Istokovics, A., Ichise, N., Matsuyama, H., Okuyama, H. and Kawasaki, K. (2000). Purification and characterization of a catalase from the facultatively psychrophilic bacterium *Vibrio rumoiensis* S-1(T) exhibiting high catalase activity. *J Bacteriol* **182**: 1903-9.

Zhao, J., Song, L., Li, C., Ni, D., Wu, L., Zhu, L., Wang, H. and Xu, W. (2007). Molecular cloning, expression of a big defensin gene from bay scallop *Argopecten irradians* and the antimicrobial activity of its recombinant protein. *Mol Immunol* **44**: 360-8.

Zingales, B., Stolf, B. S., Souto, R. P., Fernandes, O. and Briones, M. R. (1999). Epidemiology, biochemistry and evolution of *Trypanosoma cruzi* lineages based on ribosomal RNA sequences. *Mem Inst Oswaldo Cruz* **94 Suppl 1**: 159-64.