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Article *in* Experimental Parasitology · November 2003

DOI: 10.1016/j.exppara.2003.12.012 · Source: PubMed

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The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species

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Received 2 June 2003; received in revised form 10 December 2003; accepted 12 December 2003

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*

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; Lester and Davis, 1981). *P. marinus* shares structural and molecular characteristics with both the Apicomplexa and the Dinozoa (Goggin and Barker, 1993; Levine, 1978; Siddal et al., 1997), and recent molecular analyses indicate that *Perkinsus* is basal to, but not within, the dinoflagellate lineage (Saldarriaga et al., 2001), 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,

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0014-4894/$ - see front matter © 2003 Published by Elsevier Inc.
doi:10.1016/j.exppara.2003.12.012

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 °C 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 °C.

2.2. Reactive oxygen exposures

Cultured *P. marinus* trophozoites, washed once in PBS500 (500 mM NaCl, 20 mM $NaPO_4$, pH 7.2) and suspended to a final OD_{600} 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 μM/min superoxide (results not shown). The theoretical yield of superoxide from 25 mU/ml is 25 μM/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 2× stocks of trophozoites with 2× solutions of either H_2O_2 or HOCl. ROS exposures were terminated by pelleting cells by centrifugation at 450*g*, 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 2× stock of H_2O_2 or HOCl to 50 μl of a 2× 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 (10,000*g*), 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).[1] *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.

[1] *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.

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 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 compared to fish leukocytes (Bramble and Anderson, 1998). Allowing that fish hemocytes generate no more superoxide than human neutrophils, *P. marinus* may potentially tolerate the level of superoxide created by oyster hemocytes.

In comparison to *P. marinus*, *V. splendidus* showed greater sensitivity to the X/XO system. Treatments with XO at of 5, 25, 100, and 200 mU/ml, resulted in decreases in CFUs of 12, 51, 60, and 73%, respectively (Fig. 1B). Except for 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* trophozoites showed less than 10% loss of viability with exposure to H_2O_2 up to 8 mM, although the slight reduction of viability at 4 and 8 mM was statistically significant

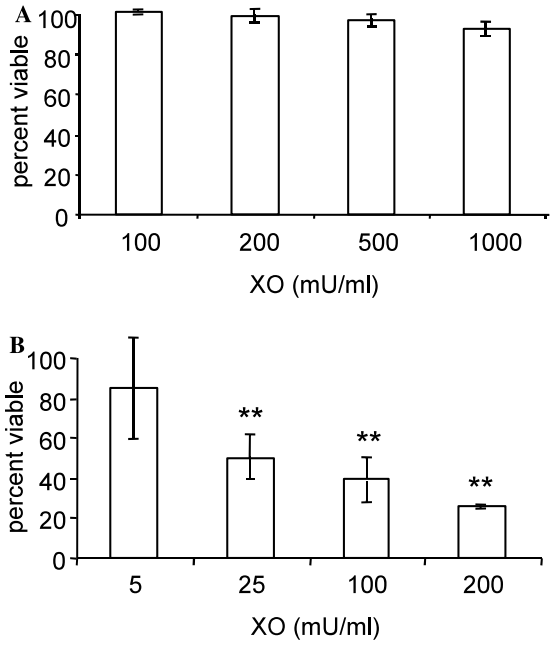

Fig. 1. Percent viability after exposure to ROS generated by the X/XO system. (A) Percent viability of *Perkinsus marinus* trophozoites after a 1 h exposure to increasing amounts of XO. (B) Percent viability of *Vibrio splendidus* after a 1 h exposure to increasing amounts of XO. Viability measurements as in Section 2; error bars are SD. $^{**}p < 0.005$ versus untreated controls.

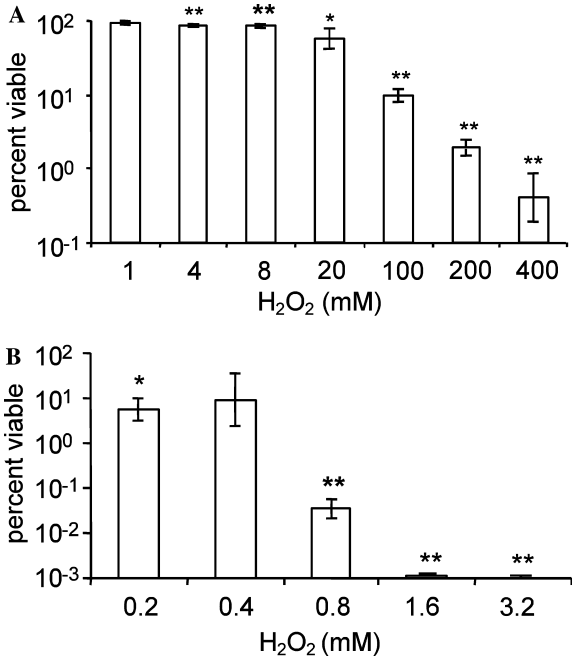

Fig. 2. Percent viability after exposure to H_2O_2. (A) Viability of *Perkinsus marinus* trophozoites after a 1 h exposure to increasing amounts of H_2O_2. (B) Viability of *Vibrio splendidus* after a 1 h exposure to increasing amounts of H_2O_2. Viability measurements as in Section 2; error bars are SD. $^{**}p < 0.005$, $^{*}p < 0.05$ versus untreated controls.

($p < 0.005$). 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 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 2.5×10^6 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 μM 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, 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 10^8 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%.

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,

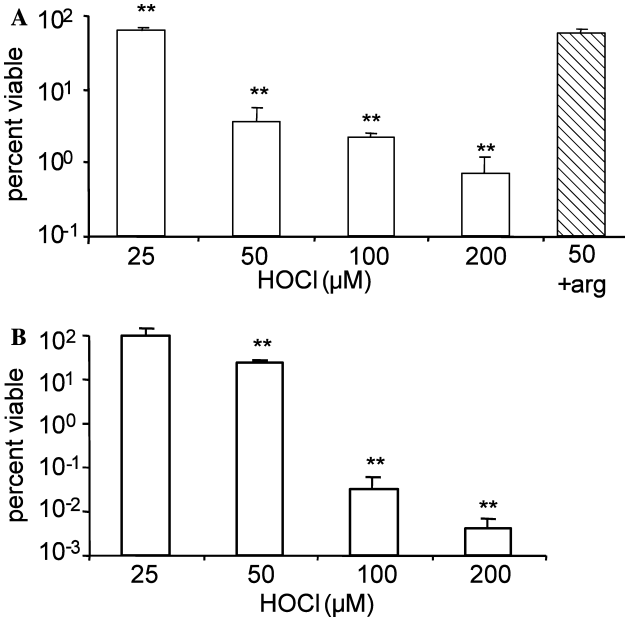

Fig. 3. Percent viability after exposure to HOCl. (A) Viability of *Perkinsus marinus* trophozoites after 1 h exposure to increasing amounts of HOCl. Shaded column represents a parallel treatment with both 50 μM HOCl and 10 mM arginine. (B) Viability of *Vibrio splendidus* after 1 h exposure to increasing amounts of HOCl as in Section 2. Viability measurements as in Section 2; error bars are SD. $^{**}p < 0.005$ versus untreated controls.

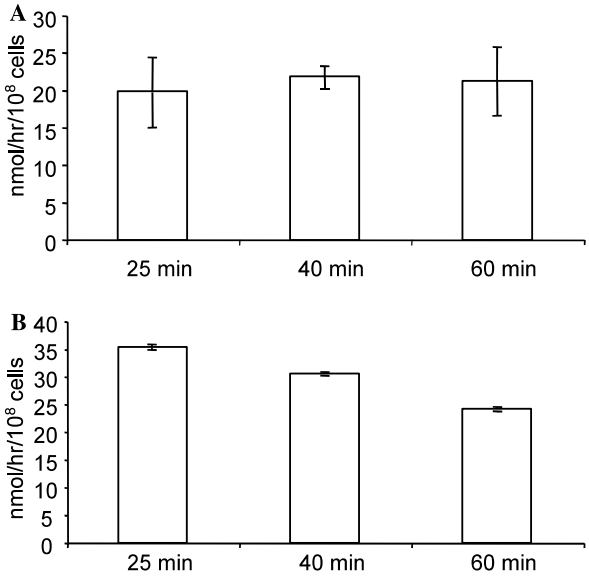

Fig. 4. Hydrogen peroxide removal by live cells. The amount of H_2O_2 removed by live cells was measured at 25, 40, and 60 min, and the rate of H_2O_2 removal (nmol/h/10^8 cells) then calculated for each timepoint. Each timepoint is an average and SD of triplicate samples. (A) *Perkinsus marinus*; (B) *Vibrio splendidus*.

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, 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 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 (ΔA_{290} of $-0.062\ min^{-1}$), but not in *V. splendidus* extracts (ΔA_{290} 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

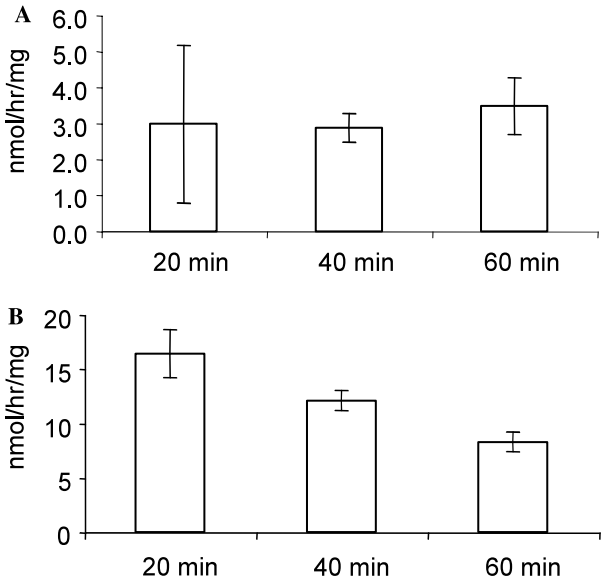

Fig. 5. Hydrogen peroxide removal by dialyzed extracts. The amount of H_2O_2 removed by dialyzed cell extracts was measured at 25, 40, and 60 min, and the rate of H_2O_2 removal (nmol/h/mg protein) then calculated for each timepoint. Each timepoint represents the average and SD of triplicate samples.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

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.

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).

Acknowledgments

We thank Drs. R. Colwell and A. Huq (COMB, UMBI) for providing *Vibrio* strains. This work was supported by Grant NA06RG0101 awarded by NOAA, Oyster Disease Research Program, Sea Grant College.

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