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ORIGINAL ARTICLE

Protozoan–bacterial symbiosis in a deep-sea hydrothermal vent folliculinid ciliate (*Folliculinopsis* sp.) from the Juan de Fuca Ridge

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Abstract

This study provides a first description of the morphology of Blue Mats: sessile, colonial folliculinid ciliates (*Folliculinopsis* sp.) that create dense bright blue carpets in certain Juan de Fuca Ridge vent fields and at vents elsewhere. In one area of widespread venting, for example, Blue Mats occupied approximately 70% of the substratum. The ultrastructure of the Blue Mat ciliates was investigated in samples from Axial Volcano on the Juan de Fuca Ridge using conventional scanning electron microscopy and thin section transmission electron microscopy. These *Folliculinopsis* sp. ciliates secrete and dwell in tubes (loricae). The loricae were colonized by both coccoid and filamentous bacteria-like structures. Greater densities of coccoid- and short-rod-shaped bacteria were found between rows of cilia on the ciliate body (zooid) and especially on the peristomal lobes (arm-like extensions typical to folliculinid ciliates). A coccoid bacterial morphotype (within and independent of a vacuole) was located throughout the ciliate cytoplasm. Groups of this organism clustered within vacuoles were regularly distributed along the ciliate cortex. Electron dense, vacuole-bound features characterized by stacked membranous structures were also found within the ciliate cytoplasm. These results suggest the existence of at least an endosymbiosis between *Folliculinopsis* sp. ciliates and bacteria at hydrothermal vents. The chemolithoautotrophic nature of these symbiotic bacteria remains to be confirmed. To our knowledge, this is the first report of a protozoan–bacterial symbiosis at vents, as well as the first reported symbiosis in folliculinid ciliates.

Problem

Protozoa have a cosmopolitan distribution and play an integral role in the decomposition of organic matter, in nutrient cycling and in the maintenance of energy flow within both terrestrial and aquatic ecosystems (Anderson 1988; Atkins *et al.* 2000). They can be of low abundance but widespread in environments that only marginally suit their survival or can rapidly colonize and exploit microenvironments that more optimally satisfy their needs (Anderson 1988). Although relatively little is known about the potential ecological significance of protozoan assemblages in hydrothermal vent environments, many protozoa can tolerate reducing conditions and are likely candidates for survival in these marine ecosystems. Ciliated protozoa have been studied at shallow water vents (Medvedev 1991a,b) and a few studies have explored the diversity of foraminifera and flagellates, as well as flagellate tolerance to pressure, sulfide and metal concentrations at deep-sea hydrothermal vents (Jonasson *et al.*

1995; Jonasson & Schröder-Adams 1996; Atkins *et al.* 1998, 2000, 2002).

To date, there are no reported endemic protozoa species at hydrothermal vents. Small & Gross (1985) published the first summary of protistan organisms at 21° N on the East Pacific Rise hydrothermal vents in which, based on Small & Lynn's (1985) recognized classes, the authors found specimens representing 14 families, 15 genera and at least 20 species. The most abundant protozoa reported in their study were sessile peritrich and folliculinid ciliates (Small & Gross 1985). Tunnicliffe *et al.* (1985) described folliculinids that formed dense blue colored protozoan mats adjacent to hydrothermal venting on Axial Volcano of the Juan de Fuca Ridge. Twenty years later, dense carpets of bright blue folliculinids still occur on Axial Volcano, and are now known from other vent sites in the northeast Pacific, indicating that these ciliates are a recurrent component of the vent fauna in this region. Van Dover *et al.* (1988) report that folliculinid ciliates and foraminifera were the most abundant taxa found on long-term recruitment arrays (3.3 years) left at Clam Acres on the East Pacific Rise and concluded that these protozoa must play an important role in the vent community. A recent study on eukaryotic diversity and microcolonizers of Mid-Atlantic Ridge hydrothermal 'niches' offers the idea that ciliates and flagellates may initiate the colonization process in hydrothermal vent systems (Lopez-Garcia *et al.* 2003). It has been suggested elsewhere that marine folliculinids (and at least one freshwater folliculinid species) are early colonizers of virgin surfaces (Prime-Habdija & Matoničkin 2005).

Both freshwater and marine species of folliculinid ciliates exist (Andrews 1923) and can be found colonizing solid substrata or as epibionts on aquatic plants and mobile invertebrates (Andrews 1914; Aladro-Lubel & Martínez-Murillo 1999; Fernandez-Leborans 2003; Prime-Habdija & Matoničkin 2005). In the Indo-Pacific and Gulf of Aqaba (Red Sea) dense clusters of the only known pathogenic folliculinid, *Hallofolliculina corallasia* cause a coral-killing disease, skeleton eroding band, by settling onto and becoming embedded in living coral skeleton while secreting their black-grey loricae (Antonius & Lipscomb 2000; Winkler *et al.* 2004). At Axial Volcano vents, while we have mostly observed folliculinids as extensive blue mats colonizing basalt surfaces, we have also found the blue loricated folliculinids attached to pycnogonids, vestimentiferan tubes and limpet shells (A. Kouris, unpublished observations).

Recent interest in theories of symbiogenesis and the common occurrence of symbioses at hydrothermal vent environments have prompted extensive investigations of endosymbioses (or endocytobioses) in protozoa (Fokin 2004). The widespread distribution, local abundance and depleted carbon isotopic signature ($\delta^{13}C$ $-33‰$) (A. Kouris, unpublished results) of the Blue Mat ciliates in hydrothermal vent fields along the Juan de Fuca Ridge suggest that these folliculinids may have a symbiotic food source (see Childress *et al.* 1987). Although symbiotic relationships between prokaryotes and protozoa are widespread in marine, freshwater and terrestrial environments, there is yet no direct evidence for symbiosis between protozoa and bacteria at hydrothermal vents. Such symbioses have been suggested in reference to the Blue Mats (Small & Gross 1985; Rosati 2002) but remain unconfirmed. Symbioses between chemolithoautotrophic sulfur-oxidizing bacteria and protists are common in other environments characterized by abundant reduced sulfur compounds (Ott *et al.* 2005). The primary aim of the present study was to use scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to determine whether or not the Blue Mat folliculinids hosted symbiotic bacteria. The possibilities of both external and internal symbioses were investigated. We also used TEM to characterize the macronucleus ultrastructure in order to identify organisms to the genus level (Fauré-Fremiet 1936). Relatively few folliculinids have been studied using electron microscopy (Mulisch *et al.* 1993) and, to our knowledge, no other folliculinid ciliate is reported to host symbiotic bacteria.

Material and Methods

Samples were collected during a 2004 New Millennium Observatory (NeMO) submersible expedition to the hydrothermal vent fields of Axial Volcano on the Juan de Fuca Ridge. The pump-operated suction sampler on the ROPOS ROV was used to sample folliculinid ciliates at Marker N3 vent (depth = 1550 m). Samples were immediately fixed in 3% glutaraldehyde–cacodylate buffer (2.0 M Cacodylic Acid adjusted to pH 7.4, 12.5 ml glutaraldehyde and 0.45 M saccharose per 100 ml of buffer) on shipboard and stored in this solution until needed. Samples were post fixated in osmium and dehydrated in a series of alcohol baths up to 100%. For SEM, samples were gold coated and viewed in a Jeol JSM 840A. For TEM, samples were embedded in Epon resin and serial sections were made with a diamond knife. Ultrathin sections were stained with uranyl acetate for seven minutes, removed and rinsed with 50% alcohol, placed on droplets of lead citrate for seven minutes and again rinsed with ultra pure water. Ultrathin sections were viewed in a Phillips 201 TEM.

Results

Distribution and general morphology

The blue color typical of many folliculinid loricae was pronounced and vibrant around hydrothermal vents

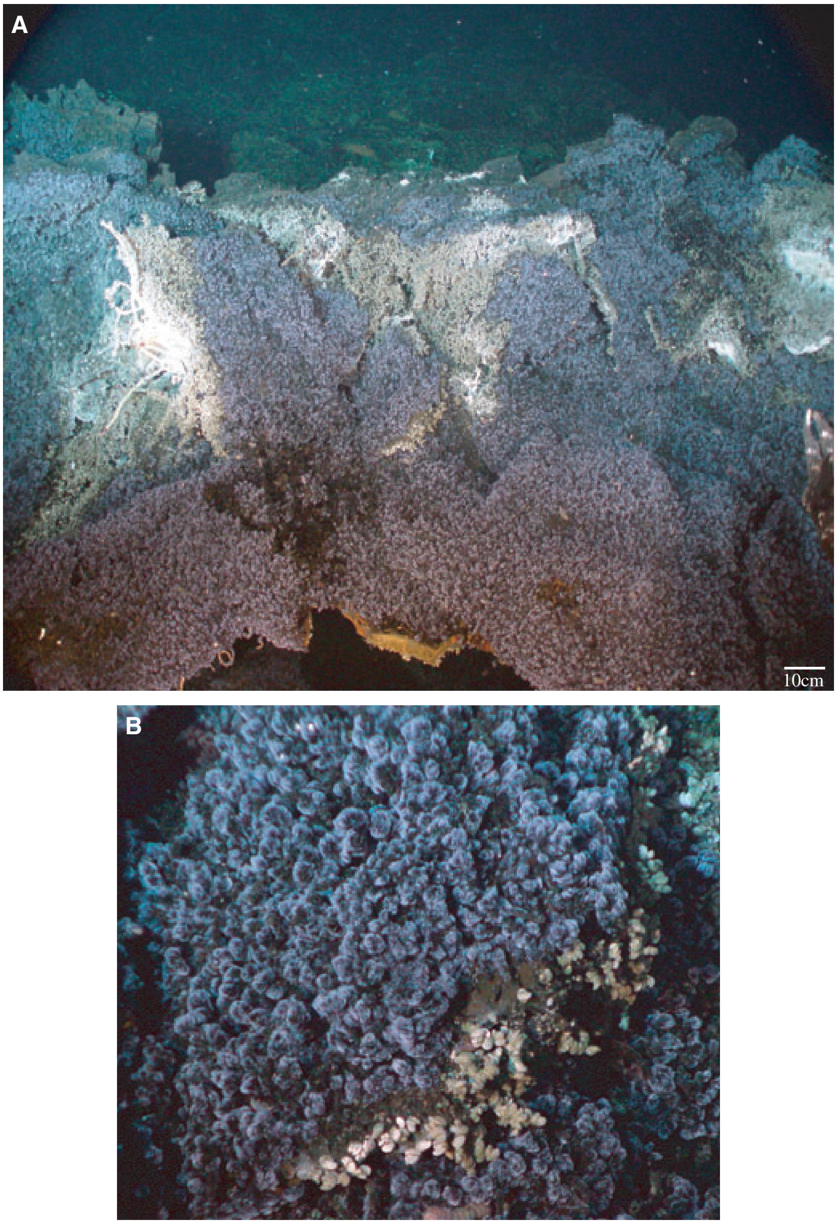

Fig. 1. *Folliculinopsis* sp. *in situ* Digital Still Camera (DSC) photographs: Blue Mat folliculinids attached to the basalt and to each other at Marker N3 vent, Axial Volcano, Juan de Fuca Ridge; A: Bare basalt visible in the background while *Folliculinopsis* sp. ciliates carpet the foreground; B: Folliculinids stacked closely together in typical 'bouquet' type formations.

because these ciliates were densely packed beside each other in large colonies. In an area of widespread venting, for example, where vent openings were 50–100 cm apart over an area of 10–20 m^2 or more, Blue Mats occupied approximately 70% of the substratum (Fig. 1A). Like other folliculinids, the blue mat ciliates secrete and dwell in chitinous sheaths, the loricae, attached to the substratum and to each other (Figs 1 and 2).

They initially secrete an ampula (sac-like structure) that adheres to a solid substratum and then construct the tube-like lorica that is arranged in a series of concentric spirals ending with a terminal everted lip (Andrews 1923). At the posterior end, the ciliate cell (zooid) splits into two arm-like extensions (peristomal lobes) that extend out of the lorica (Andrews 1923) (Fig. 3A). Under a light microscope, the zooid was purplish-red while the lorica was blue-green. The loricae ranged from 300 to 2000 μm in length.

SEM – extracellular bacteria

Irregularly scattered filamentous bacteria that were several tens of micrometers long and slightly <1 μm in diameter fouled the lorica surface (Fig. 3B). Occasionally, loricae were heavily encrusted with bacterial filaments and probably mineral deposits. No apparent attachment structures

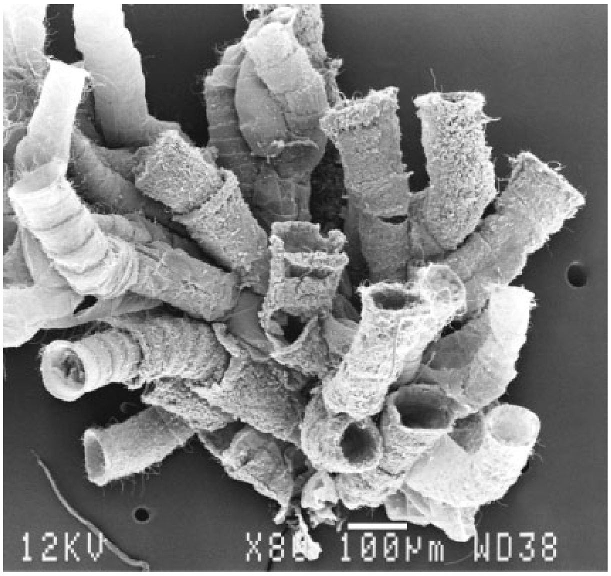

Fig. 2. *Folliculinopsis* sp. scanning electron microscopy. A cluster of blue mat folliculinids.

for bacteria were visible on the loricae. The oblong-shaped peristomal lobes were covered by rows of somatic cilia (kineties) (Fig. 3A–C). Short-rod and coccoid-shaped bacteria (average size is 0.9 μm in length) were densely piled between rows of kineties on the peristomal lobes (Fig. 3C). The concentration of these bacterial morphotypes was so high that the surface of the zooid and the base of the cilia were not visible in SEM (Fig. 3D).

TEM – intra- and extra-cellular bacteria and macronuclei

The microorganisms that were growing between rows of cilia did not appear to be inserted in the cortex, that region of the cell that is in contact with the environment on one side and the endoplasm on the other (Fisher 1996). TEM did not show any structures on the outer cortex that may serve as attachment sites for epibiotic bacteria.

In the cytoplasm, immediately adjacent to the cortex, regularly distributed vacuoles were filled with multiple (from two to over thirty) coccoid-shaped bacterial morphotypes (Fig. 4A–C). In general, the zooid was highly vacuolated. Many vacuoles near the axis of the cell appeared empty although a few were filled with digested matter (Fig. 4A). Intact individual bacterial morphotypes (both vacuolated and non-vacuolated) were ubiquitously distributed in the cell, but not necessarily interconnected. Unlike mitochondria and nuclei, intact individual bacterial morphotypes were, in the majority of instances, surrounded by a halo (Fig. 4). Another vacuole-bound feature within the cell was much more electron dense than the vacuole-encased coccoid microorganisms (Fig. 4D). These electron-dense membranes were organized in stacked structures. They were slightly smaller than individual vacuolated bacteria and only occurred singly within their encasing vacuole.

In ultra-thin sections, the ciliate macronuclei were large, generally oval shaped and beaded (Fig. 4B).

Discussion

Identification of folliculinid ciliates can be complicated by the several transformations that these organisms undergo during their life history. Individual studies may allocate different species names to the same ciliate. The single well-preserved folliculinid in Small and Gross' study was classified as *Metafolliculina* sp. based on the morphology of its lorica (Small & Gross 1985). Lorica length alone however, is not enough to classify a ciliate taxonomically. Fauré-Fremiet (1936) proposed a classification system that divides the folliculinids into two groups based on the shape of their macronucleus. As their macronucleus is beaded, the blue mat folliculinids reported in this study would, by Fauré-Fremiet's system, belong to the genus *Folliculinopsis* (Protoctista – Ciliophora – Polyhymenophora – Spirotricha – Heterotrichida – Coliphorina – Folliculinidae – Folliculinopsis) (Costello *et al.* 2001, 2004). This genus has been accepted (see Mulisch 2001; Costello *et al.* 2004). Mulisch *et al.* (1993) argue that the organization of the peristome is a more conservative trait than lorica and macronucleus shape. We agree with the latter authors that ultrastructure studies on type species of folliculinids are needed to address differing opinions on their classification. While our study considers the blue mat folliculinid from an ecological (symbiotic) point of view, a purely morphologically descriptive study may reveal that this vent ciliate is a new species. Here we will refer to the blue mat ciliate as *Folliculinopsis* sp. based on our observation of the beaded macronucleus.

Extracellular bacteria

Rosati (2002) suggests that as ectosymbiotic associations between ciliates (both aerobic and anaerobic) and bacteria are frequently observed, these relationships must be mutually advantageous for both organisms. Advantages to the ciliates (hosts) include using bacteria as a food source, as a chemical defense against potential predators repelled by a 'shell' of bacteria, as a source of reduced organic carbon or as a means to reduce toxicity in the immediate surroundings of the ciliates (Rosati 2002; Kicklighter *et al.* 2004). Desbruyères *et al.* (1998) rank both a 'biological interface' such as a tube and epibiotic bacteria amongst

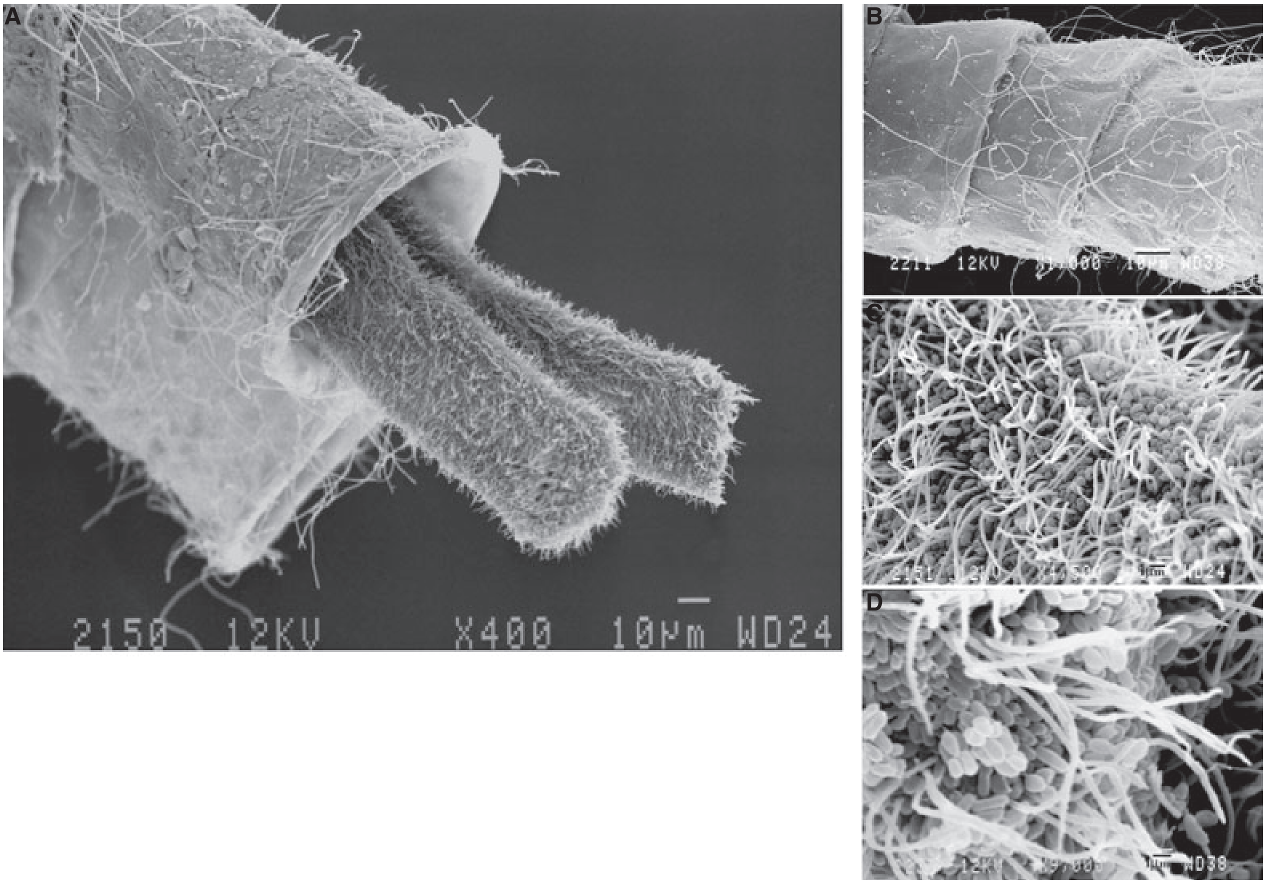

Fig. 3. *Folliculinopsis* sp. scanning electron microscopy. A: Extended *Folliculinopsis* sp. with peristomal lobes protruding from the mouth of the lorica. B: Scattered filamentous and coccoid bacterial morphotypes on the ridged lorica surface. C: Longitudinal rows of cilia on the peristomal lobes. Coccoid and short-rod bacterial morphotypes fill the spaces between the rows of cilia. D: High magnification of densely clustered coccoid and short-rod bacterial morphotypes between rows of cilia on the peristomal lobes.

the top defense mechanisms against the toxicity of the hydrothermal vent environment, this is the case for polychaete *Alvinella pompejana*, found at vents on the East Pacific Rise.

Examination of morphological adaptations by the host to accommodate epibiotic bacteria can provide a basis for distinguishing between biofouling and an epibiotic association that may be advantageous to the host. At hydrothermal vents, *Rimicaris exoculate* bresiliid shrimp (Mid-Atlantic Ridge) are extensively covered by populations of episymbiont filamentous bacteria attached to their hosts by holdfast organelles (Gebruk *et al.* 1993; Segonzac *et al.* 1993; Wirsen *et al.* 1993). Similarly, *A. pompejana* have morphological adaptations such as dorsal epidermal expansions and cuticular protrusions that appear to facilitate epibiont attachment to the host (Gaill & Hunt 1991; Desbruyères *et al.* 1998). The filamentous bacteria distributed on the *Folliculinopsis* sp. lorica appear to be more a case of biofouling than symbioses. The number of filamentous bacteria is not greater on the surface of the ciliate lorica than it would be on any other surface within a venting environment. Colonization of the lorica is patchy and irregular and there are no apparent morphological adaptations of the lorica surface to accommodate ectosymbionts.

More equivocal diagnostic features of epibiosis (*versus* biofouling) are attributes of the epibionts themselves, such as the regularity of microbial colonization of metazoan or protozoan surfaces. In contrast with apparent biofouling of the *Folliculinopsis* sp. lorica, microbial growth on the ciliate body (zooid) is more characteristic (in terms of density of bacterial epibionts) of other proposed ectosymbioses such as in *Kentrophoros* spp. ciliates. Large clusters of regularly distributed coccoid-shaped bacteria-like organisms found between rows of *Folliculinopsis* sp. cilia, and especially on the peristomal lobes, suggest an ectosymbiosis in these vent folliculinids. In part, the short-rod-shaped bacteria-like organisms stand on end like in the ciliate *Kentrophoros* spp. and in the nematode *Laxus* spp. (Ott *et al.* 1995, 2005). Free-living bacteria could attach to the peristomal lobes

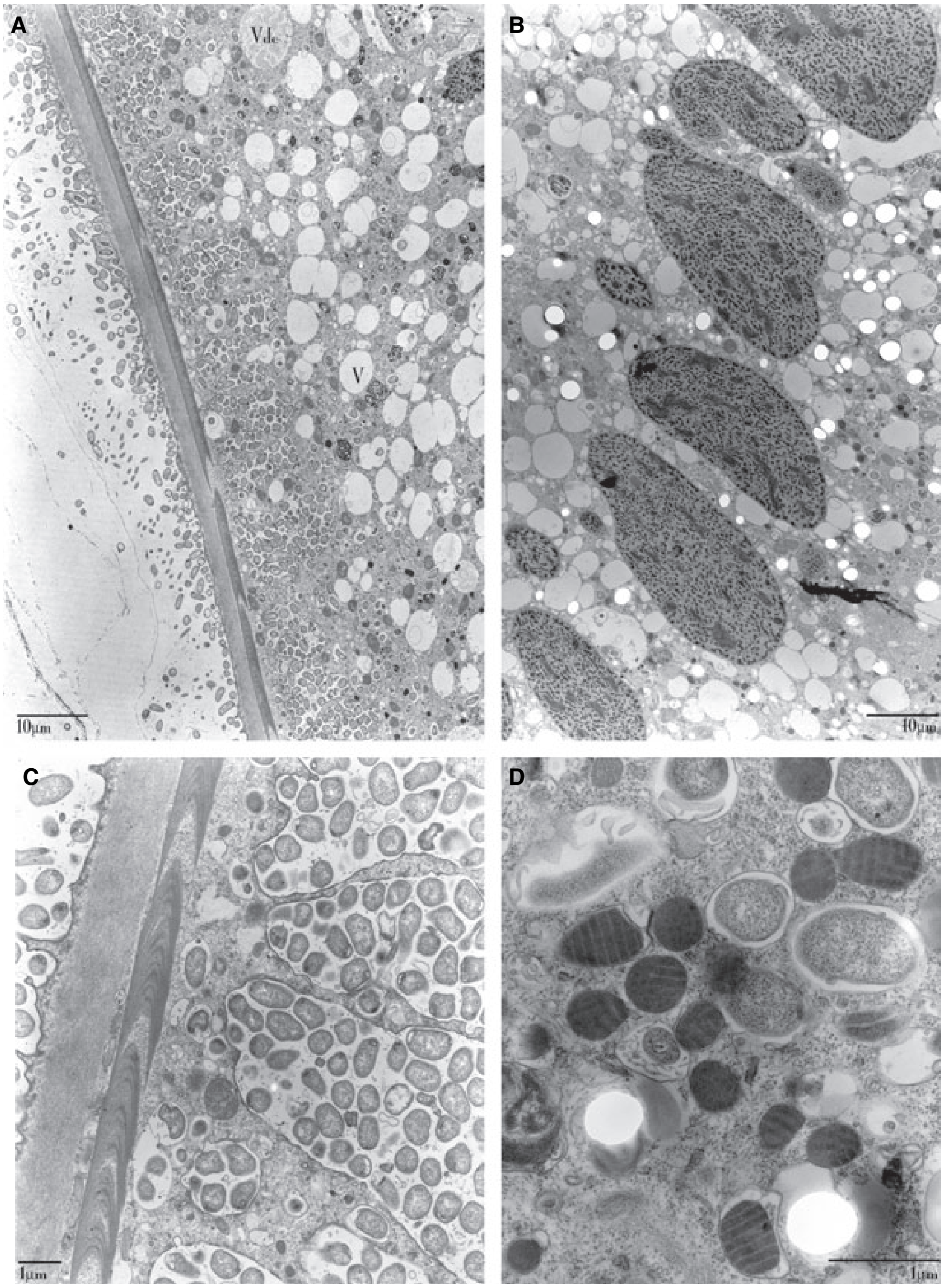

Fig. 4. *Folliculinopsis* sp. transmission electron microscopy. A: General view: Cortex separating the ciliate cytoplasm from external bacterial morphotypes. Inside cortex, along the row of somatic ciliature, vacuoles are filled with intact coccoid bacterial morphotypes. Empty vacuoles (V) and vacuoles with degraded material (Vde). B: Oblong beaded macronuclei. C: Coccoid bacteria laden vacuoles along somatic ciliature and cortex. D: Individually vacuolated, electron dense features with stacked membranes.

when the zooid is extended outside the lorica. Metachronism (coordinated beating of cilia that creates waves) may result in the circulation of nutrients from the environment to the ectosymbiotic bacterial 'guests' on the host ciliates. *Zoothamnium niveum*, a sessile, colonial peritrich ciliate colonizing mangrove roots in the Carribean, hosts bacterial epibionts (Ott *et al.* 1998) on the surface of the zooid that are identical to bacterial morphotypes found within the food vacuoles inside the host cytoplasm. As in the case of *Z. niveum*, the bacterial epibionts on the blue mat zooid may contribute to the nutrition of the Blue Mat ciliates (Bauer-Nebelsick *et al.* 1996; Ott *et al.* 1998).

Intracellular bacteria

The simple occurrence of bacteria inside the cells of colonial or other ciliates is itself not diagnostic of any symbiotic relationship. Folliculinids, like other ciliates, are known bacterivores and like other large protozoa can ingest up to 50% of their cell volume per hour (Fenchel 1987). They are capable of greatly extending their bodies outside of their loricae and can create currents using their peristomal lobes to gather bacteria or other food items for ingestion. Food particles, including bacteria, are shunted into the cytostome which is found at the base of the peristomal lobes. They are then packaged into a vacuole secreted by the ciliate host. The vacuole pinches off at the cytostome and enters into the cytoplasm along a canal (Andrews 1946). Potential bacterial endosymbionts can also enter the cytoplasm by this pathway (Fokin 2004). The widespread occurrence of ciliate–prokaryote mutualisms may have previously been overlooked although studies are now indicating that these associations have important 'ecological effects' and 'evolutionary implications' (Vannini *et al.* 2003). Fokin (2004) reports that ciliates without bacterial symbionts are more the exception than the rule and that ciliates are 'pre-adapted' to be suitable hosts. Ingestion by phagocytosis, for example, means that microorganisms can be ingested as food particles and then escape digestion (Fisher 1996).

According to Fokin *et al.* (2003), to date, nearly 60 types of bacteria have been found in ciliates including those distributed in the cytoplasm (34), macronuclei (14), micronuclei (5) or perinuclear space (6). Symbionts can satisfy their nutritional needs as well as gain shelter within the host ciliate intracellular environment (Fisher 1996).

Several characteristics of the occurrence of intracellular bacteria in *Folliculinopsis* sp. lead us to propose the existence of an endosymbiosis. The first of these characteristics is the occurrence of bacteria in regularly distributed vacuoles. Görtz (2002) reviews ciliate–bacterial symbiotic relationships reported in the literature and explains that once phagocytosed, bacteria are 'attacked by acidification, oxidative burst and lysosomal enzymes' but they can nevertheless 'prevail' in the ciliate cytoplasm either naked or in host secreted vesicles (known as either a symbiontophorous vesicle or a symbiontrophic vacuole). Although bacterial endosymbionts can occupy any area of the cytoplasm, they would be 'safest' in compartments that are devoid of the digestive activity of lysozomes such as the cortex, the nuclear apparatus the perinuclear space and the mitochondria (Fokin 2004). As shown in our TEM images, most vacuolated bacterial endosymbionts are evenly distributed along the *Folliculinopsis* sp. ciliate cortex. Although digestion of bacterial food sources is also evident (because of the presence of lysozomes), the majority of bacteria in the *Folliculinopsis* sp. cytoplasm are intact.

Given that the stacked membranous, electron dense features in the *Folliculinopsis* sp. cytoplasm are similar in size to the vacuolated bacterial endosymbionts, they may in fact be methanotrophs. Similar electron-dense features observed in the folliculinid *Lagotia minor* cytoplasm have been interpreted as pigment granules (Mulisch *et al.* 1993). These latter features, however, lack the stacked membranous structure. Methanotrophic bacterial symbionts found in *Bathymodiolus* mussels at deep-sea hydrothermal vents and cold seeps have characteristic stacked membranous structures inside their symbionts' cells (Fujiwara *et al.* 2000). Methanotrophic endosymbionts and dual symbioses are known in vent invertebrates (Endow & Ohta 1989; Fisher *et al.* 1993; Distel *et al.* 1995; Duperron *et al.* 2006). The presence of a coccoid bacterial morphotype within the *Folliculinopsis* sp. cytoplasm, as well as a possible second methanotrophic form is not unusual as ciliate cells are known to exhibit multiple bacterial infections and multiple symbioses (Görtz 2002; Fokin 2004). The chemolithoautotrophic nature of these symbiotic bacteria remains to be confirmed. Complementary molecular analysis could be performed to identify the types of microorganisms associated with the Blue Mats.

Summary

Symbioses are a common feature at hydrothermal vents and also in ciliated protozoa. While bacteria appear to foul the Blue Mat lorica surface, regular colonization of microorganisms between rows of somatic cilia, and especially on the peristomal lobes, may indicate an ectosymbiosis. Within the *Folliculinopsis* sp. cytoplasm, the regular distribution of coccoid bacteria adjacent to the cortex, an area devoid of lytic degredation of food bacteria, suggests an endosybiosis. Electron-dense, vacuole-bound features characterized by stacked membranous structures were also found within the ciliate cytoplasm. Morphological examination alone cannot confirm whether these latter features are methanotrophs or pigment granules. To our knowledge, this is the first report of a protozoan–bacterial symbiosis at vents, as well as the first reported symbiosis in folliculinid ciliates.

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