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Peroxisome Proliferation in Foraminifera Inhabiting the Chemocline: An Adaptation to Reactive Oxygen Species Exposure?¹

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Abstract

Certain foraminiferal species are abundant within the chemocline of marine sediments. Ultrastructurally, most of these species possess numerous peroxisomes complexed with the endoplasmic reticulum; mitochondria are often interspersed among these complexes. In the Santa Barbara Basin, pore-water bathing Foraminifera and co-occurring sulfur-oxidizing microbial mats had micromolar levels of hydrogen peroxide, a reactive oxygen species that can be detrimental to biological membranes. Experimental results indicate that adenosine triphosphate concentrations are significantly higher in Foraminifera incubated in 16 μM H_2O_2 than in specimens incubated in the absence of H_2O_2 . New ultrastructural and experimental observations, together with published results, lead us to propose that foraminiferans can utilize oxygen derived from the breakdown of environmentally and metabolically produced H_2O_2 . Such a capability could explain foraminiferal adaptation to certain chemically inhospitable environments; it would also force us to reassess the role of protists in biogeochemistry, especially with respect to hydrogen and iron. The ecology of these protists also appears to be tightly linked to the sulfur cycle. Finally, given that some Foraminifera bearing peroxisome-endoplasmic reticulum complexes belong to evolutionarily basal groups, an early acquisition of the capability to use environmental H_2O_2 could have facilitated diversification of foraminiferans during the Neoproterozoic.

Keywords

Anoxia; *Beggiatoa*; Blake Ridge; cold seep; hydrogen peroxide; hydrogen sulfide; microbial mat; Neoproterozoic; Santa Barbara Basin; Soledad Basin

The anoxic-oxic interface and associated reduction-oxidation (redox) zone are sites at which a great variety of biogeochemical reactions occur (e.g. Brune, Frenzel, and Cypionka 2000; Hulth et al. 2005; Preisler et al. 2007). In organic-rich sedimentary settings where the overlying waters are depleted of oxygen, redox gradients are particularly steep (e.g. Røy, Huettel, and Jørgensen 2005), and hydrogen sulfide can often reach considerable concentrations within one centimeter of the aerated sediment surface (e.g. Bernhard,

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Visser, and Bowser 2003; Visser, Beukema, and van Gernerden 1991). The dynamic redox processes occurring in this chemocline likely challenge the physiology of the aerobic and microaerophilic microbial eukaryotes inhabiting such extreme environments. For example, because hydrogen sulfide inhibits cytochrome-*c* oxidase, a critical aerobic respiratory enzyme, it is unclear how some aerobes are able to survive sulfide-enriched environmental conditions, even though some are known to do so (e.g. Grieshaber and Völkel 1998; Hagerman 1998). Possible mechanisms employed by aerobes to overcome hydrogen sulfide exposure are the incorporation of sulfur-oxidizing bacterial symbionts (e.g. Ott, Bright, and Bulgheresi 2005), reversible sulfide binding to blood, mitochondrial sulfide oxidation, and/or reliance on anaerobic metabolism (reviewed in Grieshaber and Völkel 1998).

Besides hydrogen sulfide, the chemocline has additional chemical constituents that are potentially hazardous to aerobes. More specifically, the spontaneous chemical oxidation of hydrogen sulfide produces reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), sulfide radical ($HS^{\cdot}$), hydroxyl radical ($HO^{\cdot}$), and hydrogen peroxide (H_2O_2) (Tapley, Buettner, and Shick 1999). ROS are also formed as byproducts of normal metabolic reactions in most organisms (reviewed in Genestra 2007). Prolonged ROS exposure can seriously damage many biological molecules such as proteins, lipids, and nucleic acids, but cells normally have protective mechanisms by which to minimize the effect of such oxidative stress (e.g. Abele et al. 1998; Genestra 2007; Lesser 2006; Sigler et al. 1999). Because H_2O_2 is an uncharged molecule, it is less reactive than most ROS and can cross biological membranes via diffusion and/or through channel proteins such as aquaporins (Bienert, Schjoerring, and Jahn 2006; Schrader and Fahimi 2006).

The organelle complement of most eukaryotic cells includes peroxisomes or peroxisome-like structures such as the glyoxysome of plants (Titorenko and Rachubinski 2001a). Peroxisomes serve a variety of metabolic functions, including the conversion of H_2O_2 to water and oxygen (reviewed in Masters and Crane 1995; Titorenko and Rachubinski 2001a). This conversion typically is mediated by catalase, a metalloenzyme containing four porphyrin heme (iron) groups that react with hydrogen peroxide, which often appears as a crystalline core in peroxisomes (e.g. Masters and Crane 1995).

Foraminifera (rhizarian protists, Adl et al. 2004) are presumed aerobes, given their consumption of oxygen (Hannah, Rogerson, and Laybourn-Parry 1994; Linke et al. 1995; Nomaki et al. 2007), yet observations from the past two decades suggest that at least some species are facultative anaerobes (Bernhard 1993; Bernhard, Habura, and Bowser 2006; Geslin et al. 2004; Moodley et al. 1997; Risgaard-Petersen et al. 2006), and are capable of surviving considerable periods of sulfide enrichment (e.g. at concentrations exceeding 10 μ M for weeks; Bernhard 1993; Moodley et al. 1998). In certain cases, foraminiferal populations abound in sulfide-enriched habitats, typically when trace amounts of oxygen are present (e.g. Bernhard, Sen Gupta, and Borne 1997; Bernhard et al. 2000).

Although metazoans that inhabit sulfidic environments such as hydrothermal vents and hydrocarbon seeps generally have specialized organs, biochemistry, and symbionts to permit their survival (reviewed in Grieshaber and Völkel 1998; Somero, Childress, and Anderson 1989; Van Dover and Lutz 2004), protists must encompass in their single cell all of the adaptations necessary to allow their inhabitation of these seemingly adverse environments. While many foraminiferal species that inhabit sulfidic environments have bacterial associates that are considered to be putative symbionts (e.g. Bernhard et al. 2000; Bernhard 2003) and/or sequestered chloroplasts (e.g. Bernhard and Alve 1996; Bernhard and Bowser 1999; Bernhard 2003), a review of published reports and our new observations indicate that peroxisomes are abundant in most chemocline Foraminifera. This contribution presents

details regarding foraminiferal peroxisome occurrence and association with other organelles, and poses an hypothesis as to how these aerobic protists physiologically cope with ROS.

MATERIALS AND METHODS

New data for this communication are reported for *Buliminella tenuata* and *Nonionella stella* collected in Santa Barbara Basin (SBB, centered on 34°16'N, 120°02'W) from water depths of between ~ 580–598 m. These sediments supported copious filaments of the sulfur-oxidizing bacterium *Beggiatoa* (e.g. Bernhard et al. 2003). Additional observations are presented for other foraminiferal species collected from 550 m in the Soledad Basin (off Baja Mexico, 25°12.03'N, 112°43.00'W) and from 2,155 m depth at Blake Ridge (off South Carolina, 32°29.623'N, 76°11.467'W; Table 1). The sediments collected from Soledad Basin supported populations of the sulfur-oxidizing bacterium *Thioploca* (Bernhard and Buck 2004; Prokopenko et al. 2006) and those from Blake Ridge supported a mat of sulfur-oxidizing bacterium, most likely *Arcobacter* (Robinson et al. 2004) and were from an area of active methane seepage (Van Dover et al. 2003).

For ultrastructural analyses, specimens were preserved in 3% glutaraldehyde buffered with 0.1 M Na-cacodylate (pH 7.2) within 1 h of sample collection. Specimens were embedded and stained by standard procedures (e.g. Bernhard and Bowser 1999), and then cut as either ultrathin (90 nm) or semi-thick (250 nm) sections. After staining with uranyl acetate followed by lead citrate, ultrathin sections were examined with either Philips 301, Zeiss EM910, or Zeiss EM902A transmission electron microscopes; some semi-thick sections were examined with the Wadsworth Center's High Voltage Electron Microscope (HVEM).

Two methods were used to confirm the identity of suspected peroxisomes. To determine the spacings within lattices of the crystalline cores of suspected peroxisomes, we obtained high-magnification micrographs of orthogonally sectioned cores. Multiple measurements were made of the width of each core, along with determining the number of crystalline lattices per core. The spacing was calculated as the number of lattices divided by the lattice width. Additionally, some *Buliminella tenuata* specimens from the SBB were processed for diaminobenzidine (DAB) cytochemistry, following procedures of Fok and Allen (1975).

Due to the logistical complexities involved with executing H_2O_2 analyses at sea, we opted to determine whether Foraminifera are exposed to hydrogen peroxide in situ by measuring $[H_2O_2]$ in microcosms constructed from SBB sediments and bottom waters. Sediments were placed, within 5 min of collection, in replicate HDPE containers so that ~ 1–2 cm covered the bottom of the container; SBB bottom water was added to fill the container, which was then tightly capped. These microcosms were maintained in darkness at ~ 5 °C during transport and in the laboratory. The microbial community in some of these containers remained relatively unchanged for weeks to months (JMB, pers. observ.). Approximately 1–2 mo after collection, dialysis cassettes (3,500 mwco) were filled with nitrogen-purged artificial seawater and positioned at the sediment-water interface of each microcosm, and allowed to equilibrate for ~ 1 wk. After the cassette was removed from the microcosm, an aliquot of its contents was analyzed spectrophotometrically for $[H_2O_2]$ using the procedure of Peña et al. (2001). To verify that Foraminifera were living in such conditions, we isolated some specimens, recorded test dimensions, and performed individual extractions for adenosine triphosphate (ATP) following standard procedures (Bernhard 1989; DeLaca 1986). The live-dead threshold (i.e. concentration of ATP per unit shell vol.) invoked was 415 ng ATP mm⁻³ (Bernhard and Reimers 1991), a value originally calculated for the SBB foraminiferal community.

Experiments were conducted to assess foraminiferal response to enriched hydrogen peroxide in terms of cellular energy, i.e. [ATP]. Incubations were done at four concentrations in a nitrogen-flushed glove bag housed in a cold room at 7 °C, approximately the ambient SBB bottom-water temperature (~ 6 °C; http://www.calcofi.org/newhome/data/recent_data.htm). The glove bag also housed a stereo-dissecting microscope to allow ATP extractions without introducing oxygen to the system (Bernhard and Alve 1996). After each 2.5-h incubation, ATP and H₂O₂ analyses followed procedures noted above; dissolved oxygen concentrations were determined using the microwinkler method (Broenkow and Cline 1969) and hydrogen sulfide concentrations were determined using the method of Cline (1969). For each of these experiments, 3–5 *B. tenuata* and 24–26 *N. stella* were extracted individually.

RESULTS

Ultrastructural observations

A striking feature of *Buliminella tenuata* was the presence of numerous peroxisomes associated with smooth endoplasmic reticulum (P-ER) (Fig. 1). The peroxisomal crystalline core was identified in *B. tenuata* from SBB as catalase by measurement of the lattice spacings (Fig. 2). The spacing ranged from 7.9 to 9.8 nm, averaging 8.8 nm (± 0.7 nm; $n = 8$ peroxisomal cores), which is consistent with the known catalase lattice spacing of 8.75 nm (Wrigley 1968). DAB cytochemistry also indicated that the cores of organelles associated with ER in SBB *B. tenuata* contain catalase (Fig. 3). In some micrographs, the peroxisomal membrane appears contiguous with the ER (Fig. 4).

Peroxisome-ER fields appeared quite extensive in *Buliminella tenuata* (Fig. 5) as well as *Nonionella stella* (Fig. 6), with the same complex often noted in multiple serial sections. Complexes occurred in many chambers of polythalamous species, and in *N. stella* and *B. tenuata* often existed in the foraminifer's youngest chamber (i.e. nearest the aperture and accessible to the surrounding environment). Mitochondria were seen interspersed among peroxisomes and ER, commonly appearing to be enveloped by the P-ER complex (Fig. 7). Additionally, P-ER complexes are often noted at the periphery of large vacuoles (Fig. 8). Such vacuolar associations have been observed in *B. tenuata* (from SBB), *B. elegantissima* (from Blake Ridge seep), and *Buliminella* sp. (from Soledad Basin; Bernhard and Buck 2004). Less extensive P-ER complexes were also noted in terminal-chamber frothy cytoplasm of *Globobulimina pacifica* and *Uvigerina peregrina* collected from the San Pedro Basin (Goldstein, S. T. pers. commun.).

A review of the literature shows that specimens of other foraminifers from low-oxygen, sulfide-enriched habitats possess P-ER complexes (Table 1). For example, specimens of *Nemogullmia longevariabilis* and *Gloioegullmia eurystoma*, two allogromiid species collected between 30 and 100 m in Gullmar Fjord, were reported to contain P-ER complexes (Nyholm and Nyholm 1975). Although the physicochemical conditions at the time of collection of these species were not reported, below ~ 50 m, the Fjord is stagnant for much of the year, with water exchange typically occurring every winter (Filipsson and Nordberg 2004); it is therefore possible that the P-ER bearing specimens were collected from the chemocline. A species of *Nemogullmia* also occurs in the Oxygen Minimum Zone (OMZ) off Pakistan (Gooday, A. J., pers. commun.), further implicating the inhabitation of the chemocline by this genus. Specimens of *Uvigerina peregrina* and *Globobulimina pacifica* also contain P-ER (Goldstein, S. T., pers. commun.); these may have been collected from the chemocline since they were obtained from another California borderland basin which occurs in the Oxygen Minimum Zone.

Survivorship response to H₂O₂ exposure

Only SBB microcosms that supported visible growth of the filamentous sulfur-oxidizing bacterium *Beggiatoa* were used for [H₂O₂] determinations because they most closely approximated in situ redox conditions. At the time of measurement, the [H₂O₂] values were as high as 9 μ M and substantial proportions of the *B. tenuata* and *N. stella* populations were alive in each of the three microcosms from which specimens were analyzed (Table 2). Additionally, a few specimens of two other foraminiferal species (*Bolivina spissa* and *Loxostomum pseudobeyrichi*, both of whose ultrastructures are unknown) were living in 2 μ M H₂O₂.

In the short-term experiments to assess the effects of H₂O₂ on foraminiferal ATP concentration, the [ATP] in both *B. tenuata* and *N. stella* was positively correlated with increasing [H₂O₂], up to 16 μ M (Fig. 9). The [ATP] in *N. stella* exposed to 16 μ M H₂O₂ was significantly higher than that in specimens incubated without H₂O₂ (ANOVA, $p = 0.026$); the population of *B. tenuata* was numerically insufficient to permit statistical analysis. The dissolved [O₂] ranged from ~ 2 to 4 μ M, and [H₂S] from ~ 0.9 to 1.0 μ M, in these experiments.

DISCUSSION

Peroxisomes occur as solitary organelles in benthic foraminifera from aerated environments (Goldstein and Richardson 2002; Goldstein, S. T., unpubl. data) and in planktonic foraminiferans (Anderson and Lee 1991; Anderson and Tuntivate-Choy, 1984). The presence of abundant P-ER complexes in benthic Foraminifera inhabiting chemocline sediments suggests that peroxisomes are important adaptations that allow foraminiferal inhabitation of the chemocline.

Peroxisomes, hydrogen peroxide, and environmental oxidative stress

In mussels and fish, peroxisome proliferation has been used successfully as an indicator of ROS-induced oxidative stress resulting from environmental organic contaminant exposure (Cajaraville and Ortiz-Zarragoitia 2006; Ortiz-Zarragoitia, Trant, and Cajaraville 2006). Peroxisome proliferation is rapid, on the order of a few days, in mussels subject to such pollution and reversible subsequent to remediation (Cajaraville et al. 2003). Clearly, the environment influences peroxisomal distributions, at least in certain marine taxa. The observation that foraminiferal [ATP] increased with increasing [H₂O₂], up to 16 μ M (Fig. 3), suggests that peroxisome-laden foraminiferans are active during exposure to such conditions, and may even benefit from such contact.

The H₂O₂ exposures survived by Foraminifera in our experiments exceed those considered typically harmful or lethal to some metazoans (e.g. Abele-Oeschger, Oeschger, and Theede 1994; Mischke, Terhune, and Wise 2001). Surprisingly little has been published on the effects of hydrogen peroxide on protists, other than parasites. Both *Tritrichomonas foetus* (a cattle urogenital tract parasite) and *Perkinsus marinus* (an oyster parasite) can survive [H₂O₂] exposures that are orders of magnitude higher than the experimental concentrations used here, although significant mortality did ultimately occur in response to incubations with H₂O₂ in the millimolar range (Mariane et al. 2003; Schott et al. 2003). An association between peroxisomes and mitochondria, along with ER, was noted for the thecate amoeba *Ovulinata parva* (Anderson, Rogerson, and Hannah 1996), but the ecological range of this species apparently does not include the chemocline.

While concentrations of H₂O₂ in oligotrophic surface waters are typically < 100 nM (Yocis, Kieber, and Mopper 2000), marine coastal surface waters range between 30 to 400 nM (reviewed in Abele-Oeschger et al. 1997). Rain events can raise [H₂O₂] to μ M levels in

surface waters for brief periods (Hanson, Tindale, and Abdel-Moati 2001) and intertidal sandflats typically have concentrations between $\sim 1\text{--}4\ \mu\text{M}$ in the summer (Abele-Oeschger et al. 1997). Little has been published about H_2O_2 in marine chemocline sediments, especially those from the aphotic zone. In such deep-water chemocline settings, data are sparse regarding hydrogen peroxide concentrations, sources, and residence times. In marine surface waters at pH ~ 7.8 , the reaction rate between H_2O_2 and either hydrolyzed Fe^{2+} or organically complexed Fe^{2+} is $3.1 \times 10^4\ \text{M}^{-1}\text{s}^{-1}$ or $\sim 5 \times 10^3\ \text{M}^{-1}\text{s}^{-1}$, respectively (Rose and Waite 2003). In the absence of data directly comparable to ours, we can approximate the residence time of H_2O_2 in SBB pore waters, given that Fe^{2+} ranges from ~ 0 to $50\ \mu\text{M}$ in the surface cm of SBB pore waters (Reimers et al. 1996). Using the H_2O_2 reaction rate determined by Rose and Waite (2003), we find the half-life of H_2O_2 in SBB pore waters to range from 0.04 to 138 s. Importantly, a byproduct of this Fenton reaction is hydroxyl radical, which is highly dangerous to cells (see below). Alternatively, values for the H_2O_2 residence times in Canadian freshwater lakes (Häkkinen, Anesio, and Granéli 2004) can also be used, along with SBB pore-water iron concentrations (Reimers et al. 1996); we estimate a range for H_2O_2 residence time of ~ 1 to 2.5 d in SBB pore waters inhabited by Foraminifera. The half-life for dark decay of H_2O_2 in Atlantic waters is even longer, 5.5 d (Yuan and Shiller 2001). Although the complexation of iron and hydrogen peroxide involves complex catalytic cycling and a precise turnover rate/residence time of H_2O_2 in SBB is not presently known, the rate of H_2O_2 supply in SBB pore waters is potentially both large and important to the biological community. Presumably, similar redox conditions exist in the pore water of all sulfur-oxidizing microbial mats considered here (Table 1).

Proposed model to explain the association of P-ER and mitochondria

The observed association between ER and peroxisomes is readily attributable to the fact that ER contributes to the formation of peroxisomes (Hoepfner et al. 2005; Titorenko and Rachubinski 2001b). As noted, the ER can appear continuous with peroxisomal membranes (Fig. 4).

To potentially explain the observed proliferation of peroxisomes, their intracellular distributions, and their co-occurrence with mitochondria in chemocline Foraminifera, we hypothesize that the oxygen resultant from the conversion of both environmentally and metabolically produced H_2O_2 into water and oxygen is used for foraminiferal respiration (Fig. 10). Foraminifera could thereby utilize oxygen that is recycled from breakdown of both abiological and biological hydrogen peroxide. More specifically, after pore-water H_2O_2 crosses foraminiferal cell membranes, either in the final chamber/apertural region or as vacuolar contents (see below), it would be broken down by peroxisomal catalase (Fig. 10). Additionally, intracellularly produced H_2O_2 , which results from normal metabolic processes of the mitochondria (Lesser 2006) and peroxisomes (reviewed in Fritz et al. 2007; Titorenko and Rachubinski 2001b) would be converted by the peroxisomes to water and oxygen. In chemocline settings, the latter metabolic contributions of H_2O_2 are estimated to be much smaller than the contribution from environmental H_2O_2 resultant from the oxidation of hydrogen sulfide. Chloroplasts, which are sequestered by some foraminiferal species with P-ER complexes (e.g. Bernhard and Alve 1995; Bernhard and Bowser 1999), can also produce H_2O_2 from metabolic processes (Foyer, Lelandais, and Kunert 1994; Ivanov and Khorobrykh 2003).

The subcellular distribution of P-ER complexes suggests that environmental chemistry influences their abundance. More specifically, the occurrence of P-ER in youngest chambers may be because the cytoplasm in those chambers has greater exchange with the environment compared to the cytoplasm of older chambers. Additionally, the vacuoles with abutting P-ER may help deliver H_2O_2 to the peroxisomes, given that intrashell cytoplasm actively roils (McGee-Russell 1974; Travis and Bowser 1991), and assuming active uptake of seawater

into these vacuoles, as hypothesized by Erez (2003). Thus, the dense occurrence of peroxisomes in regions of the Foraminifer with accessible environmental exchange (i.e. younger chambers and at the periphery of vacuoles) could act as a biochemical filter, facilitating the breakdown of environmental H_2O_2 , and thereby protecting the endoplasm from this ROS. In support of this assertion, the free-living amoeba *Chaos carolinensis* accumulated H_2O_2 in its large vacuoles during times of stress (i.e. starvation); it released O_2 when it was exposed to KCN (Deng, Kohlwein, and Mannella 2002). Those authors suggested that the oxygen was generated due to the release of vacuole-bound H_2O_2 and subsequent reaction with cytoplasmic catalase (Deng et al. 2002); such a mechanism mirrors a portion of our model, i.e. that vacuolate hydrogen peroxide serves as a source of recycled oxygen to chemocline foraminiferans.

If chemocline foraminiferans were to rely strictly on oxygen from the conversion of environmental H_2O_2 , would a sufficient supply of oxygen be available for their survival via respiration? Assuming that the $[\text{H}_2\text{O}_2]$ range measured in the SBB microcosms represents a typical range of in situ concentrations (Table 2) and that all environmental H_2O_2 is converted to O_2 , then $\sim 1\text{--}4.5\ \mu\text{M}$ O_2 would be available for respiration. Using these $[\text{O}_2]$ and published respiration rates of foraminiferans from various benthic habitats ($\sim 1\text{--}14\ \text{nmol individual}^{-1}\ \text{day}^{-1}$; Hannah et al. 1994; Linke et al. 1995; Nomaki et al. 2007), we deduce that even dense populations of foraminiferans would be able to reside in SBB pore waters. For example, an assemblage of $100\ \text{specimens cm}^{-3}$ would consume $0.1\text{--}1.4\ \mu\text{M}$ $\text{O}_2\ \text{day}^{-1}$. Because at least some of these species have recently been shown to respire nitrate via denitrification (Risgaard-Petersen et al. 2006), additional metabolic pathways are available for their energy production.

Why would chemocline Foraminifera rely on intra-test respiration when they inhabit pore waters with detectable dissolved oxygen? Because mitochondria are transported throughout pseudopodial networks of Foraminifera (Doyle 1935), respiration can occur outside the organism's test (albeit intracellularly). Given the minute concentrations of available oxygen in the environment and high surface area of a foraminifer's pseudopodial networks, the reliance of the organism on respiration within these pseudopodia would be a reasonable expectation. It is also reasonable that ROS likely inhibit extension of the network because lipid peroxidation results in changes in membrane fluidity and in membrane damage due to the formation of byproducts such as aldehydes and hydrocarbons (Lesser 2006). Importantly, we are not asserting that Foraminifera fail to use environmental oxygen when it is available, but are suggesting that the proposed auxiliary source can significantly augment the amount of accessible oxygen when conditions prohibit a sufficient harvest from the environment. A further consideration is that other aerobes, which are abundant in the SBB microbial mat sediments (e.g. other protists and meiofaunal metazoans; Bernhard et al. 2000), compete for the meager oxygen that is typically present in the pore waters. It will be interesting to determine if any of these other organisms utilize oxygen derived from H_2O_2 .

Two additional scenarios exist to potentially explain the peroxisome proliferation in chemocline foraminifers. For example, as noted, in the presence of Fe^{2+} , the Fenton reaction also converts H_2O_2 to hydroxyl radical, which is highly damaging. Lipid peroxidation caused by hydroxyl radicals is particularly detrimental to mitochondria, affecting both ATP production and enzyme activity (Lesser 2006). Thus, peroxisomes may protect the mitochondria from hydroxyl radicals. Furthermore, in Foraminifers that sequester chloroplasts (e.g. *Stainforthia fusiformis*, Bernhard and Alve 1996; *Nonionella stella*, Bernhard and Bowser 1999; Leutenegger 1984), an additional source of hydroxyl radicals may be chloroplasts (Liu et al. 2004). Neither alternative is mutually exclusive, however, to the posed hypothesis that mitochondria utilize oxygen resultant from hydrogen peroxide breakdown. Additionally, in both metazoans and plants, ROS are also known to play a

critical role in cell signaling for processes such as apoptosis (i.e. programmed cell death; reviewed in Bienert et al. 2006; Schrader and Fahimi 2006); it is unclear what role ROS might have in protistan cell signaling.

Biogeochemical, ecological and evolutionary implications

As recently noted by Preisler et al. (2007), we have yet to fully characterize the processes mediated by microbes that occur in the redox zone of marine sediments. Because protists are major contributors to sedimentary chemocline microbial communities (e.g. Bernhard et al. 2000; Bernhard and Buck 2004; Buck and Barry 1998; Fenchel et al. 1995), it is also important to elucidate the role that these microbial eukaryotes play in the biologically mediated processes of such dynamic biogeochemical settings. For example, our observations suggest that Foraminifera could have substantial effects on iron and hydrogen cycling given their catalase content. In addition, if our hypothesis extends to other aerobic eukaryotes, the volume of sediments that is feasibly habitable must be revised significantly upward for protists and perhaps even metazoans.

Because P-ER complexes are known from monothalamous taxa (*Gloioigullmia*; *Nemogullmia*; Nyholm and Nyholm 1975), which are modern representatives of initial foraminiferal diversification (Pawlowski et al. 2003), it is reasonable that the ability to proliferate peroxisomes evolved early in foraminiferal history.

The timing for the origin of foraminiferan protists is uncertain, although molecular clock estimates indicate an origin between ~ 0.7 and 1.2 Bya (Pawlowski et al. 2003), a period which roughly corresponds to the Neoproterozoic (~ 0.545–1.0 Bya). In the Neoproterozoic, the deep ocean likely displayed widespread anoxia and sulfide enrichment (e.g. Canfield 1998; Condie, Des Marais, and Abbott 2001), atmospheric pCO₂ was as high as 120,000 ppm (Caldeira and Kasting 1992), and massive melting of the extensive “Snowball Earth” glaciers (Hoffman et al. 1998; Kirschvink 1992) may have caused biologically significant concentrations of H₂O₂ to arise in the oceans and atmosphere (Liang et al. 2006). Because certain early-evolving foraminiferans survive considerable periods of anoxia and sulfide enrichment (Bernhard et al. 2006), high pCO₂ (Bernhard et al., in press), and, as demonstrated here, micromolar concentrations of hydrogen peroxide, accumulating evidence suggests that foraminiferan protists had the cellular machinery to allow inhabitation of all inferred Neoproterozoic marine habitats.

Exceptions and future directions

In sum, mounting evidence indicates that chemocline foraminiferans typically have P-ER complexes that are often associated with mitochondria; we hypothesize that the peroxisomes and mitochondria use environmental hydrogen peroxide as a substrate for oxidative phosphorylation. This hypothesis can be tested experimentally in a variety of ways. For example, fluorogenic substances such as JC-9 (Invitrogen Molecular Probes) that differentially react depending on mitochondrial membrane potential, could be employed, although this approach would be challenging for species that sequester or ingest chloroplasts, which autofluoresce at similar wavelengths. Cerium cytochemistry could be executed to establish the subcellular location of hydrogen peroxide; catalase and/or other enzyme activities could be measured during differential redox exposures. Pharmacological approaches could be used to test the response of Foraminifera to catalase inhibitors such as aminotriazole (Allen and Whatley 1978). Finally, we await development of methodologies to identify distribution and concentrations of environmental hydrogen peroxide at scales relevant to these protists.

We note that certain chemocline Foraminifera may appear anomalous to the H₂O₂/P-ER model. In the limited number (1–2 specimens each) of conspecifics examined from cold seeps, some foraminiferans (e.g. *Bolivina spissa*, *Epistominella pacifica*, *Loxostomum pseudobeyrichi*, *Praeglobobulimina spinescens*) had peroxisomes but lacked extensive P-ER complexes (Bernhard, Buck, and Barry 2001). Whereas silled basins like SBB have large volumes of stagnant, oxygen-poor water that is only replenished periodically (e.g. Reimers et al. 1990), oxygen replenishment in smaller-scale habitats such as seeps and vents may occur quickly, perhaps precluding the necessity of those foraminiferans to rely on environmental H₂O₂. Interestingly, peroxisome-endoplasmic reticulum complexes are not seen in an undescribed endobiont-bearing allogromiid foraminiferan inhabiting anoxic, sulfidic sediments below the chemocline (Bernhard et al. 2006). This allogromiid must have other, as yet unidentified, mechanisms for coping with ROS exposure or the anoxia prevents ROS exposure. Clearly, additional studies are required to better understand physiological adaptations of Foraminifera to “extreme” habitats.

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Fig. 1–4.

Transmission electron micrographs of semi-thick (250-nm thick) sections of Foraminifera from the chemocline, showing peroxisomes (p). **1.** Extensive complex with endoplasmic reticulum (*Buliminella tenuata*, SBB). **2.** Catalase core (*B. tenuata*, from SBB). **3.** Complex with endoplasmic reticulum associated with large vacuoles (v) in *B. tenuata* (from SBB), after DAB cytochemistry; m, mitochondria. **4.** Membrane confluence between peroxisome (p) and endoplasmic reticulum (er; *B. tenuata*, from SBB). Scale bars: 1, 3 = 1 μ m; 2, 4 = 100 nm.

Fig. 5-8.

Transmission electron micrographs of chemocline Foraminifera, showing peroxisomes. **5.** Extensive complex with endoplasmic reticulum in the youngest chamber of *Buliminella tenuata* (from SBB); vacuoles are also visible. Test (t, shell) would have been in upper right. **6.** Large P-ER complex in *Nonionella stella* (from SBB); c, sequestered chloroplast; l, lipid; v, vacuole. **7.** Ring complex with endoplasmic reticulum and associated mitochondria (m; *B. tenuata*, from SBB). **8.** Complex with endoplasmic reticulum associated with peripheries of vacuoles (v) (*Buliminella elegantissima*, from Blake Ridge *Arcobacter* mat). Section thickness: 5-7 = 250 nm; 8 = 90 nm. Scale bars: 5 = 10 μm , 6, 7 = 1 μm , 8 = 0.5 μm .

Fig. 9.

Scatter plots showing foraminiferal [ATP] versus [H₂O₂]; data presented for both live (circles) and dead (triangles) specimens. Equations refer to live specimen data. **A.** *Buliminella tenuata*. **B.** *Nonionella stella*.

Fig. 10. Schematic of proposed foraminiferal recycling of oxygen from environmentally and biologically produced hydrogen peroxide. Depicted is a calcareous foraminiferan, but peroxisome-endoplasmic reticulum complexes have also been noted in agglutinated and thecate Foraminifera. Major pathways are indicated by thick arrows. See text for details.

Table 1

Foraminiferal species observed to have peroxisome-endoplasmic reticulum complexes. Also listed is the site, type of microbial mat, approximate water depth from which examined specimens were recovered, and reference where P-ER and/or other ultrastructural characteristics were first noted for that species.

Species	Site	Mat type	Depth (m)	Reference
<i>Bolivina pacifica</i>	SBB	<i>Beggiatoa</i>	576	JMB, unpubl. data
<i>Bolivina</i> cf. <i>subadvena</i>	Soledad Basin	<i>Thioploca</i>	550	This contribution
<i>Buliminella elegantissima</i>	Blake Ridge seep	<i>Arcobacter</i>	2155	This contribution
<i>Buliminella tenuata</i>	Monterey Bay seeps	<i>Thioploca</i>	906, 1003	Bernhard, Buck, and Barry (2001)
	SBB	<i>Beggiatoa</i>	~580 – 598	This contribution
<i>Buliminella</i> sp.	Soledad	<i>Thioploca</i>	550	This contribution
<i>Globobulimina pacifica</i>	San Pedro Basin	unknown	710	Goldstein and Corliss (1994) ^d
<i>Globobulimina</i> sp.	Monterey Bay seep	<i>Thioploca</i>	1003	Bernhard et al. (2001)
<i>Glottogullmitia eurystoma</i>	Gullmar Fjord	Unknown	30 – 100	Nyholm and Nyholm (1975)
<i>Nemogullmitia longevitabilis</i>	Gullmar Fjord	Unknown	30 – 100	Nyholm and Nyholm (1975)
<i>Nonionella stella</i>	SBB	<i>Beggiatoa</i>	~580 – 598	Bernhard and Reimers (1991)
<i>Stainforthia fusiformis</i>	Drammensfjord	<i>Beggiatoa</i> ^b	45	Bernhard and Alve (1996)
<i>Uvigerina peregrina</i>	San Pedro Basin	unknown	710	Goldstein and Corliss (1994) ^d
<i>Virgulinitella fragilis</i> ^c	Cariaco Basin	<i>Beggiatoa</i>	244	Bernhard (2003)

^ap-ER observed in unpublished micrographs by S. T. Goldstein

^bAlve, E., pers. commun.

^cOnly ER fields were visible but fixation and staining were suboptimal so peroxisomes and ER may co-exist

Table 2

Percent (%) living of those *Nonionella stella* and *Buliminella tenuata* individuals from which ATP was extracted; number in parentheses indicates number of specimens of that species extracted per microcosm. The Period header denotes the number of days between sample collection from the Santa Barbara Basin and [H₂O₂] determination and ATP extractions.

[H ₂ O ₂] (μM)	Period (days)	<i>N. stella</i> % live (n)	<i>B. tenuata</i> % live (n)
2.0	58	55 (29)	100 (4)
7.6	59	20 (50)	100 (1)
9.0	47	53 (19)	50 (4)