RUNNING TITLE: Microaerophilic ectobiont-bearing foraminiferan

An ectobiont-bearing foraminiferan, *Bolivina pacifica*, that inhabits microxic pore waters: Cell-biological and paleoceanographic insights

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SUMMARY

The presence of tests (shells) in foraminifera could be taken as an indicator that this protist taxon is unlikely to possess ectosymbionts. Here, however, we describe an association between *Bolivina pacifica*, a foraminiferan with a calcareous test, and a rod-shaped microbe (bacterium or archaeon) that is directly associated with the pores of the foraminiferan's test. In addition to these putative ectosymbionts, *B. pacifica* has previously undescribed cytoplasmic plasma membrane invaginations (PMIs). These adaptations (i.e., PMIs, ectobionts), along with the clustering of mitochondria under the pores and at the cell periphery, suggest active exchange between the host and ectobiont. The *B. pacifica* specimens examined were collected from sediments overlain by oxygen-depleted bottom waters (0.7 μM) of the Santa Barbara Basin (SBB; California, USA). An ultrastructural comparison between *B. pacifica* from the SBB and a congener (*Bolivina* cf. *B. lanceolata*) collected from well-oxygenated sediments (Florida Keys) suggests that PMIs, ectobionts, and peripherally distributed mitochondria are all factors that promote inhabitation of microxic environments by *B. pacifica*. The calcitic $\delta^{13}C$ signatures of *B. pacifica* and a of co-occurring congener (*B. argentea*) that lacks ectobionts differ by >1.5‰, raising the possibility that the presence of ectobionts can affect incorporation of paleoceanographic proxies.

INTRODUCTION

The Foraminifera are rhizarian protists with an important fossil record whose extant members are, based on available biological evidence, aerobes. All living foraminifera examined to date possess mitochondria (reviewed in Anderson and Lee, 1991; Goldstein, 1999), and oxidative energy metabolism has been demonstrated experimentally in the model foraminiferan *Allogromia* (Hofer *et al*., 1972; Travis and Bowser, 1988). The numerical density of most benthic species of foraminifera is highest in oxygenated surface sediments (e.g., Rathburn *et al*., 1996). Furthermore, ecological studies indicate that, along with food availability, oxygen has governed benthic foraminiferal distribution in modern and ancient oceans (e.g., Jorissen, 1999). Given these observations, foraminiferal success in environments containing only trace concentrations of dissolved oxygen (reviewed by Bernhard and Sen Gupta, 1999) is enigmatic.

It has yet to be established how foraminifers in microxic (i.e., trace to 0.1 ml/L; sensu Bernhard and Sen Gupta, 1999) environments obtain sufficient oxygen to respire, although a variety of mechanisms has been postulated. Among these possibilities are: adaptations in test structure, so as to facilitate oxygen transport within the test (Verhallen, 1986); aggregation of mitochondria at the cell periphery (Leutenegger and Hansen, 1979); and reliance on anaerobic metabolic pathways for limited periods (e.g., Bernhard and Reimers, 1991; Moodley *et al*., 1997; Risgaard-Petersen *et al*., 2006).

Certain benthic foraminifera are abundant in sulfide-enriched settings (e.g., Bernhard *et al*., 2006a). While we continue to learn more about foraminiferal adaptations to oxygen depletion and microxia, the effects of hydrogen sulfide -- a potent inhibitor of critical aerobic respiratory enzymes -- on foraminifera have less often been studied. Problems confronting

aerobic inhabitants of oxygen-depleted, sulfidic habitats, include the potential lack of an electron acceptor and possible exposure to compound(s) that inhibit critical cellular processes.

Endosymbionts have long been known to aid aerobic inhabitants of sulfidic habitats (e.g., reviewed by Stewart *et al*., 2005). One of the best studied symbioses of marine organisms inhabiting a sulfidic chemocline are hydrothermal vent tube worms and hydrocarbon seep bivalves, in which endobionts oxidize hydrogen sulfide to allow continued aerobic respiration (e.g., also reviewed in Stewart *et al*., 2005). Other organisms, including protists, inhabiting chemocline habitats have endobionts (e.g., Fenchel and Finlay, 2008), although except for methanogens in some ciliates (e.g., Finlay and Fenchel, 1993), the identity and physiology of endobionts in protists in sulfidic habitats are largely unknown.

Foraminifera are promiscuous hosts of endosymbionts. In well-aerated environments, many planktonic foraminifera and some shallow-water so-called "larger" foraminifera have long been known to harbor photosynthetic endosymbionts (reviewed by Hemleben *et al*., 1989; Lee and Anderson, 1991; Hallock, 1999). Certain chemocline benthic foraminifera are known to support endobionts (e.g., Bernhard 1993; Bernhard and Reimers 1991; Bernhard *et al*., 2000; 2006a; Bernhard, 2003). To date, only one report documents ectobionts on a single specimen of the foraminifer *Uvigerina peregrina* (Bernhard *et al*., 2001), although there is precedence for the occurrence of ectobionts in the protistan supergroup Rhizaria, to which foraminifera belong: one member of the foraminiferan sister taxon, *Gromia*, was previously shown to harbor microbial ectobionts while inhabiting low-oxygen pore waters (0.2 ml/L; Gooday and Bowser, 2005).

Here we describe the cellular ultrastructure of the foraminifer *Bolivina pacifica* collected from an oxygen-depleted, often sulfidic region of the Santa Barbara Basin (SBB; California, USA). The specimens examined consistently harbored microbial (eubacteria or archaea, *sensu*

Pace, 2006) ectobionts associated with pores in the test. We compare the ultrastructure of *B. pacifica* to the congener *Bolivina* cf. *B. lanceolata*, collected from well-aerated sediments of the Florida Keys, and identify pathways that could be employed by *B. pacifica* to maximize oxygen transport to mitochondria. We also examine the stable isotope signatures in tests of *B. pacifica* and the co-occurring congener *B. argentea* (which lacks microbial ectobionts). Together, our results further define foraminiferal adaptations to life in low-oxygen settings, and provide a cautionary note to paleoceanographers using the stable carbon isotope signatures of *Bolivina* tests to infer past oceanographic conditions.

RESULTS

Bolivina pacifica morphology and ultrastructure

At the time of specimen collection for cytological examination, the overlying bottom-water oxygen concentration ($[O_2]$) in the SBB was detectable, but quite low, at 0.7 μM (~ 0.02 ml O_2/L). The surface of the box core from which the specimens were obtained was brown and had visible, but rare, *Beggiatoa* colonies on its surface (Fig. 1A). Syringe cores taken from the box core revealed that the brown layer extended to at least 1-cm depth (Fig. 1B). The four replicate box cores from the site that were profiled with microelectrodes, approximately one year later when bottom-water oxygen was also <1 μM, all had detectable, but low, oxygen in pore waters, to a sediment depth of at least 1 cm (data not shown).

Bolivina pacifica, which were obtained from the surface 1 cm, had well-developed pores in most of its test (Fig. 2A); areas that lacked discernible pores include the proloculus, sutures, and the apertural portion of the two youngest chambers. The average diameter of the pores was 2.9±0.4 μm (n = 40 pores). Closer examination of decalcified specimens using SEM revealed

that the pore plugs, which are thickenings of the organic lining situated under the pores (Leutenegger and Hansen, 1979), were commonly vested with rod-shaped microbes (Fig. 2B-D). Approximately 41% of the pores had one or occasionally two, rod-shaped ectobionts (n=123 pore plugs counted in five SEM micrographs of multiple specimens; Fig. 2C). SEM showed that the average length of the ectobionts was 1.6±0.3 µm (n= 32 ectobionts). Higher-magnification examination of individual pore plugs indicated that their surface was veiled with fine fibrils that were not visible on non-plug regions of the surface (Fig. 2D). Ectobionts were not observed elsewhere on the cell.

Examination of *Bolivina pacifica* by TEM revealed intact Golgi bodies, peroxisomes, endoplasmic reticulum (Fig. 3), and mitochondria with tubular cristae (Fig. 4-7). Pore plugs were composed of two distinct layers: (*i*) a microporous plate, and (*ii*) an overlying granulofibrillar basal plate (sensu Leutenegger and Hansen, 1979) decorated with surface fibrils. Tubular plasma membrane invaginations (PMIs) were typically seen beneath, but not between, the pores (Figs. 4, 5). Higher magnification views showed that these PMIs were in close apposition with mitochondria (Fig. 5), which appeared concentrated under the pore plugs (Fig. 4E, 6). To illustrate this point, Figure 6 show portions of a transect across one specimen. In this example, four times more mitochondria were found in the cytoplasm within 10 µm of the exterior test wall compared to the cell interior. Finally, TEM confirmed that ectobionts were often associated with the exterior surface of the basal plate (Fig. 4A-B). Unlike SEM, however, up to four ectobionts were seen, together with debris (Figs. 4E, F). TEM also revealed that the average length of the ectobionts was 1.8±0.3 µm (n= 5 ectobionts) in full transverse section. In some cases, ectobionts appeared to be enveloped by fibrils and amorphous matrix (cf. Figs. 4A-D). Pore plugs without visible ectobionts had mitochondria, fibrils, and PMIs (Fig. 7).

Comparisons to congeners

The foraminiferal specimens examined from the Florida Keys and those from the SBB are congeners: at the gross morphologic scale, they meet all criteria for classification in the genus *Bolivina* (Loeblich and Tappan, 1987; Revets, 1996). Unlike *B. pacifica*, however, the *Bolivina* cf. *B. lanceolata* specimens, which were collected from a well-aerated environment, did not have ectobionts, PMIs, pore-plug associated mitochondria, or pore-associated fibrils (Fig. 8). The average pore diameter of *Bolivina* cf. *B. lanceolata* was 0.9±0.1 μm (n = 8 pores from three specimens).

The $\delta^{13}C$ of tests of live *Bolivina pacifica* was -2.67‰ (n=1 analysis of 50 pooled specimens) and that of co-occurring live *B. argentea* was -1.06‰ (n=3 analyses, each of 8 pooled specimens). The $\delta^{18}O$ of *B. pacifica* tests was within 0.02‰ of the mean *B. argentea* carbonate value (2.33‰; n=3 pooled analyses).

DISCUSSION

Because representatives of all other major SBB taxa support ectobionts (e.g., flagellates such as *Calkinsia aureus*; ciliates such as *Metopus verrucosus*; metazoan meiofauna such as the nematode *Desmodora masira* and the polychaete *Xenonerilla bactericola*; Bernhard *et al*., 2000; Müller *et al*., 2001) and because many foraminifera are known to harbor endobionts (e.g., Bernhard, 1993; 2003; Richardson and Rütlzer, 1999), there is a strong likelihood that ectobionts would occur on at least one foraminiferal species inhabiting SBB.

The presence of intact organelles in the cytoplasm of *Bolivina pacifica* demonstrates that these foraminiferal specimens were alive at the time of collection, when bottom-water oxygen was nearly undetectable (0.7 μM O_2). Although one might expect a steep dissolved oxygen

gradient in these sediments (e.g., Glud *et al*., 1994), the porosity of SBB sediments is very high (>90%, Reimers *et al*., 1990) and evidence of microbioturbation in SBB sediments exists (e.g., Pike *et al*., 2001; Bernhard *et al*. 2003), thereby making it likely that trace amounts of dissolved oxygen occur in the surface cm where these foraminifera were living. Thus, *B. pacifica* is able to inhabit a microxic environment (Bernhard and Sen Gupta, 1999). Furthermore, the co-occurrence of *Beggiatoa,* albeit in low abundance, suggests that *B. pacifica* tolerates sulfidic conditions or that the *Beggiatoa* oxidizes the hydrogen sulfide (using either oxygen or nitrate, e.g., Fossing *et al*. 1995; McHatton *et al*. 1996), keeping it below *B. pacifica*'s tolerance threshold. Although the bottom waters of the SBB have temporally variable oxygen and sulfide concentrations that affect the community structure and abundance of the benthos (Bernhard *et al*., 2003), *B. pacifica* is generally absent when bottom-water oxygen is undetectable (Bernhard and Reimers, 1991; Bernhard *et al*., 2006a), common when $[O_2]$ ~1-15 μM, and rare when $[O_2]$ >20 μM (reported as *B. seminuda*, Bernhard, 1990; Bernhard *et al*., 1997; Fig. 9). Along the sampled depth transect and associated oxygen gradient, the maximum abundance of *B. pacifica* in the SBB was 56.3 specimens·cm^{-3}, when overlying bottom-water oxygen was 1.2 μM, at a water depth of 578 m (Bernhard *et al*., 1997). In the SBB, therefore, the highest abundances of *B. pacifica* typically occur on the edge of the basin, and/or when oxygen is limited but detectable.

Although fixation and embedding procedures differed between the SBB and Florida Keys *Bolivina* species (see Experimental Procedures), we are confident that ectobionts, PMIs, pore-associated mitochondria, and fibrils would have been observed in our *Bolivina* cf. *B. lanceolata* specimens if they displayed these cellular adaptations.

Ectobionts

The consistent association of ectobionts with the pore plugs of *Bolivina pacifica,* contrasted with the lack of ectobionts on non-pore regions of the plasma membrane, indicates that the microbes are not environmental contaminants arising from the preparation procedure. We therefore conclude that the ectobionts are symbionts. Lacking direct demonstration that metabolic exchange occurs between the foraminiferal host and its microbial associates, the relationship between *B. pacifica* and its ectobionts must, however, be considered a putative symbiosis (as indeed are all currently described foraminifer-bacterial endobiont associations). It is of course alternatively possible that the ectobionts are commensals, or even parasites. We have not yet established whether the ectobiont taxon also occurs as a free-living population in the SBB sediments, or whether it exclusively inhabits *B. pacifica* pore plugs. Given the relatively low density of ectobionts, de novo synthesis and supply of essential vitamins is plausible while substantial transfer of organic carbon to the host is unlikely.

Few foraminiferal species from well-oxygenated, deep-sea habitats have been examined for their cell biology, and thus also for ectobionts. In those cases (e.g., *Bulimina mexicana, Buliminella tenuata*, *Globobulimina pacifica*, *Uvigerina peregrina*; Bernhard et al., in preparation), we have not seen ectobionts as observed in *B. pacifica*. Quite a few foraminiferal species from the redoxcline have been examined for their cytology (e.g., Bernhard and Reimers, 1991; Bernhard *et al*., 2000; 2001; 2006a), and none have exhibited consistent ectobionts. Importantly, however, the ultrastructure of only a few percent of extant benthic foraminiferal species has been examined. In sum, we believe that *B. pacifica* is a rarity, although we expect to find more foraminifera with ectobionts in the future (ectobionts have recently been observed on specimens of *C. wuellerstorfi* from hydrocarbon seeps; JMB upubl).

The identity and physiology of the *Bolivina pacifica* ectobionts have not yet been established. The candidate microbes include both aerobic and anaerobic chemolithoautotrophs (such as certain sulfide oxidizers) and chemoorganoheterophs (regular "heterotrophs"). Unfortunately, the ectobionts lack distinctive sub-cellular structures that could provide an initial clue to phylogeny. The ectobionts of the ciliates *Metopus contortus* and *Caenomorpha levanderi* and the endobionts of the foraminifer *Virgulinella fragilis*, all of which inhabit similar sulfide-enriched environments, are reported to be sulfate-reducing bacteria (Fenchel and Ramsing, 1992; Tsuchiya *et al*., 2006). Although it would be peculiar for a presumptive aerobe to have symbionts that produce a metabolic byproduct (i.e., hydrogen sulfide, e.g., Smith *et al*., 1977) toxic to the host, mitochondria are known to have mechanisms to detoxify sulfide (reviewed by Bagarinao, 1992). Conversely, given that the symbionts of many organisms in sulfide-rich environments are sulfur/sulfide oxidizing bacteria (e.g., Goffredi and Barry, 2002; Ott *et al*., 2004), the ectobionts of *B. pacifica* could be sulfide oxidizers or sulfur oxidizers. Alternatively, given the example of some nematodes that harbor denitrifying bacterial ectosymbionts (Hentschel *et al*., 1999), the *B. pacifica* ectobionts could be denitrifiers; in support of this idea are recent observations that some non-symbiont-bearing foraminifera from similar habitats perform complete denitrification (Risgaard-Petersen *et al*., 2006; Høgslund *et al*., 2008). Dedicated studies to determine the molecular phylogeny and physiological capabilities of the microbial partner in this foraminifer-ectobiont consortium should include fluorescent in situ hybridizations (FISH), to confirm any identity implied by 16S rDNA sequencing.

The fibril structures on the surface of the pores could be appendages of the ectobionts termed pili or fimbriae, which are known to promote cell adhesion to abiotic and biotic surfaces (e.g., Gohl *et al*., 2006), and typically play an important role in biofilm formation for Gram-

negative bacteria (e.g., Klausen *et al*., 2006). The amorphous material that sometimes appears to envelope the ectobionts is probably an extracellular polymeric substance (EPS; also known as exopolysaccharide) that comprises a significant proportion of bacterial biofilms (e.g., Wimpenny *et al*., 2000). Biofilms have been postulated to be important regulators of symbioses between soil bacteria and leguminous plants (Skorupska *et al*., 2006), and some anaerobic ciliates are known to have firmly attached sheaths, perhaps composed of EPS, that harbor ectosymbionts (e.g., Finlay *et al*., 1991).

Mitochondrion-Pore Associations

Bolivina from well-aerated sediments (i.e., *Bolivina* cf. *B. subexcavata* from San Pedro Basin, CA, Leutenegger and Hansen, 1979; *Bolivina* cf. *B. lanceolata* from the Florida Keys, this study) lack well-defined mitochondrion-pore associations; also, the mitochondria are more evenly distributed throughout the cytoplasm. Interestingly, however, *B. argentea*, another congener that like *B. pacifica* inhabits oxygen-deficient sediments, was previously recognized to have pore-associated mitochondria (Leutenegger and Hansen, 1979). Thus, *Bolivina* appears to be plastic with regard to mitochondrial distribution; the configuration seems to depend on oxygen availability and/or some other factor(s) that co-vary with oxygen concentration (e.g., hydrogen sulfide concentration). It is important to note, however, that mitochondria in *B. pacifica* are not exclusively associated with the pores. The phenomenon of pore-associated mitochondria is also exhibited by some other genera that inhabit oxygen-depleted environments (i.e., *Buliminella, Loxostomum*; Leutenegger and Hansen, 1979), although mitochondria in other foraminiferans (e.g., *Stainforthia fusiformis*; Bernhard and Alve, 1996) are not exclusively associated with pores.

Leutenegger and Hansen (1979), postulating that mitochondrial clusters under pores cause an oxygen diffusion gradient across the pores into the cytoplasm, inferred a respiratory function for the pores. Indeed, uptake of the soluble dye neutral red through pores of one foraminiferal species was thought to indicate that pores play a role in gas exchange (Berthold, 1976). Importantly, however, foraminifera from more strongly sulfidic regions of the SBB lack peripheral mitochondria (e.g., Bernhard *et al*., 2006a), perhaps because these foraminifera have alternative adaptations to the more severe reducing conditions (e.g., Bernhard *et al*., 2006a; Bernhard and Bowser, 2008).

The presence of ectobionts and/or PMIs with associated mitochondria could explain why tests of certain benthic foraminifera that inhabit oxygen-depleted pore waters have large pores (e.g., Moodley and Hess, 1992), contrary to earlier assumptions about oxygenation levels and pore size (reviewed by Boersma and Mikkelsen, 1990). Our comparison of the *B. pacifica* and *Bolivina* cf. *B. lanceolata* suggests that larger pores may provide an adaptive advantage for associations with ectobionts. Further studies of the ectobiont and mitochondrial distributions with respect to pore diameter are required in additional taxa, before this morphologic attribute can be considered a useful and reliable micropaleontologic proxy.

Plasma Membrane Invaginations

Except in the case of *Bolivina pacifica*, the published micrographs of cytoplasm from *Bolivina* congeners inhabiting either oxygen-depleted or aerated environments (this study; see also Leutenegger and Hansen, 1979) show no evidence of ectobionts or PMIs. To our knowledge, PMI-like structures have not been previously reported in other protists (A.G.B. Simpson, pers. comm. 2007) or other foraminifera, although conduits in the extracellular matrix

of a SBB flagellate (*Calkinsia aureus*) have very recently been postulated to facilitate metabolic exchange between ectobionts and host (Yubuki *et al*., 2009).

Pores are considered to be far more permeable to dissolved gases and ions than are carbonate foraminiferal tests (Berthold, 1976). Nevertheless, pores provide a considerable barrier to oxygen diffusion, given the relatively slow diffusion rates of oxygen across membranes, as compared to diffusion through water (Ivanov et al., 2004). The PMIs can be likened to oxygen transport channels, which are hypothesized to exist due to oxygen's low permeability coefficient in membranes (Ivanov et al., 2004). On the basis of (i) the relatively thick structures at pore terminations (i.e., the basal and microporous plates; Figs. 3-5), (ii) the low permeability of oxygen through membranes compared to water, and (iii) the intimate structural dispositions of mitochondria and PMIs, it is reasonable to postulate that the PMIs relate to oxygen transport.

If the ectobionts are aerobes, then they must be competing with foraminiferal mitochondria for oxygen. Perhaps, the PMIs promote the coexistence of aerobic ectobionts and mitochondria in oxygen-limited environments such as the SBB, by allowing more efficient oxygen partitioning between these constituents, permitting a sufficient supply to the mitochondria. If the ectobionts are anaerobes, then competition for oxygen should not occur, but the PMIs could nevertheless facilitate efficient delivery of oxygen to the mitochondria beneath the pore plates.

To assess the potential competition between the mitochondria and hypothetically aerobic ectobionts for oxygen (which was very low—0.7 μM—at the time of sampling), we used published respiration rates and estimated oxygen consumption rates to approximate and compare the two components' oxygen demands. For *Bolivina pacifica,* a respiration rate of 0.9 (±0.3)×10^{-}

^{9} mol O_2 day^{-1} $individual^{-1}$ was calculated for specimens collected from aerated pore waters at 1430 m (Nomaki *et al*., 2007). This rate, along with values for surface area and pore density taken from SBB *B. pacifica*, was used to estimate that the mitochondria under each pore require 1.3×10^{-13} mol O_2 day^{-1}, assuming a lack of respiration in pseudopodia. Oxygen consumption rates for sulfide-oxidizing bacteria have rarely been published, so we used a rate of 6.9×10^{-16} mol O_2 day^{-1} μm^{-3} (calculated from Nelson *et al*., 1986 and unpubl. data, D.C. Nelson, pers. comm. 2008), along with measurements from our SEM micrographs, to estimate that the average *B. pacifica* ectobiont (0.6225 μm^3, n=14 ectobionts) requires 4.3×10^{-16} mol O_2 day^{-1}. Thus, even if a given pore has up to four ectobionts, as seen by TEM, the foraminiferal respiration demand at each pore exceeds the demand of ectobionts by about three orders of magnitude. The inference is that mitochondria require an efficient means of obtaining sufficient oxygen to support foraminiferal aerobic respiration.

The *B. pacifica* abundance (Fig. 9) and respiration rate (Nomaki *et al*., 2007) can be used to estimate that when bottom-water oxygen is ~ 1μM, the *B. pacifica* population consumes 0.05 μmol O_2 cm^{-3} day^{-1}. Thus, *B. pacifica* are using about one twentieth of the bottom-water oxygen. Diffusion or active pore-water exchange by metazoan meiofauna and ciliates (Aller and Aller, 1992; Glud and Fenchel, 1999) presumably replenishes trace amounts of oxygen into sediments. Assuming a respiration rate for co-occurring foraminifera to be similar to that of *B. pacifica* and using published foraminiferal densities (Bernhard *et al*., 1997), the total oxygen demand of foraminifera at this site is approximately 0.3 μmol O_2 cm^{-3} day^{-1}. The plethora of additional aerobes in SBB sediments (Bernhard *et al*., 2000; 2003), however, likely gives rise to considerable competition for oxygen in these pore waters, lending yet more support to the idea that *B. pacifica* mitochondria require an efficient mechanism for oxygen uptake.

Paleoceanographic Implications

The stable isotope signatures of *Bolivina* tests are often used for paleoceanographic reconstructions (e.g., Kennett *et al*., 2000; Stott *et al*., 2002; Hill *et al*., 2003; Holsten *et al*., 2004; Douglas and Staines-Urias, 2007). However, different species of *Bolivina* from the same sample can have considerably different carbon isotopic signatures (e.g., Stott *et al*., 2002; Holsten *et al*., 2004). The $\delta^{13}C$ signature in carbonate of most calcareous foraminifera differs from the $\delta^{13}C_{DIC}$ of the pore waters bathing the specimen at the time of collection (e.g., McCorkle *et al*., 1990; 2008). These discrepancies have been attributed largely to microhabitat effects, where the scales of sampling may be insufficient to assess fidelity of proxy incorporation, and to poorly understood biological mechanisms termed "vital effects" (e.g., McCorkle *et al*., 1990; 2008). Available data suggest the magnitude of vital effects to be species-specific (e.g., Hintz *et al*., 2006; McCorkle *et al*., 2008). Vital effects likely have multiple causes, such as calcification rate, diet, migration patterns, that may or may not be consistent within any given population or between populations.

The two *Bolivina* species (*B. pacifica* and *B. argentea*) analyzed here for isotopes both have pore-associated mitochondria (Leutenegger and Hansen, 1979, this study), but they differ in terms of ectobiont occurrence. Although the $\delta^{13}C$ gradients in SBB pore waters are steep, and although a difference of a few mm in calcification depth can cause isotopic differences in co-occurring foraminifera (assuming equilibrium calcification; Stott *et al*., 2002), our data indicate that differences in the $\delta^{13}C$ signatures of congeners from the same sample can instead arise from the presence/absence of ectobionts. The presence of ectobionts could cause the $\delta^{13}C$ signature to be depleted relative to the value for the surrounding pore waters, as has been observed for an endobiont-bearing foraminifer, *Virgulinella fragilis* (Bernhard, 2003). Thus, when the stable

carbon isotope signatures of *Bolivina* are used to reconstruct paleoceanographic conditions, knowledge of a species' association with ectobionts may help refine paleoceanographic interpretations.

CONCLUSIONS

Although microbial *endo*symbioses are well known in foraminifera, the present study is the first to document foraminiferal *ecto*bionts. The PMIs characterized here in *B. pacifica* may represent another novel adaptation of foraminiferan protists that promotes their inhabitation of oxygen-depleted habitats. Our finding of an intimate association between foraminifera and ectobionts allows further insights into the complex microbial interactions and physiological processes occurring in the chemocline of marine sediments, and may be important in refining paleoceanographic interpretations that utilize geochemical data of *Bolivina* tests.

EXPERIMENTAL PROCEDURES

Samples for cell biological examination were collected from laminated sediments from the SBB using a Soutar boxcorer in September 1998 (576 m water depth; 34°17.65N, 120°01.99W; California USA). Bottom-water samples were taken in a Niskin bottle that was attached to the corer frame and rigged to trigger as the corer closed at the seafloor. Bottom-water samples were analyzed for dissolved oxygen by the microwinkler method (Broenkow and Cline, 1969). Profiles for pore-water oxygen were not measured directly at the time of foraminiferal collection, but profiles from the same site are available for September 1999 (i.e., one year after foraminiferal collections). Dissolved oxygen profiles were obtained with needle electrodes using methods described by Visscher *et al*. (1991; 2002).

As soon as possible after the box core was secured on deck, various subsamples were removed and taken into an environmental room with a temperature approximating ambient bottom conditions (~6^{o}C). For this study, a 60-cc syringe core (2.5 cm inner diameter) was sectioned at 1-cm increments, to a depth of 3 cm. The sections were preserved in 3% glutaraldehyde buffered with 0.1 M Na-cacodylate (pH 7.2). In the shore-based laboratory, sediments were sieved with chilled buffer over a 63-µm screen. Specimens of *Bolivina pacifica* were isolated from the coarser fraction (i.e., >63 µm) from the 0-1 cm core interval, and were prepared for electron microscopy using our standard procedures (e.g., Bernhard *et al*., 2000), with the exception that some specimens were not completely decalcified. Some specimens for scanning electron microscopy (SEM) were decalcified in buffer prior to critical point drying. Eight specimens were examined with variable-pressure, field-emission SEM with a LEO 1550vp FEGSEM; thin (90-nm) sections of three specimens were examined by transmission electron microscopy (TEM) in a Zeiss 910 or a Zeiss EM 902A. Semi-thick (250 nm) sections of some specimens were examined using a high voltage electron microscope (HVEM); some sections were imaged with a Zeiss Axiovert 40C with attached Olympus DP70 digital camera.

For comparison, the ultrastructure of congeners from well-aerated environments was examined. *Bolivina* cf. *B. lanceolata* was isolated in March 2001 from fine-grained carbonate sediments, including the sediment-water interface of a sea-grass bed, adjacent to Little Duck Key, Florida (USA), from a water depth of <1 m. Living specimens (i.e., those with orange cytoplasm and/or visible pseudopodial activity) were selected for TEM and were prepared using high-pressure freezing followed by freeze substitution (HPF/FS) according to the protocols of Goldstein and Richardson (2002). For this study, the ultrastructure of five specimens was examined with a Zeiss EM 902A.

Co-occurring live *B. pacifica* and *B. argentea* specimens were analyzed for their stable carbon and oxygen isotope signatures. The sediment sample, which was collected from 430-m water depth in the SBB in June 2008, was incubated in the laboratory in CellTracker Green CMFDA (Invitrogen; 1 μM final concentration) for ~10 hr near in situ temperature (7°C). Fluorescent (live; Bernhard *et al*., 2006b) specimens were isolated, cleaned of adherent material, rinsed in distilled water, and air dried. Stable isotopes were analyzed from pooled specimens (*B. pacifica*, n=50; *B. argentea*, n=8) using a Kiel III Carbonate Device connected to a Finnigan MAT 253 mass spectrometer system; data are expressed relative to the Vienna Peedee belemnite (VPDB) standard.

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FIGURE LEGENDS

Figure 1. A. Photograph of intact Soutar boxcore from which specimens for TEM were isolated. Each side of the corer, which still contains overlying bottom water, is 33 cm. B. Representative syringe core (2.9-cm outer diameter) from the boxcore shown in A. Note that the brown oxidized zone extends well below 1-cm depth.

Figure 2. SEM micrographs of *Bolivina pacifica*. A. Overview of entire test, showing well-defined pores. B. Cytoplasm of a decalcified specimen, in which pores appear as elevated features. The structure at the aperture was probably a feeding bolus. C. Higher-magnification view of a decalcified specimen's plasma membrane exterior, showing elevated pore plugs, many of which are overlain by rod-shaped bacteria. D. Close-up view of two pores showing associated bacterial ectobionts and fibrous nature of the pore surface. Scale bars: A = 50 μm; B = 20 μm; C = 2 μm; D = 1 μm.

Figure 3. TEM micrographs of selected *Bolivina pacifica* organelles. A. Paired Golgi bodies. v = vacuoles. B. Peroxisomes (p) and endoplasmic reticulum (er). Scale bars: A = 1 μm; B = 0.5 μm.

Figure 4. TEM micrographs of *Bolivina pacifica* showing peripheral cytoplasm and layered pore plugs. A. Pore structure consisting of basal plate (bp), microporous plate (mp), and fibrils (f). Note the three overlying bacterial cells (b) that are enveloped by fibrous material, as well as mitochondria (m) and a plasma membrane invagination (arrowhead). B-D. Sections showing rod-like shape of ectobionts and multiple tubular plasma membrane invaginations extending

toward mitochondria. Electron-opaque material is remaining calcite of test (t). E. Lower-magnification image showing a concentration of mitochondria under a pore, in addition to pore-associated inorganic debris (d). F. Another image showing mitochondria under pore, with ectobionts and associated inorganic debris. Scale bars: A-D = 0.5 μm; E, F = 1 μm.

Figure 5. HVEM stereo-pair of *Bolivina pacifica* pore-plug ensemble and underlying cytoplasm. Stereo views emphasize multiple PMIs and underlying mitochondria. Note one especially well-developed PMI (arrowhead), leading through the basal layer into the cytoplasm; other PMIs lead into mitochondria. Scale bar = 0.5 μm.

Figure 6. Transect across *Bolivina pacifica*. A. Phase-contrast light micrograph of 0.25-um-thick section. Black bar across specimen denotes areas where HVEM micrographs were taken. B. HVEM micrograph of ectoplasm, showing pore plug and mitochondria. C. HVEM micrograph taken at the cell interior. D. HVEM micrograph of ectoplasm at the opposite side of the specimen. Scale bars: A = 50 um; B = 1 um (C, D are the same scale).

Figure 7. TEM micrograph showing cross section through an ectobiont-free pore of *Bolivina pacifica.* Note the presence of mitochondria and plasma membrane invaginations. Scale bar = 0.5 μm.

Figure 8. TEM micrographs of *Bolivina* cf. *B. lanceolata,* showing peripheral cytoplasm and pore plugs. Note the lack of PMIs and pore-associated mitochondria (m) in both A and B, and the Golgi body under the pore in B. v = vacuoles; t = test. Scale bars: A = 1 μm; B = 0.5 μm.

Figure 9. Density of *Bolivina pacifica* as a function of dissolved O_2 in overlying bottom waters at the time of sampling. Note the absence of specimens during anoxia, and the rare occurrence when $[O_2] > \sim 15$ μM. Data from Bernhard *et al*. (1997), originally reported as *B. seminuda*.

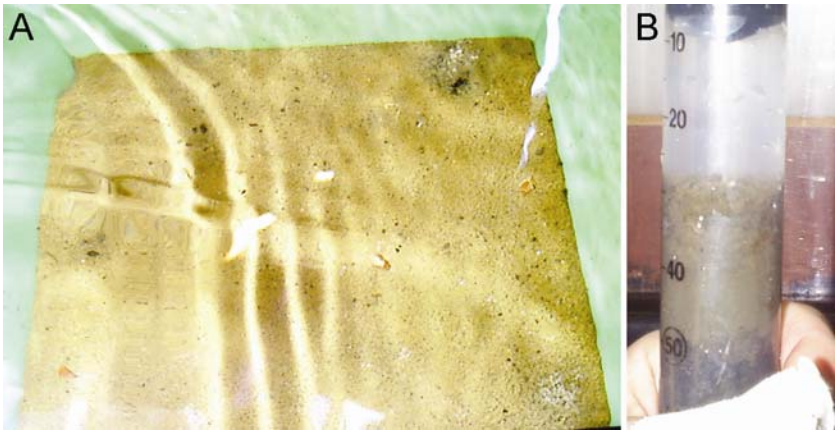
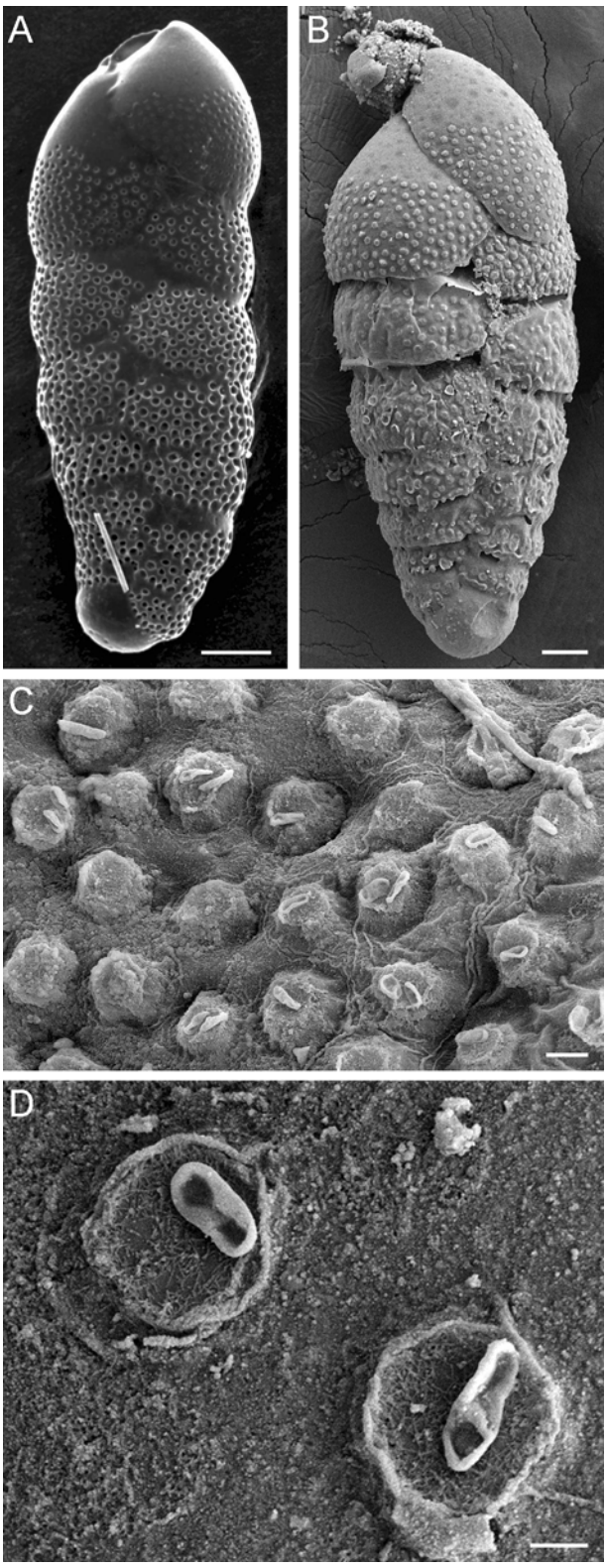


Figure 1.



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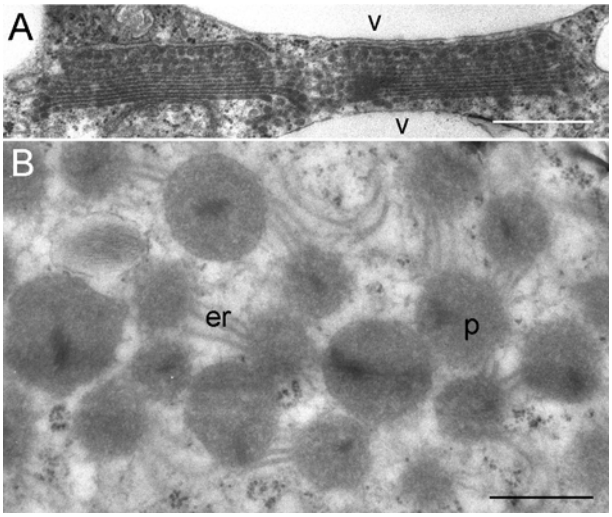

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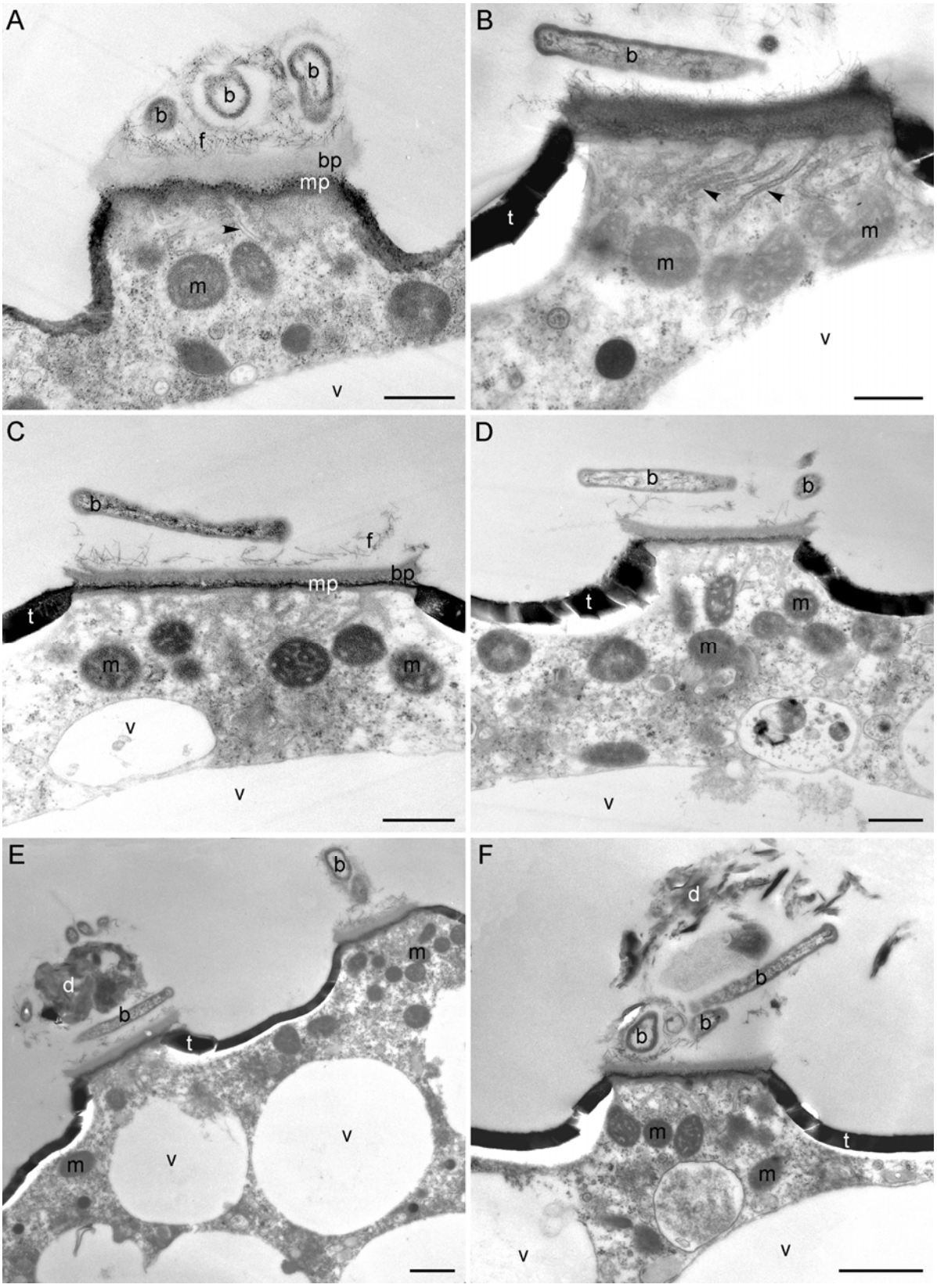
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Figure 4.

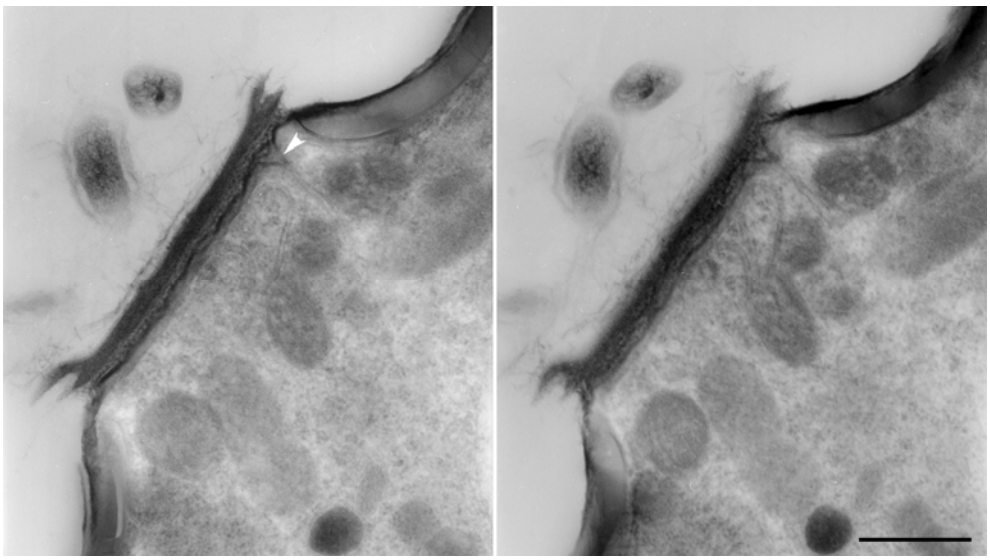
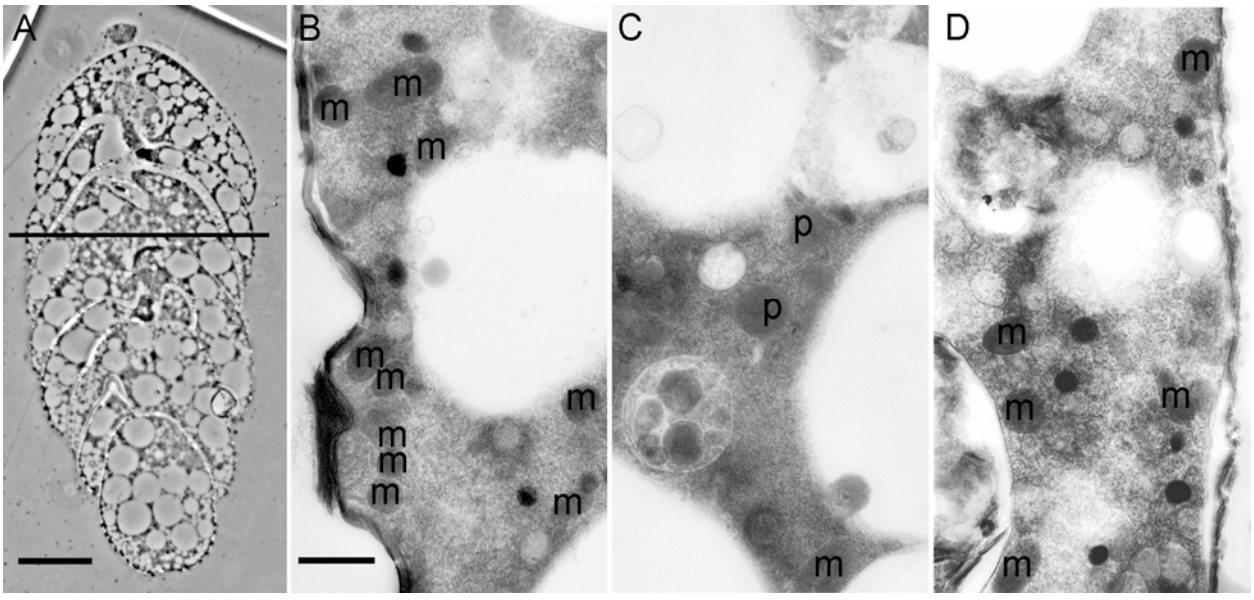


Figure 5.



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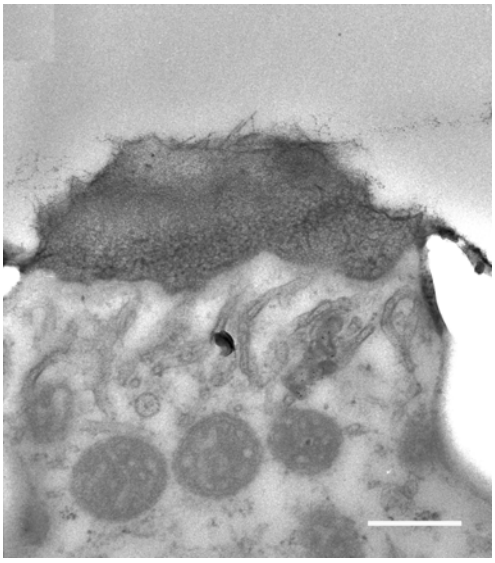
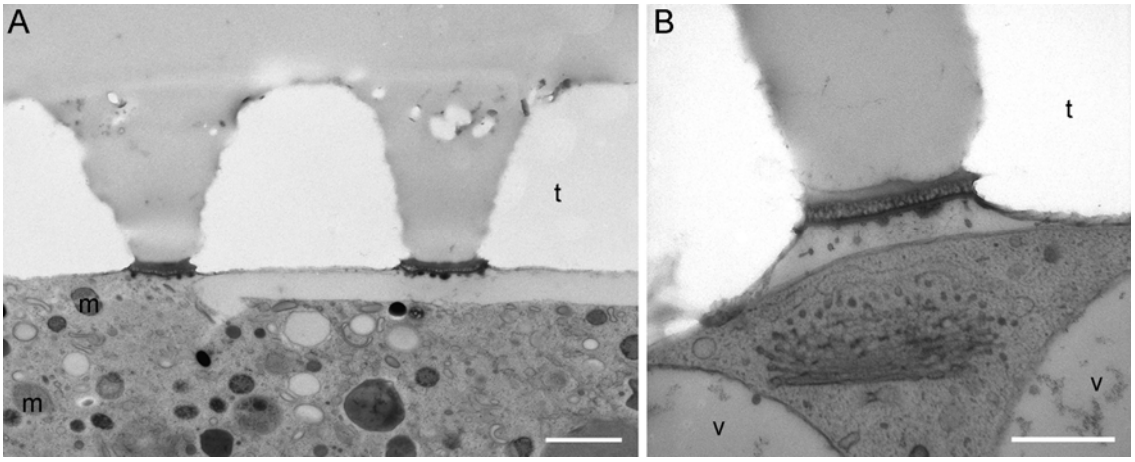


Figure 7.



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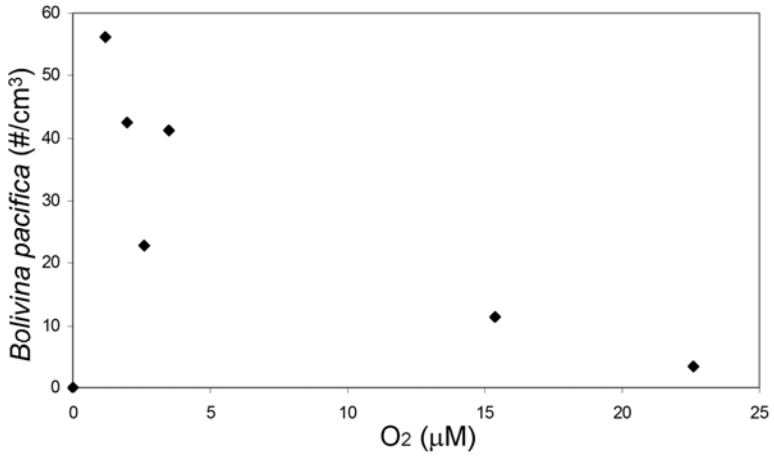

Figure 9.