Ultrastructural observations on prokaryotic associates of benthic foraminifera: food, mutualistic symbionts, or parasites?

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Abstract

Because prokaryotes (Eubacteria, Archaea) are ubiquitous in the marine realm, it may not be surprising that they are important to the diet of at least some foraminifera. Over recent decades, Transmission Electron Microscopy (TEM) has revealed that, at the ultrastructural level, additional intimate relationships exist between prokaryotes and foraminifera. For example, the cytoplasm of a variety of benthic foraminiferal species contains intact prokaryotes. Other benthic foraminiferal species support prokaryotic populations on their exterior. Some of these prokaryote-foraminifera associations are sufficiently consistent to be considered symbioses. Symbiotic relationships include beneficial associations (mutualism; commensalism) to detrimental associations (parasitism). Here, we provide a synopsis of known foraminiferal-prokaryotic symbioses and TEM micrographs illustrating many specific associations. We further comment on and illustrate additional interactions such as bacterial scavenging on foraminifera and foraminiferal feeding on prokaryotes. Documenting and understanding all of these microbial interactions will contribute to a more comprehensive knowledge of benthic marine ecology and biology.
1. Introduction

Benthic foraminifera rely on a variety of sources for nutrition: bacteria (e.g., Lee, 1980; Mojtabahid et al., 2011; Nomaki et al., 2006), algae (e.g., Anderson et al., 1991; Goldstein, 1999), Dissolved Organic Matter (DOM; Delaca et al., 1981), and even certain metazoans (e.g., Bowser et al., 1992). Another role for algae is as foraminiferal symbionts. For example, the majority of ecologically important species of extant planktonic foraminifera have algal symbionts (Kucera, 2007) and one has cyanobacterial symbionts (Bird et al., 2017). Symbiont-bearing planktonic foraminifera and larger benthic foraminifera from tropical reefs rely on photosynthetic activities of their symbionts for energy sources and enhancement of calcification (reviewed by Hallock, 1999). Symbiosis is a stable, consistent association involving biological interaction between two or more species. A symbiotic relationship can have varied impacts on the different partners. Specifically, a symbiosis can be beneficial to each partner (i.e., mutualism), beneficial to one partner but of little consequence to the other partner (i.e., commensalism), or detrimental to one partner but beneficial to the other (i.e., parasitism). Mutualism and parasitism can be considered endmembers along a continuum that includes commensalism (e.g., Ewald, 1987; Hopkins et al., 2017).

Aside from being simply a food source, bacteria may actually be indispensable to the foraminiferal diet (Lee, 1980; Muller and Lee, 1969). Over the past few decades, Transmission Electron Microscopy (TEM) has revealed additional relationships between foraminifera and prokaryotes (i.e., Eubacteria, Archaea). For example, TEM demonstrated that some benthic foraminifera have prokaryotes in their digestive vacuoles (Quinqueloculina sp., Rosalina globularis, Abyssotherma pacifica; Heeger, 1990; Lee et al., 1991) and others deposit feed, ingesting sediments with attached prokaryotes, which are presumably digested (e.g.,
Globobulimina pacifica; Goldstein and Corliss, 1994). Conversely, prokaryotes can scavenge foraminiferal carcasses (Bernhard et al., 2010b). Additional associations between benthic foraminifera and prokaryotes have been documented with TEM over the past few decades (e.g., Bernhard, 1993, 2003; Bernhard et al., 2006; Heeger, 1990; Richardson and Rützler, 1999). In some cases, prokaryotes were associated with degraded foraminiferal cytoplasm (e.g., Pyrgo murrina, Plate 29 in Heeger, 1990). Other associations between benthic foraminifera and prokaryotes appear to be stable and consistent and therefore considered symbioses. Given we know nothing regarding the interactions between these organisms, assigning symbiosis type is a challenge. We can glean much about foraminiferal biology and physiology with TEM, especially in the context of putative symbiosis between a benthic foraminifer and prokaryotes and the fitness of the host. Assessing host fitness via TEM is key to understanding if a symbiosis is mutualistic or commensal versus parasitic.

For the sake of brevity, henceforth, we refer to consistent, stable foraminiferal-prokaryotic associations as “symbioses”. Also, in general, the term “endobiont” or “ectobiont” is used when inferences about a symbiotic relationship are less than confident. From a different perspective, in situations where foraminiferal specimens are rare or difficult to obtain (e.g., deep sea, hydrocarbon seeps, polar regions, in-situ or laboratory experiments), few conspecifics are available for ultrastructural examination. In these instances, we clearly cannot demonstrate consistency among numerous conspecifics, but the documentation of singular prokaryote-host associations can contribute valuable information to the literature upon which future investigations can build.

Most cases of foraminiferal-prokaryotic symbioses involve endobionts, but some cases of foraminiferal ectobionts have been described. This contribution presents a synopsis of the
instances of foraminiferal-prokaryotic symbioses known to date (Table 1) along with images comparing and contrasting these varied associations with trophic relationships such as feeding and scavenging.

2. Materials and Procedures

Micrographs presented in this contribution were all taken at the time of original analyses. All fixation and imaging methodology as well as site information appear in the original publications, which are cited in the text describing the association illustrated in the micrograph(s). In general, sediments were fixed in TEM-grade glutaraldehyde (3% final concentration) in 0.1M cacodylic acid sodium salt buffer. Typically, specimens were isolated from buffer-rinsed sediments, and processed using Bernhard’s standard methods (e.g., Bernhard et al., 2000). Specimens of *Ammonia* sp. (phylotype T6; Hayward et al., 2004; Holzmann, 2000), *Globobulimina affinis*, and *Virgulinella fragilis* from Japan, Namibia, and New Zealand were isolated from sediments, immediately fixed in 2.5% or 3.0% seawater-buffered TEM-grade glutaraldehyde (final concentration), and subsequently transferred into filtered (0.2 µm) sea water and kept at 4°C until further processing, which followed the standard JAMSTEC protocols for foraminiferal TEM analyses (e.g., Nomaki et al., 2014; Nomaki et al., 2015; Tsuchiya et al., 2015). Unless otherwise noted, all foraminifera discussed and imaged here were considered living at the time of fixation, based on the appearance of their organelles (i.e., Bernhard et al., 2010b; Nomaki et al., 2016; Nomaki et al., 2014).

3. Results and Discussion

3.1. Generalities
Most known putative symbioses between benthic foraminifera and prokaryotes occur in hosts from oxygen-depleted habitats (e.g., Bernhard, 2003; Bernhard et al., 2000; Bernhard et al., 2006; Nomaki et al., 2014). Such habitats include naturally occurring redoxclines (geochemical gradients along which oxidation-reduction reactions occur; typically coinciding with the oxic-anoxic interface) or in lab-induced treatments manipulated to have low oxygen concentrations or anoxia. Such environments include those where the oxic-anoxic interface occurs near or coincident with the sediment-water interface (e.g., silled basins, meromictic saline lakes, hydrocarbon seeps) or deeper in sediments, in so-called deep infaunal microhabitats, where oxygen becomes depleted to zero. There have been two published reports on benthic foraminifera-prokaryote symbioses from well-aerated bottom-water environments (Richardson and Rützler, 1999; Tsuchiya et al., 2015). Both of these cases (*Spiculidendron corallicolum*; *Virgulinella fragilis* from Wellington Harbor New Zealand) are discussed in more detail below (section 3.3). Because symbiont-bearing *V. fragilis* are also found in oxygen-depleted bottom-water habitats, the occurrence of symbiont-bearing *V. fragilis* in an aerated setting (Tsuchiya et al., 2015) is especially intriguing. Dedicated investigations of foraminifera from more well-aerated environments may reveal additional instances of symbioses between benthic foraminifera and prokaryotes.

Not all benthic foraminifera recovered from anoxic habitats have symbionts. For example, although it has been shown to denitrify, *Globobulimina pseudospinescens* reportedly lacks symbionts (Risgaard-Petersen et al., 2006). Similarly, foraminifera inhabiting hydrocarbon-seep sediments typically often lack prokaryotic symbionts (Bernhard et al., 2001; Bernhard et al., 2010b).
Because few characteristic morphological traits exist in prokaryotes, differentiating between Eubacteria and Archaea using TEM is unwise. While many symbionts of metazoans are bacteria, one could argue that most benthic foraminiferal-prokaryote symbioses likely involve bacteria. However, anaerobic ciliates are known to have methanogenic archaean symbionts (e.g., Edgcomb et al., 2011; Narayanan et al., 2009) so we await discovery of a foraminiferal-archaeal symbiosis. Documenting such an association will require methods beyond TEM imaging such as genetic analyses and Fluorescent In Situ Hybridization (FISH) techniques.

3.2 Ectobionts

Because foraminifera have tests (shells) often composed of inorganic materials, it may seem counterintuitive that ectobionts could be associated with foraminiferal cells. While one might expect that prokaryotes attach to a foraminiferal test exterior, it may be surprising that prokaryotes have been documented attached to the exterior of foraminiferal pore plugs (Fig. 1), which are the organic barrier between the foraminiferal cells and the environment that occur in the pores or holes typical to most calcareous foraminiferal tests. The best described case of foraminiferal ectobionts is *Bolivina pacifica* from Santa Barbara Basin (CA, USA) (Fig. 1A-C; Bernhard et al., 2010a). The ectobiont prokaryote is rod shaped and associated with many, but not all, *B. pacifica* pore plugs (Fig 1A, C). Like many other foraminifera that inhabit oxygen-depleted sediments (e.g., Leutenegger and Hansen, 1979; see also LeKieffre et al., this volume), *B. pacifica* also has mitochondria that concentrate under pore plugs. *B. pacifica* is unique, to our knowledge, because it has specialized conduits appearing to connect the pore plug to underlying mitochondria (the so-called plasma membrane invaginations; Bernhard et al., 2010a). Because
the ectobiont-laden *B. pacifica* hosts appeared fit, we infer that this association is commensal or mutualistic.

Rod-shaped ectobionts have also been documented on *Uvigerina peregrina* pore plugs (Fig. 1D; Bernhard et al., 2001), but only in one specimen from a hydrocarbon cold seep off central California (Monterey Bay, USA). Another conspecific from that material lacked such ectobionts. Examination of additional *U. peregrina* from similar seeps will demonstrate whether or not this is a consistent association. A specimen of *Loxostomum pseudobeyrichi* collected from one of the Monterey Bay hydrocarbon seeps investigated by Bernhard et al. (2001) was noted to support a prokaryote on one of its pore plugs (Fig. 1E); such a stochastic occurrence should not be considered a symbiosis.

Prokaryotes existing on pore plugs were documented from shallow-water, tidal flat *Ammonia* sp. (phytotype T6) after an experiment that included incubation in anoxia (Fig. 2; Nomaki et al., 2014). These prokaryotes were typically rod-shaped, but not always of only one morphotype (Fig. 2). Such occurrences of pore-associated bacteria were much rarer on *Ammonia* sp. (phytotype T6) incubated in oxic conditions compared to the anoxic specimens (H. Nomaki, unpubl.). Thus, we infer that these pore-associated prokaryotes may be related to reducing conditions. Compared to the *B. pacifica* ectobionts, the *Ammonia* sp. (phytotype T6) ectobionts were much further removed from foraminiferal cytoplasm (not shown) probably because *Ammonia* sp. (phytotype T6) has a much thicker test than *B. pacifica*, causing the *Ammonia* pore plugs to be much thicker. Such observations suggest the *Ammonia* sp. (phytotype T6) ectobionts were not interacting directly with foraminifer but using the pore space as microhabitat; thus this association should not be considered a symbiosis.
Prokaryotes were noted to exist between chambers of *Rosalina globularis* from the tropics (Heeger, 1990). That brief description did not report the number of specimens examined, the consistency of this association, nor speculate on the role or function of these microbes. Until more details about this association are known, we do not consider them to be symbionts.

Prokaryotic associates were observed between the test interior and the inner organic lining (OL) of a multi-chambered biserial agglutinated foraminifer occurring in a core collected adjacent to a hydrocarbon-seep clam bed (Fig. 3; Bernhard et al., 2010b; Nomaki et al., this issue). Thus, in this instance, the prokaryotes were not endobionts, but considered ectobionts, although occurring within the confines of the test. In some regions examined with TEM, prokaryotes were absent or few (Fig. 3A), while in other areas, numerous rod-shaped prokaryotes occurred between the inner organic lining and the interior surface of the test (Fig. 3B, D), or between folds of the test (Fig. 3C). Occasionally, a prokaryote appeared attached to the organic lining (Fig. 3A,C). The association of numerous prokaryotes within the test of this agglutinated seep specimen suggests interactions between these microorganisms. While some microbes were noted in vacuoles of this specimen (Fig 3A; see also Nomaki et al., this issue), none of the prokaryotes in these vacuoles appeared to be rods. Thus, it is not clear at this time if the ectobiont prokaryotic associates were a food source. Only examination of more foraminiferal conspecifics will resolve this situation. Another instance of prokaryotes occurring inside a test but outside the OL was noted in a specimen of the calcareous *Nonionella stella* from the laminated, low-oxygen sediments of Santa Barbara Basin (see Fig. 7D in Bernhard and Reimers, 1991). In this instance, the prokaryotes were only detected in the final (youngest) chamber; additional specimens collected at different times should be examined to establish consistency of this association. *Nonionella stella* from Santa Barbara Basin also consistently has kleptoplasts.
(Bernhard and Bowser, 1999; Grzymski et al., 2002). The significance of such an association is discussed below in the context of the endobiont-bearing foraminifer *Virgulinella fragilis* (see also Jauffrais et al., this issue).

### 3.3 Endobionts

The agglutinated *Spiculidendron corallicolum*, which is an arborescent agglutinated foraminifer from coral reefs, was shown to harbor ovoid prokaryotic endobionts and algal endobionts in its cytoplasm (not shown; Richardson and Rützler, 1999; Rützler and Richardson, 1996). Richardson and Rützler (1999) retracted their assertion of algal endosymbiosis upon re-examination of their original material along with additional material. The prokaryotic endobiont was tentatively identified on a morphological basis as a nitrifying bacterium (Richardson and Rützler, 1999); molecular approaches are required to resolve this situation. The occurrence of prokaryotic endobionts in *S. corallicolum* is noteworthy because the host inhabits well aerated waters, being attached to coral rock (Rützler and Richardson, 1996). Clearly, this situation requires additional study to establish if these prokaryotic endobionts consistently occur in this foraminifer.

*Quinqueloculina* sp. (or *Q. seminula*, depending on text or caption) from organic-rich, ~20-m deep North Sea sediments reportedly has rod-shaped prokaryotes in its cytoplasm (not shown; Heeger, 1990). That report did not provide details regarding the fitness of the foraminiferal host cytoplasm and did not speculate if this was a type of symbiosis. Examination of additional specimens is warranted to determine if this is a consistent occurrence.

*Buliminella tenuata* living in the oxygen-depleted sediments of Santa Barbara Basin (California, USA) is known to harbor copious rod-shaped prokaryotic endobionts (Fig. 4A-C;
Bernhard, 1996; Bernhard et al., 2000). The endobionts of *B. tenuata* were consistently encapsulated by host membrane (Fig. 4B, C), each in a small vacuole. This, and the fact that some endobionts were noted to be dividing (Fig. 4A, C), implies a stable, likely mutualistic, symbiosis between the host and endobionts. Endobionts were distributed randomly throughout the foraminiferan cytoplasm (Fig. 4A-C), as opposed to aligning at the foraminiferan periphery or with the host’s large vacuoles (see below). Organelles such as mitochondria, digestive vacuoles, and a nucleus were well preserved in these hosts, as were vacuoles and lipids (Fig. 4A-C). Some, but not all, conspecifics of *B. tenuata* from hydrocarbon-seep sediments collected off central California also had endobionts (Bernhard et al., 2001; Bernhard et al., 2010b; Martin et al., 2010). These endobionts, however, were not encapsulated by the host’s membrane and were coccoid (Fig. 4D), not rod-shaped as in the Santa Barbara Basin *B. tenuata*. Similar coccoid endobionts were observed in some living *B. tenuata* from nearby non-seep sediments (Bernhard et al., 2010b). The reason for such plasticity in endobiont presence/absence and endobiont type is not known but could be related to type of symbiosis (commensal/mutualistic vs. parasitic; see below) and deserving of further study.

Perhaps the best-known case of benthic foraminiferan endobionts is the calcareous species *Virgulinella fragilis*, which harbors two types of endobionts: rod-shaped prokaryotes and algal chloroplasts (Bernhard, 2003; Tsuchiya et al., 2015). This dual symbiosis was first noted in specimens from the oxic-anoxic interface of the Cariaco Basin (Venezuela; Bernhard, 2003). Additional populations from Japan (Namako-Ike), Namibia (Walvis Bay), and New Zealand (Wellington Harbor) were used more recently to gain insights regarding the relationship and symbiont identification (Tsuchiya et al., 2015). Although one of the *V. fragilis* populations lives in sediments overlain by well-aerated bottom water (Wellington Harbor, New Zealand), a similar
pattern of endobiont distribution was observed in all four populations: the rod-shaped prokaryotes occur at the host periphery and chloroplasts exist internally, away from the foraminiferal periphery (Fig. 5). Although the Wellington Harbor site is now aerated, the harbor was eutrophic in the 1970s due to commercial activities that introduced organic matter to the area (Grindell and Collen, 1976; Tsuchiya et al., 2015). During this oxygen-depleted, sulfidic period, *V. fragilis* inhabited the harbor (Grindell and Collen, 1976). Presently, *V. fragilis* exists in restricted locations in the harbor (Tsuchiya et al., 2015). Although bottom water was well aerated at the time of the Tsuchiya et al. (2015) sampling, it is possible that *V. fragilis* live in organically enriched oxygen-depleted microhabitats in Wellington Harbor sediments.

Endobionts from all four *V. fragilis* populations were encapsulated by host membrane in a small vacuole, similar to the endobionts of *B. tenuata*. The rod-shaped prokaryotes had slight differences in appearance among the four populations (Fig. 6). Both the Cariaco and Japanese prokaryotes had distinct internal vacuoles (Fig. 6A,D), while the Namibian and New Zealand endobionts did not (Fig. 6B,C). Some individual prokaryotic cells were noted to be dividing in the foraminiferal cytoplasm (Fig. 5A,D; Tsuchiya et al., 2015). Because the three *V. fragilis* populations studied by Tsuchiya et al. (2015) had similar bacterial sequences, all being δ-proteobacteria, these morphological variations could be due to differences in fixation protocols, differences in environmental conditions at the time of fixation, or foraminiferal physiological status at the time of fixation. Sequence data are not available for the Cariaco *V. fragilis* prokaryotic associates. Often, mitochondria of *V. fragilis* are closely associated with the endobionts (Fig. 6; Tsuchiya et al., 2015). *V. fragilis* is known to have copious numbers of peroxisome-endoplasmic reticulum complexes (Fig. 7A), similar to other benthic foraminifera from oxyclines (Bernhard and Bowser, 2008; LeKieffre et al., this issue). Clearly the symbiosis
of *V. fragilis* and the rod-shaped bacterium is mutualistic as indicated by bacterial abundance in
the host, endobiont encapsulation, bacterial division in the host cell, and high foraminiferal
abundances. As noted above, *V. fragilis* sequesters chloroplasts (Fig. 7B). The fact that a
benthic foraminifer from the aphotic zone sequesters chloroplasts is a fascinating puzzle because
chloroplasts are photosynthetic organelles, yet these foraminifera live in darkness. This
kleptoplasty phenomenon is beyond the scope of this contribution and has been discussed
elsewhere (Bernhard and Bowser, 1999; Grzymski et al., 2002; Tsuchiya et al., 2015).
Ultrastructural examples of sequestered chloroplasts in shallow-water (photic zone) foraminifera
appear in Jauffrais et al. (this issue).

A deeply infaunal (6–7 cm) specimen of *Globocassidulina* cf. *G. biora* from shallow-
water Antarctic sediments had short rod-shaped endobionts under pore plugs (not shown;
Bernhard, 1993). Because only one specimen of *Globocassidulina* cf. *G. biora* was examined
with TEM, it is not clear if this association with prokaryotes is a consistent characteristic in this
foraminiferal species.

As noted above, coccoid endobionts have been previously documented to exist in some
benthic foraminifera (e.g., some *B. tenuata*; Bernhard et al., 2010b; Martin et al., 2010).
Coccoid-shaped endobionts are copious in an undescribed saccamminid foraminifer from
laminated sediments of Santa Barbara Basin (reported as an allogromiid in Bernhard et al., 2006
and Bernhard et al., 2012). Unlike in *V. fragilis* where endobionts occur at the host cell
periphery, in this saccamminid, the endobionts appear to line the peripheries of large “empty”
vacuoles (Fig. 8; see also Bernhard et al., 2012; Bernhard et al., 2006). Although these
endobionts are not encapsulated by the host membrane, because of the consistency of their
distribution around these large vacuoles, the saccamminid endobionts are considered mutualistic
or commensal symbionts. Clearly exchange is occurring between the endobionts and vacuoles; further discussion regarding possible interactions is beyond the scope of this contribution. To date, these endobionts have not been sequenced so their identity is unknown, although it is established that the endobionts contain the nitrite reductase gene nirK (Bernhard et al., 2012).

A similar association between endobionts and large foraminiferal vacuoles was also observed in Ammonia sp. (phylotype T6) incubated in anoxia (Nomaki et al., 2014). The Ammonia sp. (phylotype T6) endobionts were not as dense as observed in the saccamminid, but their typical association with vacuoles suggests an interaction between the endobionts and vacuole contents (Fig. 9A-C; see also Fig. 7B in Nomaki et al., 2014). These endobionts were typically found in the youngest two or three chambers of anoxia-incubated specimens (Nomaki et al., 2014; 2016), but not observed in specimens incubated in oxic conditions (Nomaki et al., 2014). Bacterial associates were also observed in Ammonia sp. (phylotype unknown) collected from naturally occurring anoxic sediments of the Wadden Sea tidal flat (Koho et al., this issue). These Ammonia sp. endobionts were not typically observed at a vacuole periphery, but were found in the cytosol and in degraded vacuoles (Koho et al., this issue).

The appearance of the Ammonia sp. endobionts varied, with rod-shaped forms (Fig. 9) as well as coccoid forms (Fig. 7B in Nomaki et al., 2014, also see Koho et al., this issue). Neither form was encapsulated in host membrane, as in V. fragilis and B. tenuata. The Nomaki et al. (2014) isotope-labeling study using 15N-labeled nitrate suggested nitrate utilization (most likely denitrification) with subsequent use of nitrate-N to amino acid synthesis, only in the specimens from anoxic incubations. Thus, the endobionts seemed to be involved in either nitrate utilization or amino acid synthesis or both of these processes. Furthermore, a subsequent incubation experiment using the same foraminiferal species but collected in a different season (i.e., March
vs. July) showed different morphotypes of possible endobionts (Nomaki et al., 2016). We suggest that the prokaryote-Ammonia sp. associations at this site are highly plastic, as noted for Buliminella tenuata, discussed above.

3.4 TEM evidence for permanent to temporary and transient symbioses

The observation that the endobionts of Ammonia sp. (phylotype T6) (Nomaki et al., 2014, 2016), the Santa Barbara Basin saccamminid (Bernhard et al. 2006; 2012), and some Buliminella tenuata (Fig. 4D; Bernhard et al. 2010b) lack encapsulation by host membrane lends further insights into the stability of prokaryote-foraminiferal relationships. Encapsulation within host membrane is a characteristic of true “permanent” symbioses in other eukaryotic taxa (e.g., molluscs such as cold-seep clams; Ikuta et al., 2016), where metabolic exchange has been identified (Kuwahara et al., 2007). Thus, we may infer that the endobionts of V. fragilis and Santa Barbara Basin B. tenuata are bona fide mutualistic and/or commensal symbioses and that the other endobiont cases described above may be transient associations such as transitions from commensal to parasitic symbioses. While the Santa Barbara Basin saccamminid had intact organelles (e.g., mitochondria, Golgi) and large vacuoles were ubiquitously lined with endobionts, the endobionts were not encapsulated by host membrane. Although the structured association of endobionts at vacuole peripheries suggests a stable beneficial relationship, the lack of encapsulation may indicate a less stable, more transient association, or parasitic relationship. The case of Ammonia sp. (phylotype T6) from the Japanese tidal flat may be a recent transient relationship because endobionts occur exclusively in the youngest 2-3 chambers in specimens exposed to experimentally manipulated anoxia, but endobionts were not found in Ammonia sp. (phylotype T6) specimens exposed to aerated conditions (Nomaki et al. 2014). Such observations
suggest the anoxia-incubated *Ammonia* sp. (phylotype T6) were stressed to a tipping point, resulting in endobiont invasion.

We do not know the foraminiferal endobiont acquisition mechanism. For example, are endobionts passed from parent to offspring (e.g., prokaryote division within the host cell, Fig. 4A,C; 5A) or are endobionts phagocytosed by each foraminiferal generation? Various shaped prokaryotes have been noted in degradation (food) vacuoles (e.g., Fig. 5C; 10A,B; Goldstein and Corliss, 1994, Nomaki et al., this issue). As already noted, there is some evidence of phagocytosis with subsequent transfer into foraminiferal cytoplasm (Fig. 10C), without digestion (see also Bernhard et al., 2010b). Such cases of a transition from degradation vacuole into foraminiferal cytoplasm are exclusively, to our knowledge, endobionts that lack encapsulation by host membrane (Fig. 10D). Additional observations indicate that foraminiferal cytoplasm of one chamber(s) can appear degraded while that in other chambers appears fit, with intact mitochondria and other organelles. For example, Figs. 10C,D show images of the same specimen of *Globobulimina pacifica*, yet the vacuoles in Fig. 10D appear degraded because they are irregular in shape and membranes of organelles such as peroxisomes are not crisp. In this case, there are many endobionts, some in degradation vacuoles and some within cytoplasm (Fig. 10D). The specimen shown in Figs. 10C,D could be interpreted to have mutualistic or commensal endobionts transitioning to detrimental endobionts (i.e., parasites; Bernhard et al., 2010b). Other instances exist where endobionts appear intact but foraminiferal organelles are degraded and barely identifiable (Fig. 10E). Sometimes the ultrastructure of rose bengal-stained benthic foraminifera clearly shows absence of identifiable eukaryotic materials (i.e., organelles) yet presence of intact prokaryotes (Fig. 10F). In these cases, the prokaryotes have various morphologies and appear to be scavenging remains of foraminiferal cytoplasm. At this time, it is
not known if prokaryotes cause foraminiferal host mortality or if prokaryotes invade after foraminiferal death.

We hypothesize that phagocytosed prokaryotes can transition into foraminiferal cytoplasm to establish a commensal or mutualistic symbiosis. The host foraminifer either does or does not digest each phagocytosed prokaryote. If, later, the host becomes stressed due, for example, to an experimental manipulation or change in environmental condition, the commensal/mutualistic endobionts then increase division rates and ultimately overpopulate the foraminiferal cytoplasm, thereby eventually killing the host. Of course, it is possible that endobiont presence in foraminiferal cytoplasm is beneficial to both endobiont and host, in which case eventually a permanent symbiosis would occur. In sum, we suggest that the environment and foraminiferal physiologic state mandate the intracellular prokaryotic community; some phagocytosed prokaryotes are digested while others can be transitioned into the cytoplasm as endobionts. If the environment changes to unfavorable foraminiferal conditions but favorable endobiont conditions, the endobionts overtake the host cell, becoming parasites. Such transitions are a topic deserving of further dedicated study.

3.5 Phylogenetic considerations

While most benthic foraminifera with prokaryotic symbionts are rotalids (Table 1), it is premature to infer that miliolids, agglutinated, and thecate forms have lower rates of such associations. Clearly this situation is an example of small sample sizes (i.e., few species examined). Assessing more species from a wide variety of families will help resolve phylogenetic trends of prokaryote-bearing smaller benthic foraminifera. If rotalids do in fact have higher incidents of symbioses, such associations may have conferred an advantage (s) and promoted their diversification over time.
4. Conclusions

Prokaryotic-foraminiferal associations are not uncommon. While some benthic foraminifera have associations with ectobionts, most foraminiferal-prokaryotic associations involve endobionts. The prokaryotes involved in symbioses with benthic foraminifera vary in morphology between different host species, with rod-shaped and coccoid morphotypes both well represented. Most, but not all, symbiont-bearing foraminiferal hosts inhabit oxycline habitats, with steep chemical gradients. In the majority of instances, additional material needs to be examined with TEM to determine stability and consistency of the prokaryotic populations and types, over time and space. *Virgulinella fragilis* is the most compelling case of bona fide symbiosis given that populations from four disparate regions of the world all have a dual symbiosis with morphologically similar rod-shaped bacterial endobionts and kleptoplasts, all similarly distributed in the host foraminifer’s cell. *Buliminella tenuata* is another compelling case because its endobionts vary morphologically depending on location, with rods being prevalent in host specimens from oxygen-depleted laminated sediments of Santa Barbara Basin while coccoid endobionts prevail in host specimens from hydrocarbon seep and non-seep sediments of Central California. Furthermore, these Central California *B. tenuata* do not universally have endobionts. Such plasticity is a topic worthy of dedicated study. Finally, another topic worthy of dedicated study is the possibility that endobionts transition from food to commensal symbionts to parasitic symbionts to scavengers after death of the host. There remains much about foraminiferal biology, physiology and ecology to be learned using TEM, especially with recently-developed correlative methods (Nomaki et al., this issue).
5. Acknowledgements

We thank Beth Richardson (University of Georgia) for sectioning FLEC-TEM specimens. HN and MT thank Katsuyuki Uematsu and Akihiro Tame (Marine Works Japan, Ltd) for their help with TEM preparations and observations of *Ammonia* sp., *G. affinis*, and Japanese, Namibian, and New Zealand *V. fragilis*. We collectively thank two anonymous reviewers for their comments on an earlier manuscript version. JMB’s contributions were funded by US NSF funding over many years, most recently NSF grant OCE-1634469, as well as the WHOI Robert W. Morse Chair for Excellence in Oceanography and The Investment in Science Fund at WHOI. MT and HN’s contributions were funded by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (no. 24340131 to MT and no. 22740340 to HN).
6. References


**Figure legends**

**Figure 1.** Benthic foraminiferal ectobionts. A. Scanning Electron Micrograph of decalcified *Bolivina pacifica* showing four circular pore plugs (pp), two with attached rod-shaped prokaryotes (*). B-E, Transmission Electron Micrographs. B-C. *B. pacifica*, showing pore plugs in cross section, with attached rod-shaped bacteria; m = mitochondrion, ol = organic lining, v = vacuole, black arrowheads = plasma membrane invagination. D. *Uvigerina peregrina*, Clam Flats seep, with two ectobionts above pore plug. E. *Loxostomum pseudobeyrichi*, Clam Flats seep, with an ectobiont above pore plug; fv = fibrillar vesicles, mvb= multivesicular bodies. Scales bars: A, C-E = 1 µm; B = 0.5 µm.

**Figure 2.** Ectobionts on *Ammonia* sp. (phylotype T6) from an anoxic experiment treatment, with three ectobionts overlying pore plug. Scales bar = 0.5 µm.

**Figure 3.** TEM micrographs of agglutinated deep-sea foraminiferal prokaryotic associates. This specimen was prepared with FLEC-TEM (see Bernhard and Richardson, 2014; Nomaki et al., this issue), so it was not osmicated. A. Single prokaryote (black and white arrow) closely associated with organic lining (ol); prokaryotes inside vacuole (black arrowhead). t = test, v = vacuole. B, D. Prokaryotes (*) occurring between test and organic lining; m = mitochondrion. C. Prokaryotes (*) occurring outside the test and one prokaryote (black arrow) closely associated with organic lining; dv= digestive vacuole. Scales bars: A,C = 2 µm; B,D = 1 µm.

**Figure 4.** TEM micrographs of *Buliminella tenuata* endobionts. A-C. Live *B. tenuata* from Santa Barbara Basin, showing rod-shaped endobionts (*). Black arrows points to dividing
endobionts (A, C). n = nucleus, m = mitochondrion, v = vacuole, dv = digestive vacuole, li = lipid droplet. D. Live B. tenuata from Clam Flats seep, showing coccoid endobionts (*). Scales bars: A = 2 µm; B-C = 1 µm; D = 0.5 µm.

Figure 5. TEM micrographs of Virgulinella fragilis, showing characteristic rod-shaped endobiont (*) distributions at foraminiferal periphery and more central chloroplast (c) occurrences. A = Cariaco Basin, Venezuela; B = Walvis Bay, Namibia; C = Namako-Ike, Japan; D = Wellington Harbor, New Zealand. n = nucleus, nu = nucleolus, m = mitochondrion, v = vacuole, dv = digestive vacuole, li = lipid droplet, t = location of former test, + = phagocytosed prokaryotes (morphologically differ from endobionts). Black arrows point to dividing endobionts. Scales bars: A,C = 5 µm; B = 2 µm; D = 10 µm.

Figure 6. TEM micrographs of Virgulinella fragilis, showing endobionts (*) in detail. A. Note: the chloroplast (c) is a composite of four plastids. m = mitochondrion, v = vacuole, t = location of former test. A = Cariaco Basin, Venezuela; B = Walvis Bay, Namibia; C = Namako-Ike, Japan; D = Wellington Harbor, New Zealand. Scales bars: A = 1 µm; B-D = 0.5 µm.

Figure 7. TEM micrographs of Virgulinella fragilis from Wellington Harbor. A. Peroxisome (p)-endoplasmic reticulum (er) complex. B. Higher magnification view of sequestered chloroplast (c). dv = digestive vacuole, li = lipid droplet. Scales bars: A-B = 1 µm.
**Figure 8.** TEM micrographs of unidentified Santa Barbara Basin saccamminid showing endobiont (*) association with large “empty” vacuoles (v); m = mitochondrion. Scales bars: A = 1 µm; B = 0.5 µm.

**Figure 9.** TEM micrographs of *Ammonia* sp. (phylotype T6) from anoxic experiment treatment showing rod-shaped endobionts (*), often in association with “empty” vacuoles (v); m = mitochondrion, li = lipid droplets. Scales bars: A = 1 µm; B-C = 0.5 µm.

**Figure 10.** TEM images appearing to show transient prokaryote-foraminiferal associations. A-B. *Globobulimina affinis* from anoxic experiment treatment showing phagocytosed bacteria (*). C-D. Live *Globobulimina pacifica* from Clam Flats seep, showing coccoid endobionts (*) in degradation vacuoles (dv), transitioning across vacuole membrane (black arrows), and in cytoplasm. Also visible are mitochondria (m) and peroxisomes (p); t = location of former test, v = vacuole. E. Dead *Bulimina mexicana* from Clam Flats seep, showing coccoid endobionts in dense cytoplasm, lacking vacuoles. Organelles are barely discernable, possibly being degraded mitochondria (m?). F. Dead *Cibicidoides wuellerstorfi* from Clam Flats seep, showing a variety of prokaryote endobionts (*) and no discernable foraminiferal organelles. pp = pore plug, t = location of former test. Scales bars: A,C-E = 1 µm; B = 0.5 µm; F = 2 µm.
Table 1. A summary of foraminifera-prokaryote associations discussed in this contribution.

<table>
<thead>
<tr>
<th>Foraminiferal species</th>
<th>Environment</th>
<th>Prokaryote traits</th>
<th>Prokaryote distribution</th>
<th>Speculated type of association</th>
<th>Encapsulation</th>
<th>Associated features and notes</th>
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<tbody>
<tr>
<td><strong>Bolivina pacifica [C]</strong></td>
<td>Silled basin / chemocline</td>
<td>Rod</td>
<td>Ectobiont (pores)</td>
<td>Commensal or mutualistic</td>
<td>NA</td>
<td>Plasma membrane invagination</td>
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<tr>
<td><strong>Agglutinated biserial form [A]</strong></td>
<td>Near hydrocarbon-seep clam bed</td>
<td>Rod</td>
<td>Between organic lining and test</td>
<td>possibly food</td>
<td>NA</td>
<td>Only one specimen examined</td>
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<tr>
<td><strong>Ammonia phykotype T6 [C]</strong></td>
<td>Tidal flat</td>
<td>Rod</td>
<td>Ectobiont (pores)</td>
<td>unknown</td>
<td>NA</td>
<td>anoxic habitat</td>
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<td><strong>Ammonia phykotype T6 [C]</strong></td>
<td>Tidal flat</td>
<td>Rod</td>
<td>Endobiont, near vacuoles, youngest 2-3 chambers</td>
<td>Temporary or parasitic</td>
<td>No</td>
<td>Anoxic incubation</td>
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<tr>
<td><strong>Ammonia phykotype T6 [C]</strong></td>
<td>Tidal flat</td>
<td>Rod</td>
<td>Endobiont, cell periphery, youngest 2-3 chambers</td>
<td>Temporary or parasitic</td>
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<td>Anoxic incubation</td>
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<td><strong>Buliminella tenuata [C]</strong></td>
<td>Silled basin / chemocline</td>
<td>Rod</td>
<td>Endobiont</td>
<td>Permanent; Mutualistic or Commensal</td>
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<td><strong>Buliminella tenuata [C]</strong></td>
<td>Hydrocarbon cold seep</td>
<td>Coccoid</td>
<td>Endobiont</td>
<td>Transient? (verging on parasitism)</td>
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<td><strong>Globocassidulina cf. G. biora [C]</strong></td>
<td>6-7cm below sediment-water interface</td>
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<td><strong>Quinqueloculina sp. saccamminid (Santa Barbara Basin) [T]</strong></td>
<td>organic rich photic zone</td>
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<td>unknown</td>
<td>unknown</td>
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<td><strong>Spiculidendron corallicolum [A]</strong></td>
<td>Coral reef</td>
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<td>Commensal or mutualistic</td>
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<td><strong>Uvigerina peregrina [C]</strong></td>
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<td>Oxic-anoxic interface</td>
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<td>Endobiont at cell periphery</td>
<td>Permanent; Mutualistic or Commensal</td>
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C= calcareous; A = agglutinated; T = thecate; NA= Not applicable
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