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

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Keywords: Transmission Electron Microscopy; rhizarian protist; commensalism; microbiome; oxygen depletion; bacteria

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; Mojtahid 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.,

56 *Globobulimina pacifica*; Goldstein and Corliss, 1994). Conversely, prokaryotes can scavenge
57 foraminiferal carcasses (Bernhard et al., 2010b). Additional associations between benthic
58 foraminifera and prokaryotes have been documented with TEM over the past few decades (e.g.,
59 Bernhard, 1993, 2003; Bernhard et al., 2006; Heeger, 1990; Richardson and Rützler, 1999). In
60 some cases, prokaryotes were associated with degraded foraminiferal cytoplasm (e.g., *Pyrgo*
61 *murrina*, Plate 29 in Heeger, 1990). Other associations between benthic foraminifera and
62 prokaryotes appear to be stable and consistent and therefore considered symbioses. Given we
63 know nothing regarding the interactions between these organisms, assigning symbiosis type is a
64 challenge. We can glean much about foraminiferal biology and physiology with TEM, especially
65 in the context of putative symbiosis between a benthic foraminifer and prokaryotes and the
66 fitness of the host. Assessing host fitness via TEM is key to understanding if a symbiosis is
67 mutualistic or commensal versus parasitic.

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

77 Most cases of foraminiferal-prokaryotic symbioses involve endobionts, but some cases of
78 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 archaeal 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

146 the ectobiont-laden *B. pacifica* hosts appeared fit, we infer that this association is commensal or
147 mutualistic.

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

156 Prokaryotes existing on pore plugs were documented from shallow-water, tidal flat
157 *Ammonia* sp. (phylotype T6) after an experiment that included incubation in anoxia (Fig. 2;
158 Nomaki et al., 2014). These prokaryotes were typically rod-shaped, but not always of only one
159 morphotype (Fig. 2). Such occurrences of pore-associated bacteria were much rarer on *Ammonia*
160 sp. (phylotype T6) incubated in oxic conditions compared to the anoxic specimens (H. Nomaki,
161 unpubl.). Thus, we infer that these pore-associated prokaryotes may be related to reducing
162 conditions. Compared to the *B. pacifica* ectobionts, the *Ammonia* sp. (phylotype T6) ectobionts
163 were much further removed from foraminiferal cytoplasm (not shown) probably because
164 *Ammonia* sp. (phylotype T6) has a much thicker test than *B. pacifica*, causing the *Ammonia* pore
165 plugs to be much thicker. Such observations suggest the *Ammonia* sp. (phylotype T6) ectobionts
166 were not interacting directly with foraminifer but using the pore space as microhabitat; thus this
167 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;

214 Bernhard, 1996; Bernhard et al., 2000). The endobionts of *B. tenuata* were consistently
215 encapsulated by host membrane (Fig. 4B, C), each in a small vacuole. This, and the fact that
216 some endobionts were noted to be dividing (Fig. 4A, C), implies a stable, likely mutualistic,
217 symbiosis between the host and endobionts. Endobionts were distributed randomly throughout
218 the foraminiferal cytoplasm (Fig. 4A-C), as opposed to aligning at the foraminiferal periphery or
219 with the host's large vacuoles (see below). Organelles such as mitochondria, digestive vacuoles,
220 and a nucleus were well preserved in these hosts, as were vacuoles and lipids (Fig. 4A-C). Some,
221 but not all, conspecifics of *B. tenuata* from hydrocarbon-seep sediments collected off central
222 California also had endobionts (Bernhard et al., 2001; Bernhard et al., 2010b; Martin et al.,
223 2010). These endobionts, however, were not encapsulated by the host's membrane and were
224 coccoid (Fig. 4D), not rod-shaped as in the Santa Barbara Basin *B. tenuata*. Similar coccoid
225 endobionts were observed in some living *B. tenuata* from nearby non-seep sediments (Bernhard
226 et al., 2010b). The reason for such plasticity in endobiont presence/absence and endobiont type is
227 not known but could be related to type of symbiosis (commensal/mutualistic vs. parasitic; see
228 below) and deserving of further study.

229 Perhaps the best-known case of benthic foraminiferal endobionts is the calcareous species
230 *Virgulinella fragilis*, which harbors two types of endobionts: rod-shaped prokaryotes and algal
231 chloroplasts (Bernhard, 2003; Tsuchiya et al., 2015). This dual symbiosis was first noted in
232 specimens from the oxic-anoxic interface of the Cariaco Basin (Venezuela; Bernhard, 2003).
233 Additional populations from Japan (Namako-Ike), Namibia (Walvis Bay), and New Zealand
234 (Wellington Harbor) were used more recently to gain insights regarding the relationship and
235 symbiont identification (Tsuchiya et al., 2015). Although one of the *V. fragilis* populations lives
236 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

283 or commensal symbionts. Clearly exchange is occurring between the endobionts and vacuoles;
284 further discussion regarding possible interactions is beyond the scope of this contribution. To
285 date, these endobionts have not been sequenced so their identity is unknown, although it is
286 established that the endobionts contain the nitrite reductase gene *nirK* (Bernhard et al., 2012).
287 A similar association between endobionts and large foraminiferal vacuoles was also
288 observed in *Ammonia* sp. (phylotype T6) incubated in anoxia (Nomaki et al., 2014). The
289 *Ammonia* sp. (phylotype T6) endobionts were not as dense as observed in the saccamminid, but
290 their typical association with vacuoles suggests an interaction between the endobionts and
291 vacuole contents (Fig. 9A-C; see also Fig. 7B in Nomaki et al., 2014). These endobionts were
292 typically found in the youngest two or three chambers of anoxia-incubated specimens (Nomaki
293 et al., 2014; 2016), but not observed in specimens incubated in oxic conditions (Nomaki et al.,
294 2014). Bacterial associates were also observed in *Ammonia* sp. (phylotype unknown) collected
295 from naturally occurring anoxic sediments of the Wadden Sea tidal flat (Koho et al., this issue).
296 These *Ammonia* sp. endobionts were not typically observed at a vacuole periphery, but were
297 found in the cytosol and in degraded vacuoles (Koho et al., this issue).
298 The appearance of the *Ammonia* sp. endobionts varied, with rod-shaped forms (Fig. 9) as
299 well as coccoid forms (Fig. 7B in Nomaki et al., 2014, also see Koho et al., this issue). Neither
300 form was encapsulated in host membrane, as in *V. fragilis* and *B. tenuata*. The Nomaki et al.
301 (2014) isotope-labeling study using ^{15}N-labeled nitrate suggested nitrate utilization (most likely
302 denitrification) with subsequent use of nitrate-N to amino acid synthesis, only in the specimens
303 from anoxic incubations. Thus, the endobionts seemed to be involved in either nitrate utilization
304 or amino acid synthesis or both of these processes. Furthermore, a subsequent incubation
305 experiment using the same foraminiferal species but collected in a different season (i.e., March

306 *vs*. July) showed different morphotypes of possible endobionts (Nomaki et al., 2016). We
307 suggest that the prokaryote-*Ammonia* sp. associations at this site are highly plastic, as noted for
308 *Buliminella tenuata*, discussed above.
309
310 3.4 TEM evidence for permanent to temporary and transient symbioses
311 The observation that the endobionts of *Ammonia* sp. (phylotype T6) (Nomaki et al., 2014,
312 2016), the Santa Barbara Basin saccamminid (Bernhard et al. 2006; 2012), and some *Buliminella*
313 *tenuata* (Fig. 4D; Bernhard et al. 2010b) lack encapsulation by host membrane lends further
314 insights into the stability of prokaryote-foraminiferal relationships. Encapsulation within host
315 membrane is a characteristic of true "permanent" symbioses in other eukaryotic taxa (e.g.,
316 molluscs such as cold-seep clams; Ikuta et al., 2016), where metabolic exchange has been
317 identified (Kuwahara et al., 2007). Thus, we may infer that the endobionts of *V. fragilis* and
318 Santa Barbara Basin *B. tenuata* are bona fide mutualistic and/or commensal symbioses and that
319 the other endobiont cases described above may be transient associations such as transitions from
320 commensal to parasitic symbioses. While the Santa Barbara Basin saccamminid had intact
321 organelles (e.g., mitochondria, Golgi) and large vacuoles were ubiquitously lined with
322 endobionts, the endobionts were not encapsulated by host membrane. Although the structured
323 association of endobionts at vacuole peripheries suggests a stable beneficial relationship, the lack
324 of encapsulation may indicate a less stable, more transient association, or parasitic relationship.
325 The case of *Ammonia* sp. (phylotype T6) from the Japanese tidal flat may be a recent transient
326 relationship because endobionts occur exclusively in the youngest 2-3 chambers in specimens
327 exposed to experimentally manipulated anoxia, but endobionts were not found in *Ammonia* sp.
328 (phylotype T6) specimens exposed to aerated conditions (Nomaki et al. 2014). Such observations

329 suggest the anoxia-incubated *Ammonia* sp. (phylotype T6) were stressed to a tipping point,
330 resulting in endobiont invasion.

331 We do not know the foraminiferal endobiont acquisition mechanism. For example, are
332 endobionts passed from parent to offspring (e.g., prokaryote division within the host cell, Fig.
333 4A,C; 5A) or are endobionts phagocytosed by each foraminiferal generation? Variously shaped
334 prokaryotes have been noted in degradation (food) vacuoles (e.g., Fig. 5C; 10A,B; Goldstein and
335 Corliss, 1994, Nomaki et al., this issue). As already noted, there is some evidence of
336 phagocytosis with subsequent transfer into foraminiferal cytoplasm (Fig. 10C), without digestion
337 (see also Bernhard et al., 2010b). Such cases of a transition from degradation vacuole into
338 foraminiferal cytoplasm are exclusively, to our knowledge, endobionts that lack encapsulation by
339 host membrane (Fig. 10D). Additional observations indicate that foraminiferal cytoplasm of one
340 chamber(s) can appear degraded while that in other chambers appears fit, with intact
341 mitochondria and other organelles. For example, Figs. 10C,D show images of the same specimen
342 of *Globobulimina pacifica*, yet the vacuoles in Fig. 10D appear degraded because they are
343 irregular in shape and membranes of organelles such as peroxisomes are not crisp. In this case,
344 there are many endobionts, some in degradation vacuoles and some within cytoplasm (Fig. 10D).
345 The specimen shown in Figs. 10C,D could be interpreted to have mutualistic or commensal
346 endobionts transitioning to detrimental endobionts (i.e., parasites; Bernhard et al., 2010b). Other
347 instances exist where endobionts appear intact but foraminiferal organelles are degraded and
348 barely identifiable (Fig. 10E). Sometimes the ultrastructure of rose bengal-stained benthic
349 foraminifera clearly shows absence of identifiable eukaryotic materials (i.e., organelles) yet
350 presence of intact prokaryotes (Fig. 10F). In these cases, the prokaryotes have various
351 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).

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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.

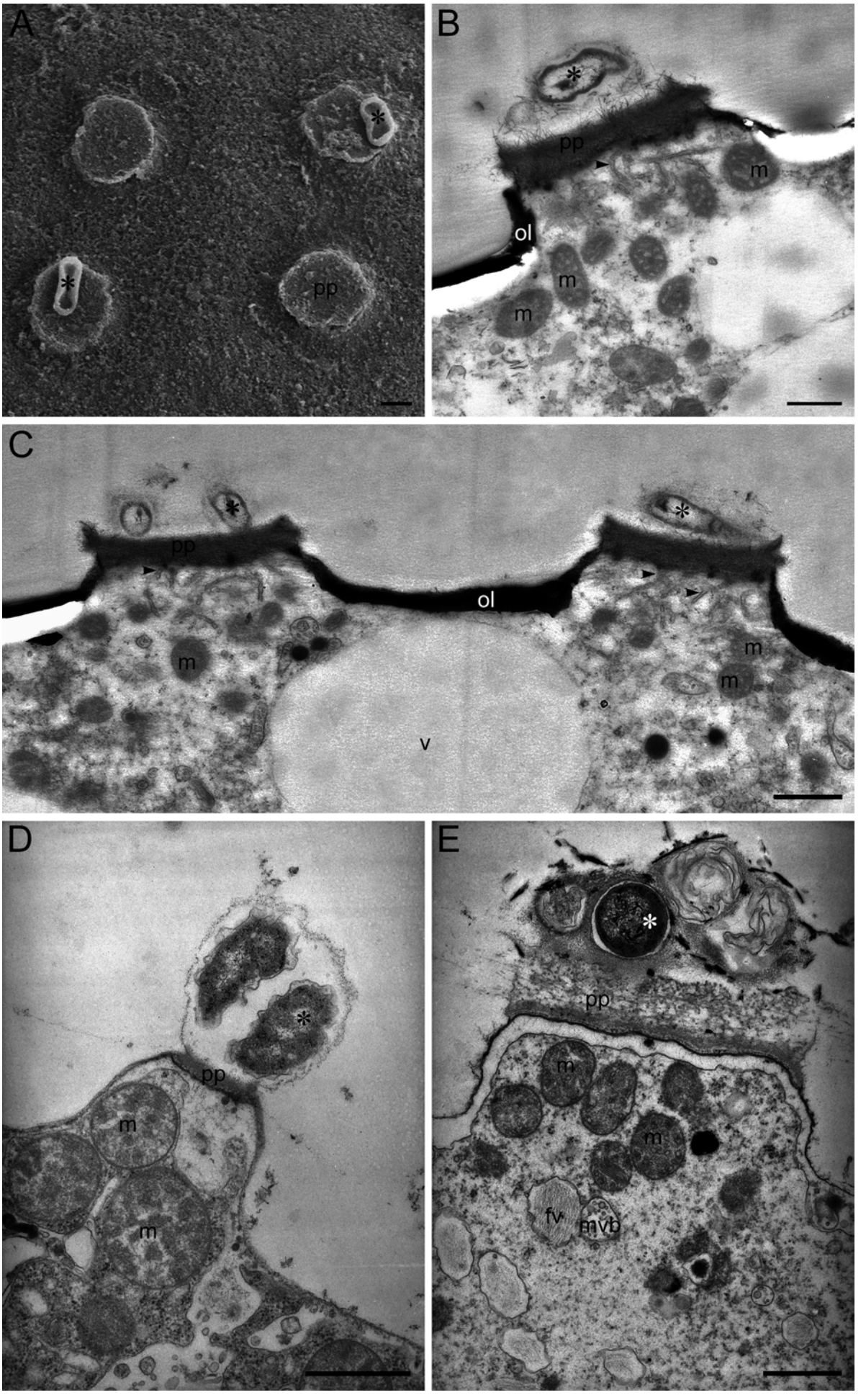
Figure 6. TEM micrographs of *Virgulinella fragilis*, showing endobionts (*) in detail. A. Note: the chloroplast (c) is a composite of four plastids. m = mitochondrion, v = vacuole, t = location of former test. A = Cariaco Basin, Venezuela; B = Walvis Bay, Namibia; C = Namako-Ike, Japan; D = Wellington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

Figure10. 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.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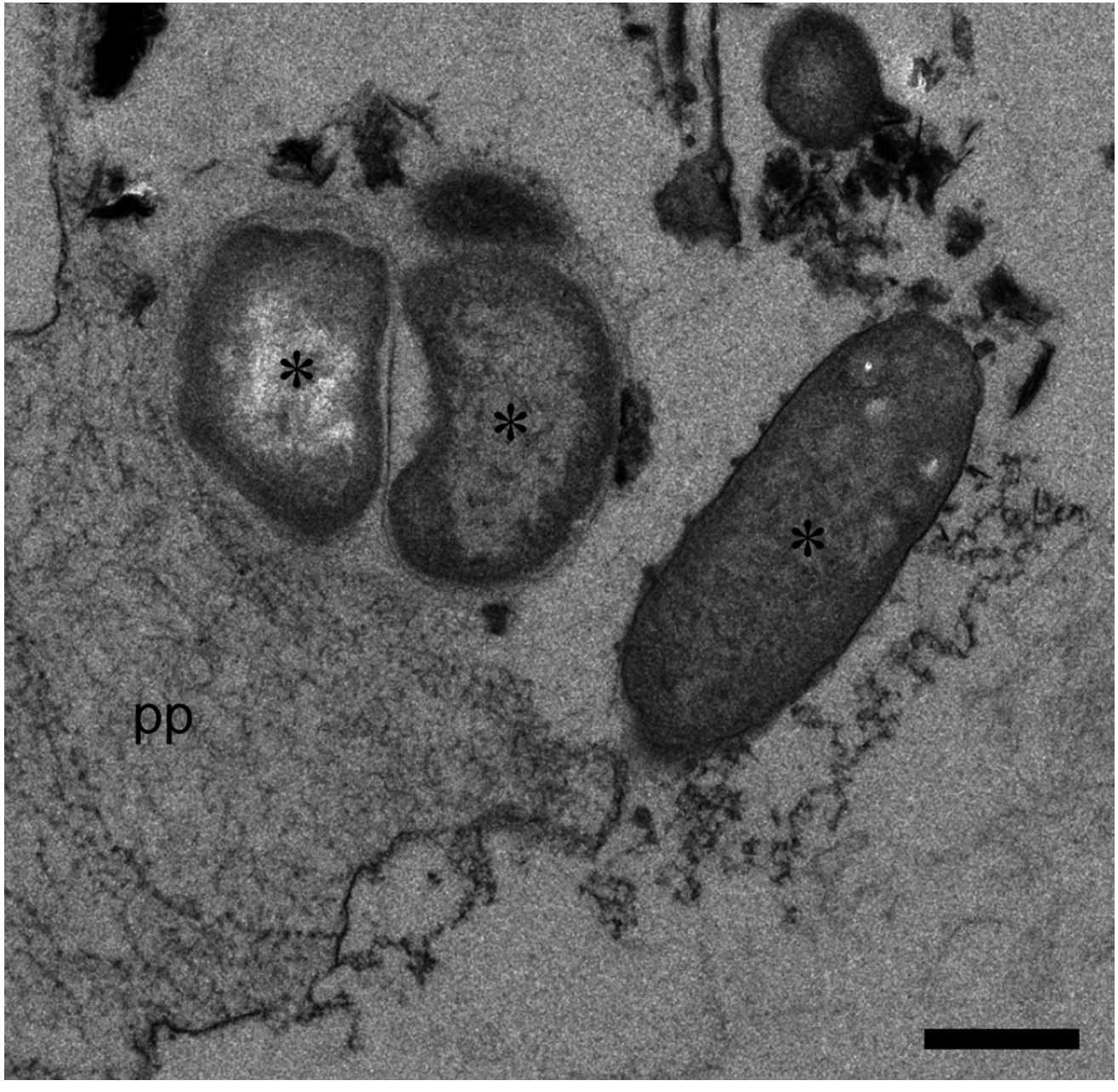
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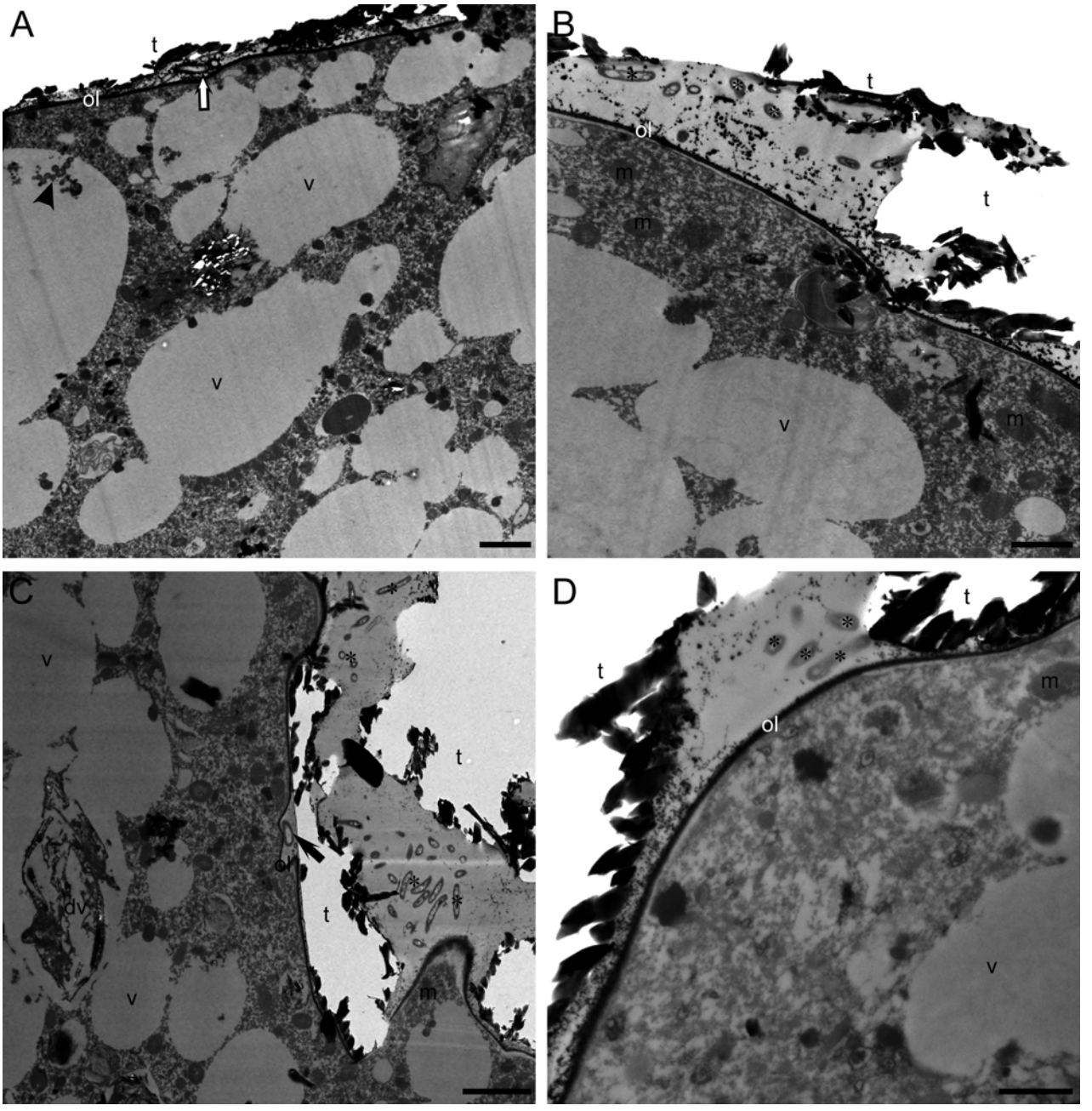
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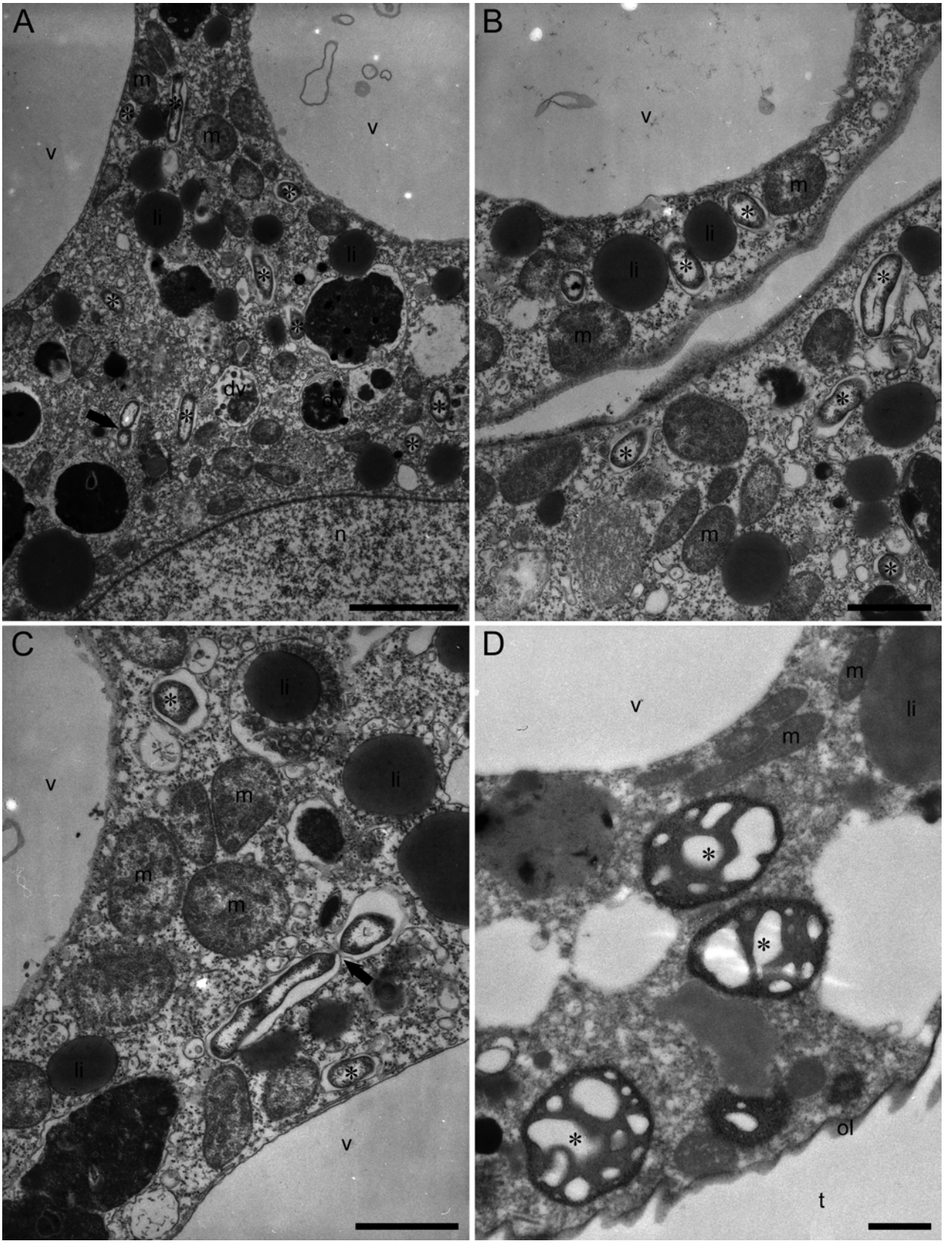
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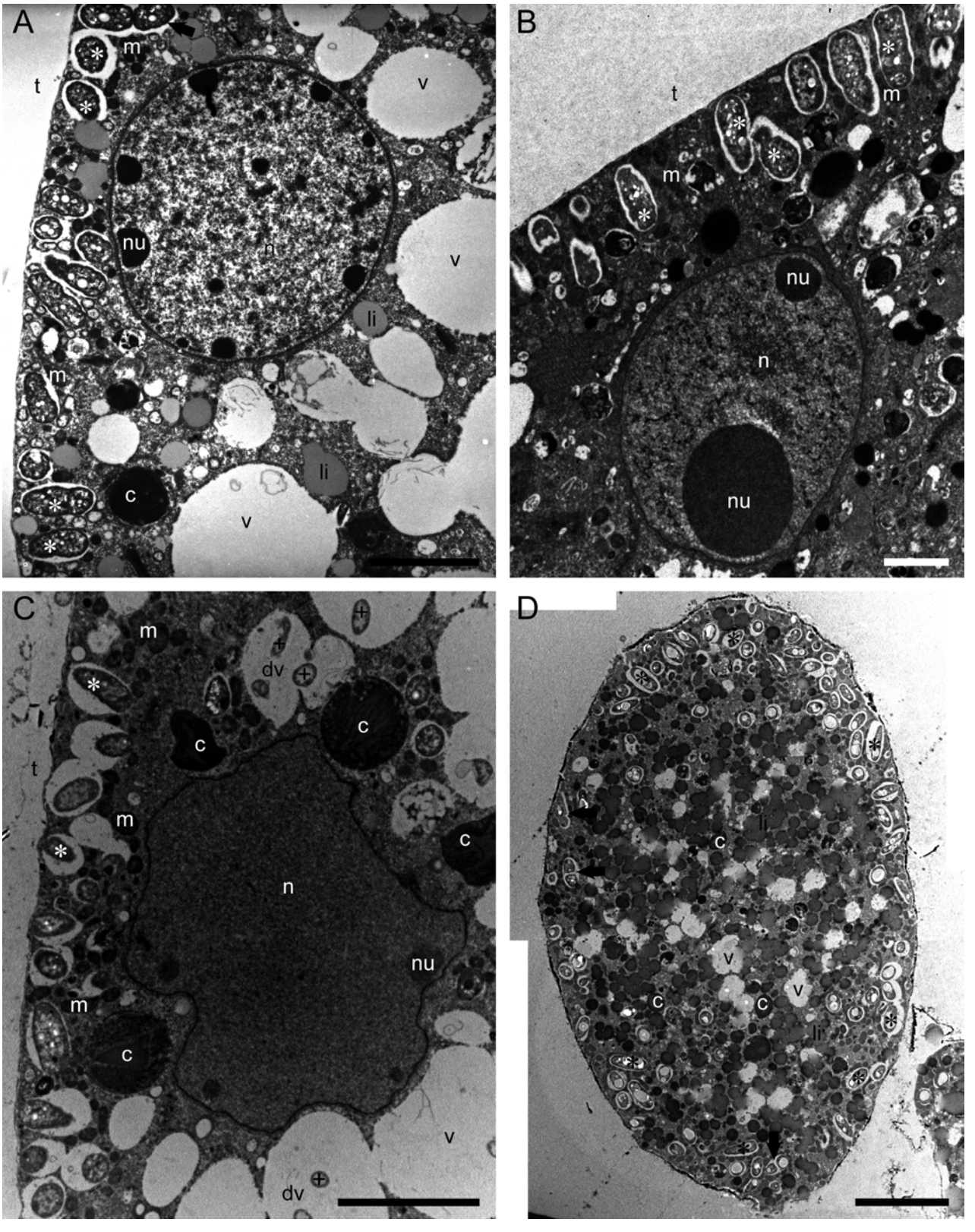
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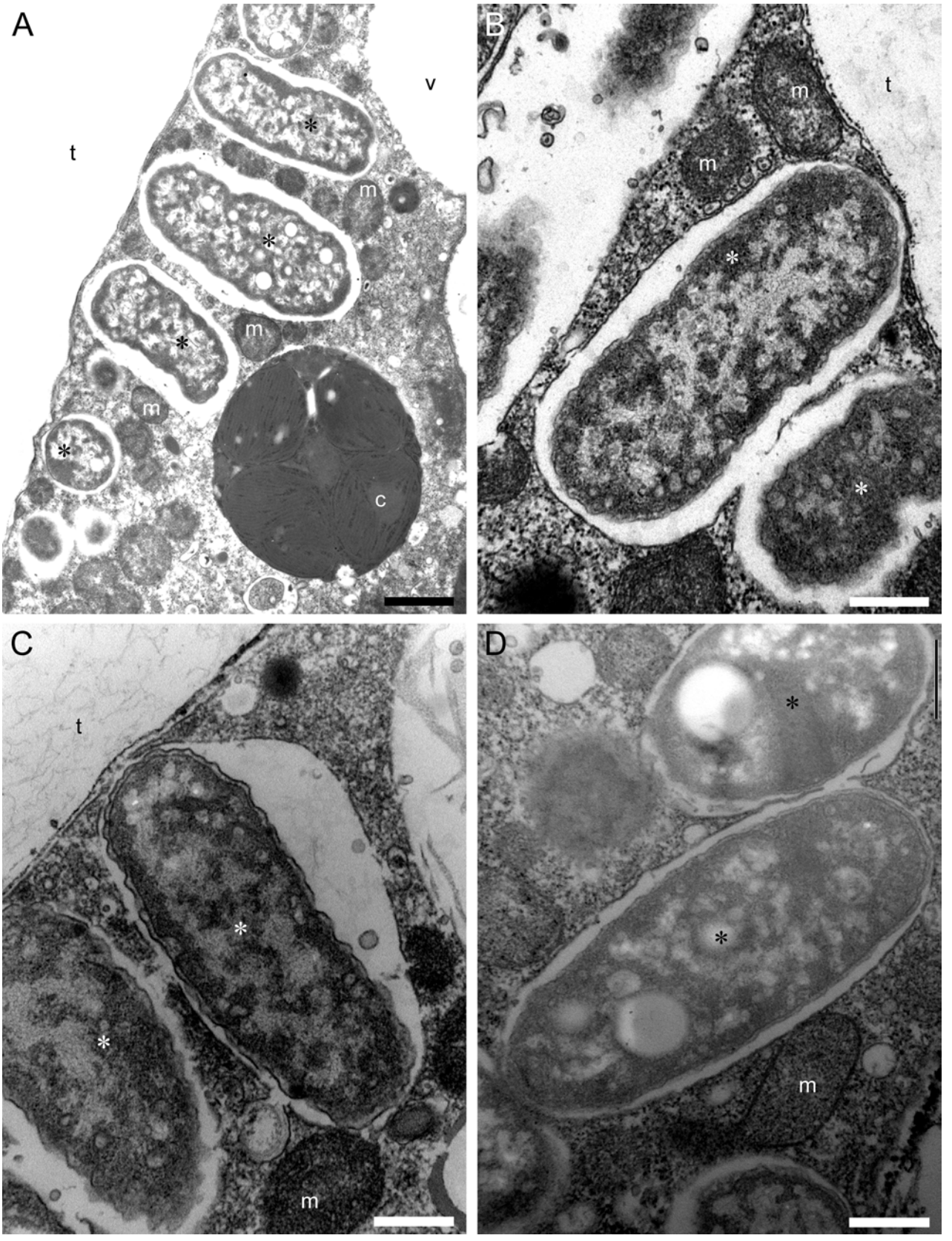
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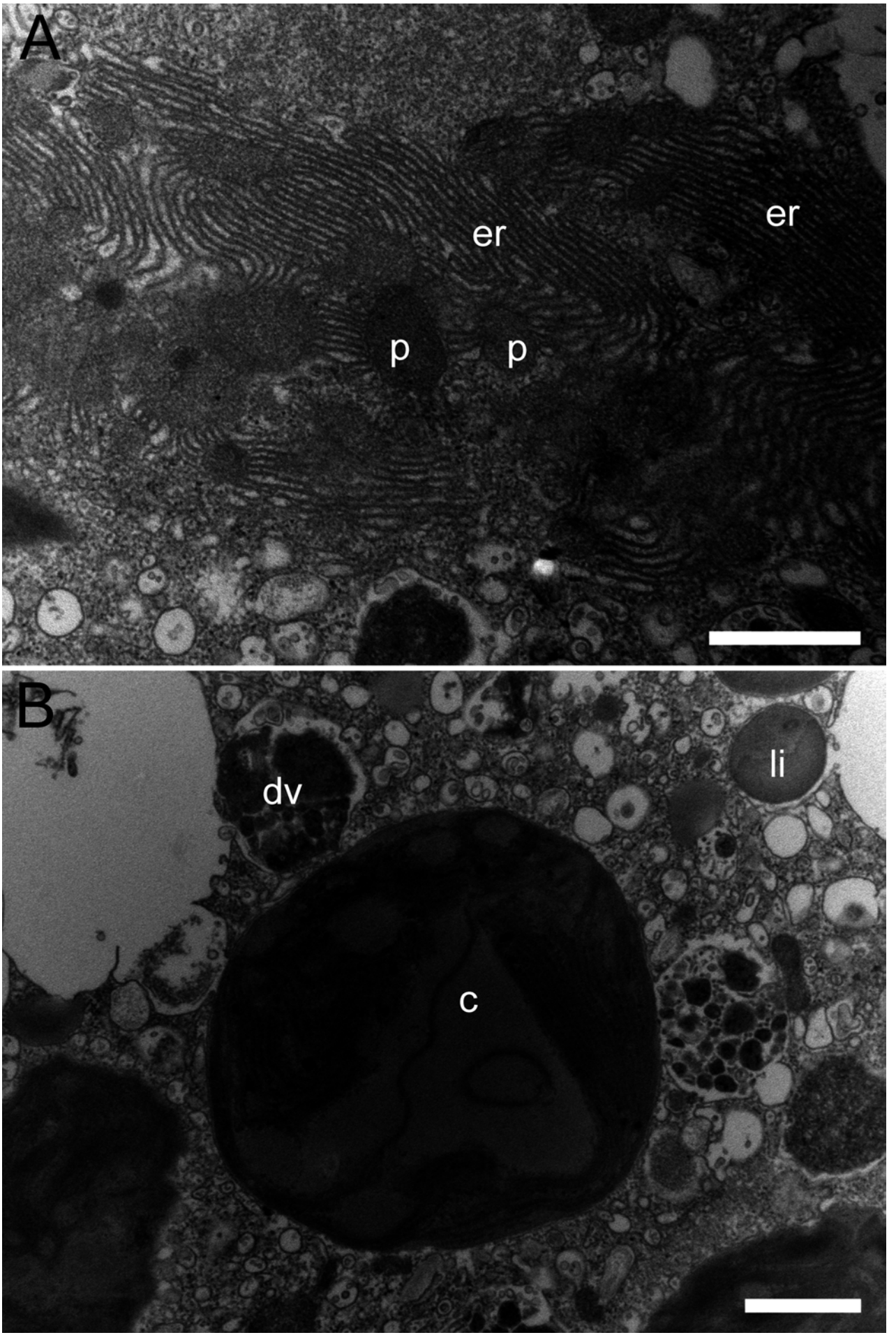
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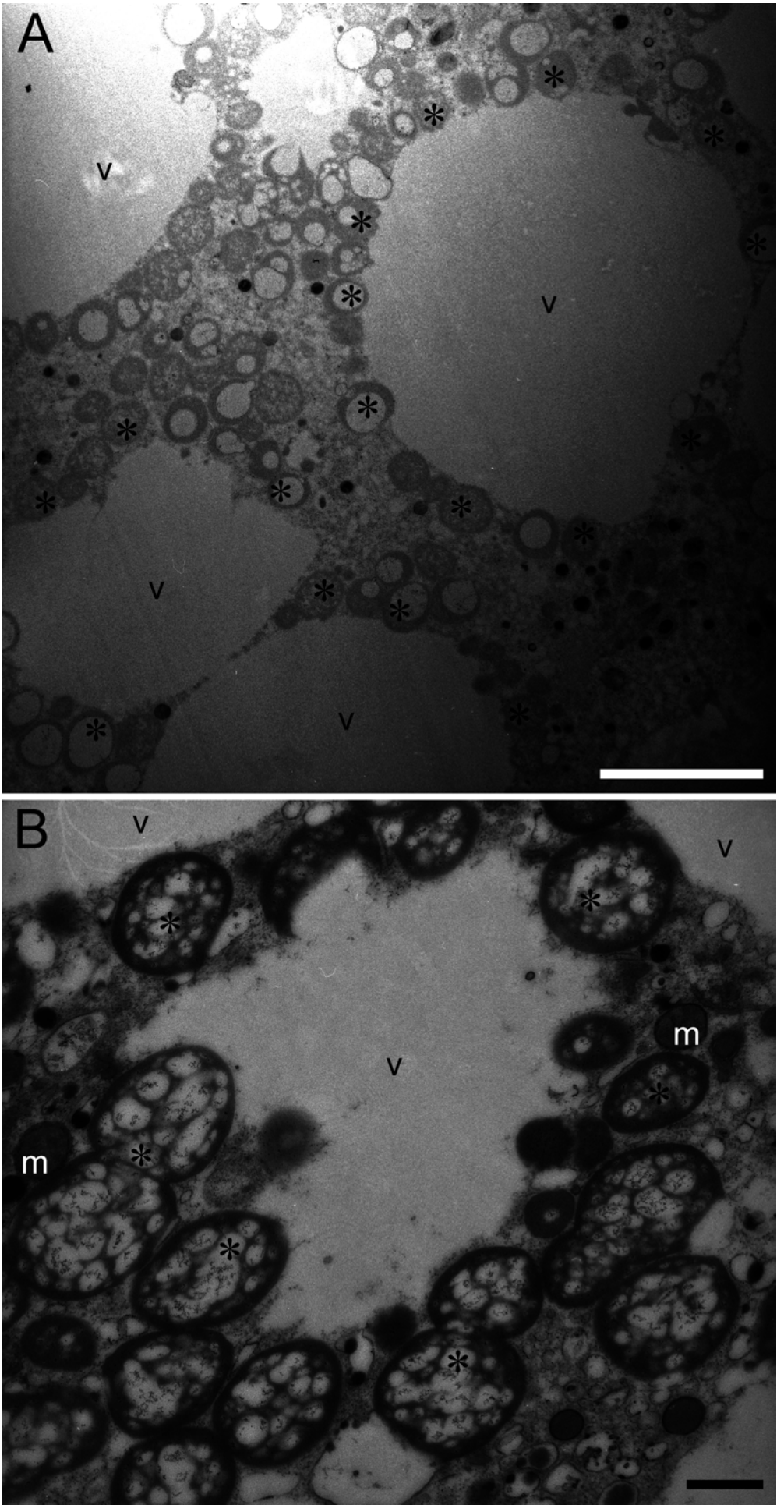
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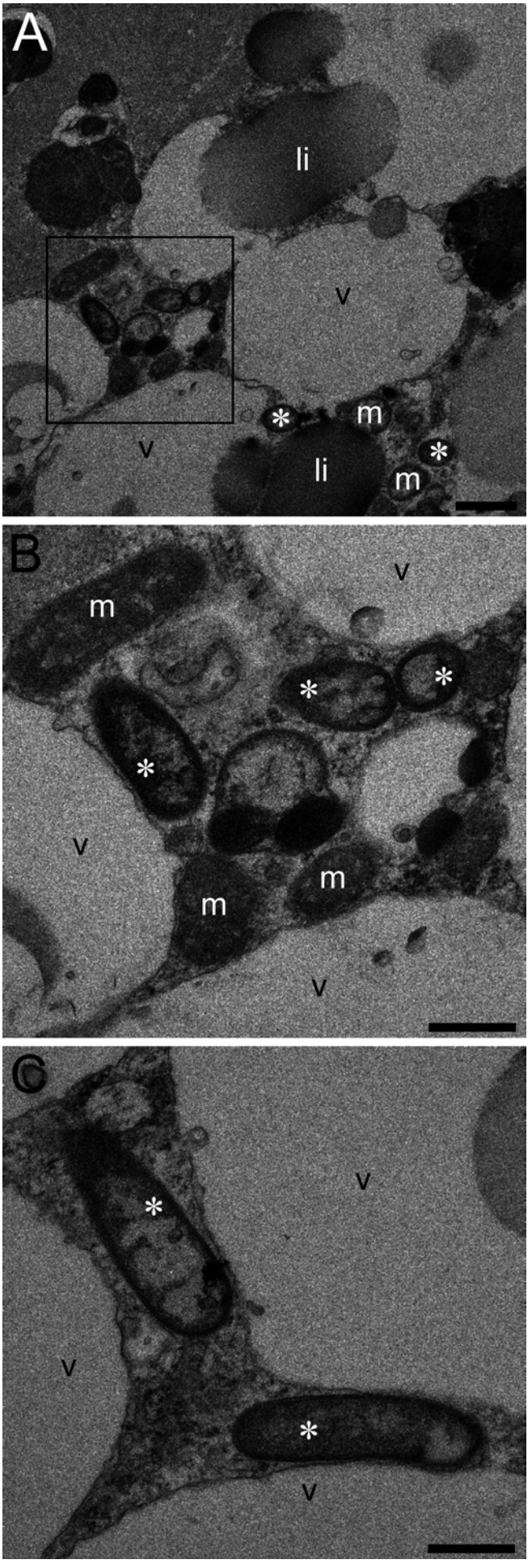
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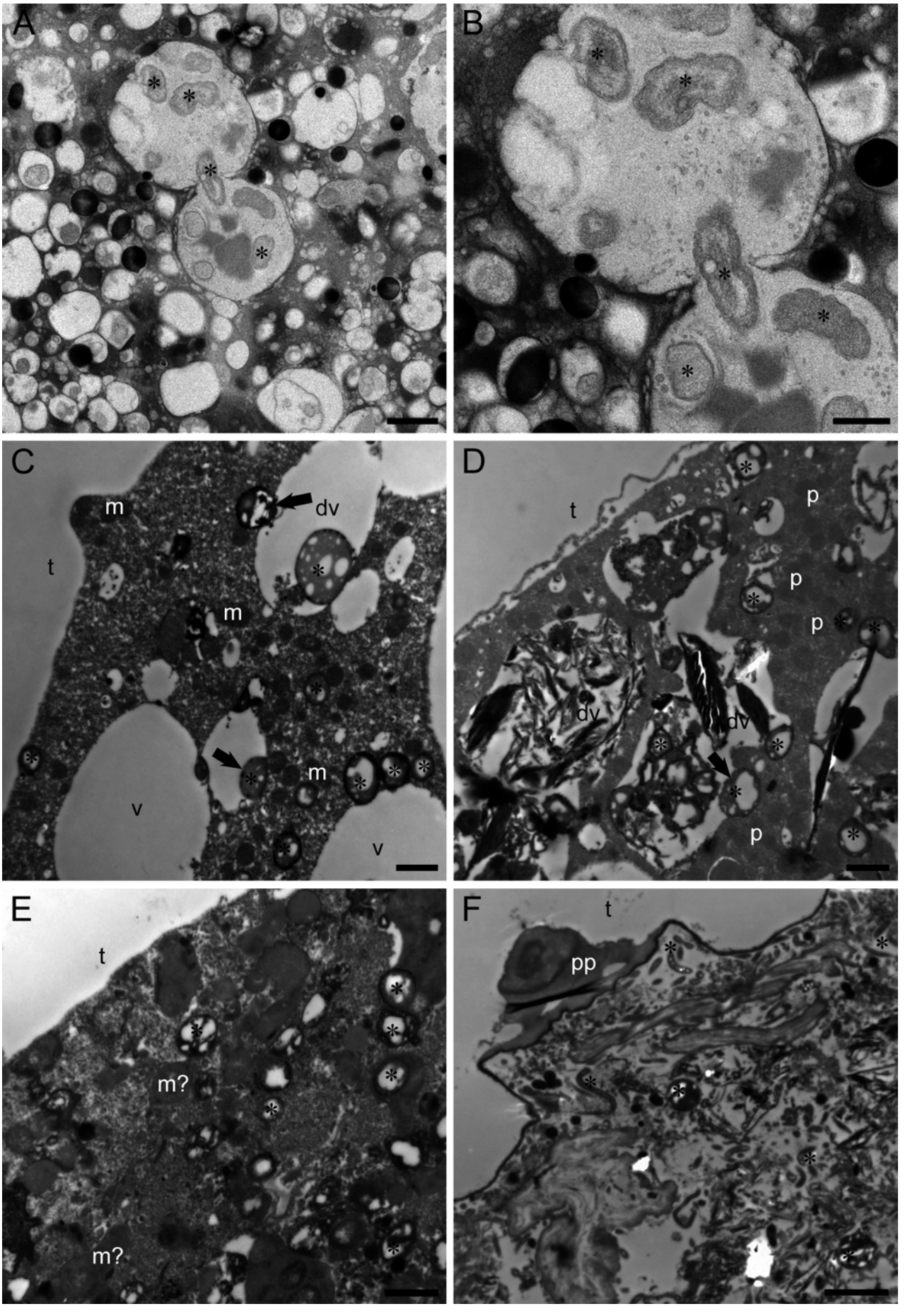
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Table 1. A summary of foraminifera-prokaryote associations discussed in this contribution.

Foraminiferal species	Environment	Prokaryote traits	Prokaryote distribution	Speculated type of association	Encapsulation	Associated features and notes
Bolivina pacifica [C]	Silled basin / chemocline	Rod	Ectobiont (pores)	Commensal or mutualistic	NA	Plasma membrane invagination
Agglutinated biserial form [A]	Near hydrocarbon-seep clam bed	Rod	Between organic lining and test	possibly food	NA	Only one specimen examined
Ammonia phylotype T6 [C]	Tidal flat	Rod	Ectobiont (pores)	unknown	NA	anoxic habitat
Ammonia phylotype T6 [C]	Tidal flat	Rod	Endobiont, near vacuoles, youngest 2-3 chambers	Temporary or parasitic	No	Anoxic incubation
Ammonia phylotype T6 [C]	Tidal flat	Rod	Endobiont, cell periphery, youngest 2-3 chambers	Temporary or parasitic	No	Anoxic incubation
Buliminella tenuata [C]	Silled basin / chemocline	Rod	Endobiont	Permanent; Mutualistic or Commensal	Yes	
Buliminella tenuata [C]	Hydrocarbon cold seep	Coccoid	Endobiont	Transient? (verging on parasitism)	No	Not present in all specimens
Globocassidulina cf. *G. biora* [C]	6-7cm below sediment-water interface	Short rod	Endobiont under pore plug	Transient?	No	Only one specimen examined
Nonionella stella [C]	Silled basin / chemocline	Rod	Between organic lining and test of final chamber	unknown	NA	Also retains kleptoplasts
Quinqueloculina sp.	organic rich photic zone	Rod	Endobiont	unknown	unknown	
saccamminid (Santa Barbara Basin) [T]	Silled basin / chemocline	Coccoid	Endobiont at vacuole periphery	Permanent; Mutualistic or Commensal	No	Endobiont has *nirK* gene
Spiculidendron corallicolum [A]	Coral reef	Ovoid	Endobiont	Commensal or mutualistic	No	
Uvigerina peregrina [C]	Hydrocarbon cold seep	Rod	Ectobiont (pores)	Opportunistic	NA	Not present in all specimens
Virgulinella fragilis [C]	Oxic-anoxic interface	Rod	Endobiont at cell periphery	Permanent; Mutualistic or Commensal	Yes	Also retains kleptoplasts

C= calcareous; A = agglutinated; T = thecate; NA= Not applicable

Figure (if any)	References
1A-C	Bernhard et al., 2010a
3	Nomaki et al., this issue; this study
2	Nomaki et al., 2014
9	Nomaki et al., 2014
-	Nomaki et al., 2016
4A-C	Bernhard, 1996; Bernhard et al., 2000
4D	Bernhard et al., 2001; Bernhard et al., 2010b; Martin et al., 2010
-	Bernhard, 1993
-	Bernhard and Reimers, 1991
-	Heeger, 1990
8	Bernhard et al., 2006, Bernhard et al., 2012
-	Richardson and Rützler, 1999; Rützler and Richardson, 1996
1D	Bernhard et al., 2001
5-7	Bernhard, 2003, Tsuchiya et al., 2015