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Bacterial Endosymbionts in the Agglutinating Foraminiferan *Spiculidendron corallicolum* Rützler and Richardson, 1996

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Abstract

The cytoplasm of the Caribbean agglutinating foraminiferan *Spiculidendron corallicolum* Rützler and Richardson, 1996, consistently contains gram-negative, non-photosynthetic bacteria that show ultrastructural similarities to marine nitrifying bacteria. In some micrographs, bacteria occur in densities as high as 21 cells per 60 μm^3 of cytoplasm, indicating that an individual foraminiferan may contain as many as 2.2×10^9 bacterial endosymbionts. The bacteria are free-living in the cytoplasm, spherical to ovoidal in shape (~0.6 × 0.7 µm), and devoid of extracellular structures. Ultrastructurally, the bacteria are characterized by a discrete fibrillar nucleoid, a carboxysome-like electron dense inclusion (100 nm), and intracytoplasmic lamellae (100 nm × length of the cell). The presence of dividing individuals within the cytoplasm and the absence of this group within the foraminiferans' digestive vacuoles, indicate that the bacteria are endosymbionts and not ingested food organisms. The discovery of prokaryotic endosymbionts in what has been considered to be a basal foraminiferal clade, indicates that endosymbiosis may have played a more significant role in the evolution and diversification of foraminiferans than has previously been suspected.

Keywords: Astrorhizidae, bacterial endosymbionts, Belize, endosymbiosis, foraminiferans

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1. Introduction

The foraminiferan *Spiculidendron corallicolum* Rützler and Richardson, 1996, is a large arborescent foraminiferan that lives attached to hard substrates in shaded fore-reef habitats throughout the Caribbean (Rützler and Richardson, 1996). The species constructs its test from siliceous sponge spicules held together by a dense fibrillar organic cement that exhibits ultrastructural similarities to spongin (Rützler and Richardson, 1996). The organism cements itself to a hard substrate at the base of the test and builds an undivided (non-septate) tubular, branching test that may extend into the water column up to 6 cm from the base (Rützler and Richardson, 1996) (Fig. 1). Live individuals have a distinct, but diffuse yellowish-brown coloration to their cytoplasm and were observed to extrude an intricate web-like pseudopodial net that hangs suspended between the distal branches of the test (S. Richardson, personal observations, August 1995).

While many foraminiferans are known to harbor algal endosymbionts, only one species, *Buliminella tenuata*, has been shown to possess bacterial endosymbionts (Bernhard, 1996). Examination of the cytoplasm of *S. corallicolum* by transmission electron microscopy (TEM) has revealed that this species is consistently associated with an intracellular gram-negative, non-photosynthetic bacterium that shows ultrastructural similarities to marine nitrifying bacteria. The purpose of this paper is to describe this association and discuss its evolutionary implications for foraminiferans.

2. Material and Methods

Live specimens of *Spiculidendron corallicolum* were collected by SCUBA from 20- to 23-m water depths on South Reef, located near the Smithsonian's Coral Reef Field Station at Carrie Bow Cay, Belize, Central America, in August 1995. Individuals were pried off coral rock with pieces of the substrate still attached and returned to shore immersed in sea water. In the laboratory on Carrie Bow Cay, specimens were examined with a Wild M3 stereo-microscope, then preserved for transmission electron microscopy (TEM) following a protocol modified from Leys (1995) and Travis and Allen (1981). Prior to fixation, specimens were submersed in calcium-free seawater (MBL Formula 134: Bidwell and Spotte, 1985) for approximately one hour at room temperature. Specimens were initially fixed for approximately 30 minutes in 2% glutaraldehyde + 1% osmium tetroxide + 5 μM EGTA + 10% sucrose in a sodium acetate buffer (pH 6.4) at room temperature, then transferred to fresh fixative (on ice) for approximately 2.5 hours. Specimens were then rinsed twice with buffer and dehydrated in a graded ethanol (EtOH) series to 70% EtOH.

Figure 1. Living individual of *S. corallicolum* of approximately 5-cm height, attached to coral rock substrate. Underwater photograph by Paul Human.

Specimens were transported from the field station to New Haven, CT, in 70% EtOH.

In the laboratory at Yale University, specimens were first decalcified in 2% ascorbic acid (in 70% EtOH) to remove the coralline algae which commonly encrust the basal portion of the test, then desilicified in 4% hydrofluoric acid (in 70% EtOH) to dissolve the sponge spicules used by the organism to construct its test. Specimens were then dehydrated in a graded ethanol series and embedded in Spurr's resin. Ultrathin sections were stained in uranyl acetate (saturated alcohol solution), and examined with a JEOL 1200 EX microscope (Department of Invertebrate Zoology, Smithsonian Institution, Washington, DC).

3. Results

General observations

The cell ultrastructure of *S. corallicolum* is characterized by a highly

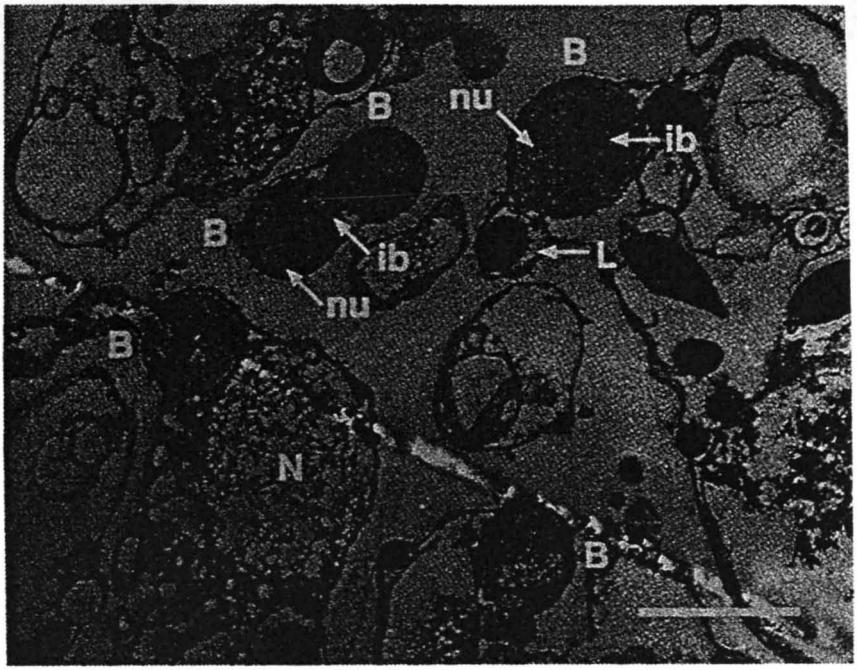

Figure 2. *S. corallicolum* cytoplasm showing bacteria (B), bacterial nucleoid (nu), inclusion body (ib), foraminiferan nucleus (N), and lysosome fusing with enlarged bacterium (L). Note: pair of bacteria in upper part of photo undergoing division by binary fission. Electron micrograph, scale bar = 1 μm.

vacuolated cytoplasm that is comprised of multiple interphase nuclei (1–1.5 μm); a golgi apparatus; small (~0.5 μm), roughly spherical mitochondria with vesicular cristae; digestive vacuoles (phagosomes or phagolysosomes); lysosomes; elongated vesicles containing a fibrillar substance; coated vesicles; microtubular arrays; and numerous non-photosynthetic gram-negative bacteria (Fig. 2). Based on observations that bacteria occur in densities as high as 21 cells per 60 μm^3 of cytoplasm, a single, 30-mm high individual of *S. corallicolum* is estimated to contain between 1.4×10^9 and 2.2×10^9 endosymbiotic bacteria.

In TEM micrographs, the bacteria appear round to elliptical in cross-section and measure approximately 0.6 × 0.7 μm (average dimensions, n=24). Extracellular structures, e.g., flagella, pili or fimbrae, capsules or slime layers, were lacking. At least three pairs of individuals (25% of the individuals censused) are undergoing division by binary fission (Fig. 2). The mode of division, a

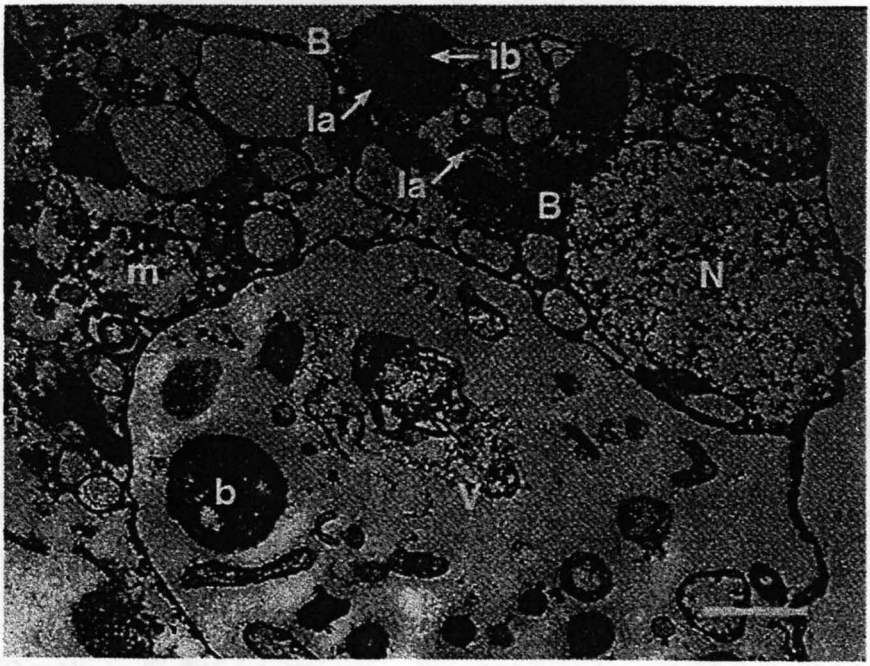

Figure 3. *S. corallicolum* cytoplasm showing bacteria (B), inclusion body (ib), intracytoplasmic lamellae (la), foraminiferan nucleus (N), mitochondrion (m), and digestive vacuole (V) containing debris and unidentified bacterium (b). Electron micrograph, scale bar = 500 nm.

constrictive process inside a rigid wall, was described by Beveridge (1989) as characteristic for gram-negative bacteria.

The bacteria were not observed to be enclosed in vacuoles, but appear to be free-living in the cytoplasm of the host cell. Nor were they found amongst the contents of the foraminiferans' digestive vacuoles, an indication that the bacteria are endosymbionts and not ingested food particles. Digestive vacuoles were observed to contain the remnants of another unknown bacterium, in addition to diatom frustrules and unidentified debris (Fig. 3). Occasionally, a lysosome appears to be fusing with the outer member of an enlarged bacterial cell (Fig. 2). We interpret this as the mechanism by which the host cell removes dead or dying endosymbionts, as it is known that lysozyme hydrolyzes the peptidoglycan layer that is an integral part of gram-negative bacterial walls (Beveridge, 1989). Host digestion of endosymbionts has been observed in numerous invertebrate-prokaryote endosymbioses; the endosymbionts, it is

hypothesized, comprise a significant source of organic material for the host organisms (Fisher, 1990; Childress and Fisher, 1992; Streams et al., 1997).

Endosymbiont ultrastructure

The bacterial cells were surrounded by an outer membrane with a knobby or undulating surface characteristic of the lipopolysaccharide membranes of gram-negative bacteria in electron micrographs (Moriarity and Hayward, 1982; Beveridge, 1989). A periplasmic space of approximately 18 nm occurs between the outer membrane and the inner bacterial membrane. Within the bacterial cytoplasm, there exists a discrete fibrillar nucleoid that may occupy up to 40% of the cross-sectional area of the cell. The nucleoid is segregated from the darker, more electron dense, ribosome-containing region of the cell by a distinct boundary (Fig. 2).

Several of the bacterial cells contain a rounded to subhedral inclusion, of moderate electron-density, and approximately 100 nm in diameter. The inclusion body is often situated in close proximity to the stacked lamellae and resembles the carboxysomes described from nitrifying bacteria, cyanobacteria, and thiobacilli (Shively, 1974; Westphal et al., 1979).

The most distinctive feature of this bacterium is the presence of a bar-like array of stacked lamellae that protrudes into the cell cytoplasm (Figs. 3 and 4). These stacks measure up to 100 nm in height and appear to extend the entire length of the cell. The lamellae are similar, both in ultrastructure and location within the cell, to the intracytoplasmic membranes of the marine nitrifying (ammonia oxidizing) bacterium *Nitrosoccocus oceanus*, a motile, free-living bacterium (Watson, 1965; Murray and Watson, 1965). *N. oceanus* can be distinguished from the bacterial endosymbiont in *S. corallicolum* by its larger size (2 μm), multiple flagella (1–22), and unusually thick cell wall comprised of four layers (Remsen et al., 1967; Watson and Mandel, 1971).

4. Discussion

Our previous work indicated the presence of potential dinoflagellate endosymbionts in the cytoplasm of *S. corallicolum* (Rützler and Richardson, 1996, Fig. 3g). These results, however, have not been duplicated in the present study. Results from the examination of the cell ultrastructure of different individuals collected in 1995, in addition to the reinterpretation of earlier micrographs (e.g., Rützler and Richardson, 1996, Figs. 3b and f), indicate that *S. corallicolum* is the host to an unidentified species of gram-negative bacterium that exhibits ultrastructural similarities to marine nitrifying bacteria.

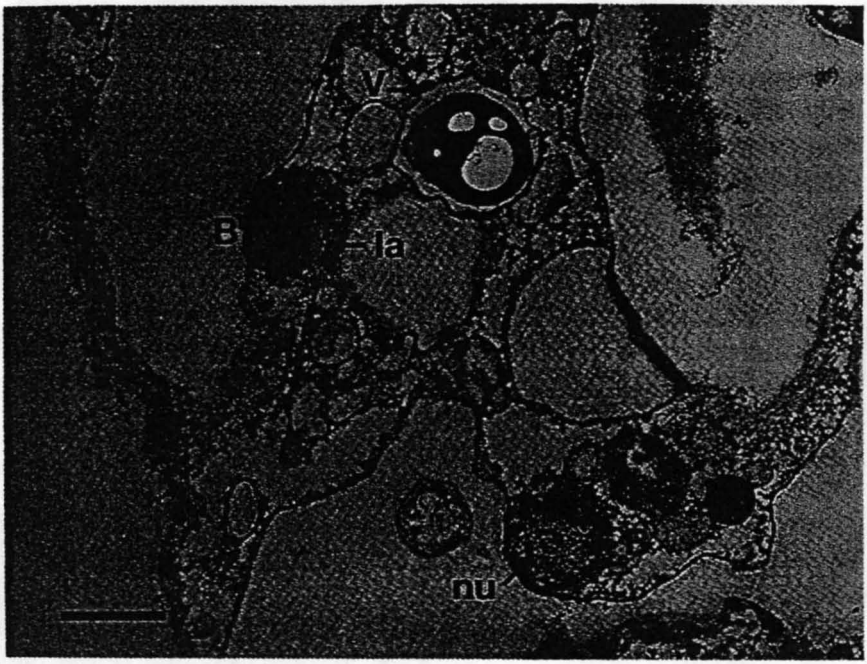

Figure 4. *S. corallicolum* cytoplasm showing bacteria (B), bacterial nucleoid (n), intracytoplasmic lamellae (la), and digestive vacuole (V). Electron micrograph, scale bar = 500 nm.

Gram-negative bacteria comprise not only the majority of prokaryotic endosymbionts documented in other protists (Ossipov et al., 1997), but the majority of bacteria dwelling in marine sediments (Moriarity and Hayward, 1982; Austin, 1988). While definitive identification of the bacterial endosymbiont in *S. corallicolum* awaits further study, it nevertheless exhibits affinities with known species of marine nitrifying bacteria at both the light and ultrastructural level. Free-living nitrifying bacteria are gram-negative, aerobic chemolithoautotrophs that require CO_2 as their carbon source and ammonium or nitrite as their energy source (Schlegel, 1993). Although the densities of free-living nitrifying bacteria in the marine environment are unknown, in culture maximum densities range from 2×10^7 to 2×10^8 cells per ml, depending on the culture medium utilized (Watson, 1963). In comparison, the bacterial endosymbiont in *S. corallicolum* is estimated to occur at a maximum density of 3.5×10^8 cells per ml, indicating that the foraminiferal cytoplasm may provide optimal growth conditions, such as an adequate supply of ammonium and CO_2 and an optimum pH. Likewise, the endosymbionts may

function to breakdown the nitrogenous waste products of the host and provide the foraminiferal cell with a supplementary, endogenous source of nutrients.

All species of marine nitrifying bacteria identified to date possess cytochromes a, b and c, which impart to their colonies a yellowish-brown color, similar to the cytoplasmic coloration observed in *S. corallicolum*. Experimental evidence shows that nitrifying bacteria have a widespread photosensitivity to light in the near-ultraviolet range (Guerrero and Jones, 1996a and b), a fact that is consistent with observations that the preferred habitat of *S. corallicolum* is in semi-shaded localities at 20–35 m water depths (Rützler and Richardson, 1996).

The only non-photosynthetic prokaryotes known to possess intracytoplasmic membranes, such as those seen in the endosymbionts of *S. corallicolum*, are nitrifying bacteria and methanotrophic bacteria (Beveridge, 1989; Schlegel, 1993). While only free-living species of nitrifying bacteria have been described from the marine realm (Murray and Watson, 1965; Watson, 1965; Watson et al., 1971; Koops et al., 1976; Jones et al., 1988), methanotrophic bacteria have been identified as endosymbionts in deep-sea sponges, mussels and gastropods found living in close proximity to hydrothermal vents and hydrocarbon seeps (Childress et al., 1986; Cavanaugh et al., 1987; Fisher, 1990; Vacelet et al., 1996; Windoffer and Giere, 1997).

Foraminiferans are known to host a diverse array of intracellular photosynthetic eukaryotic endosymbionts (Lee and McEnery, 1983; Leutenegger, 1984; Lee and Anderson, 1991); however, bacterial endosymbionts have only been documented in one other foraminiferan, the calcareous species *Buliminella tenuata*, the cytoplasm of which has been shown to contain intracellular rod-shaped bacteria with ultrastructural affinities to magnetotactic bacteria (Bernhard, 1996). West (1995) also predicted the existence of such an association based on the many ultrastructural similarities she found between nitrifying bacteria and the fibrillar bodies observed in the cytoplasm of planktic foraminiferans. Previously, the occurrence of bacteria in the cytoplasm of foraminiferans has been interpreted as ingested food (Nyholm and Nyholm, 1975b; Angell, 1980) or degenerating mitochondria (Nyholm and Nyholm, 1975a). Bacteria have also been found associated with calcareous foraminiferans inhabiting low-oxygen environments; for example, the species *Nonionella stella* has been observed to have bacteria living outside the cytoplasm, but within the organic lining of the last-formed chamber of the test (Bernhard and Reimers, 1991; Berhard, 1996; 1993). Potential cyanobacterial endosymbionts have also been reported in two species of "large," calcareous foraminiferans already known to harbor dinoflagellate endosymbionts (Lee et al., 1997).

The phenomenon of intracellular endosymbiosis in foraminiferans is usually

considered to be restricted to a polyphyletic array of derived calcareous taxa, both planktonic and benthonic (Hemleben et al., 1989; Lee and Anderson, 1991). The documentation of intracellular symbionts in *S. corallicolum*, a primitive, non-septate, tubular, agglutinated living species indicates therefore, that endosymbiosis may be a plesiomorphic character for the entire group of foraminiferans, and not just a homoplasic condition that has independently arisen in specialized groups.

Many of the life history characters which have been invoked to explain the acquisition and maintenance of endosymbionts in calcareous taxa (Lee and Anderson, 1991), are also widely distributed throughout the entire clade of Foraminifera. For example, all foraminiferans are characterized by the possession of granuloreticulose pseudopodia and the ability to ingest food particles by phagocytosis (Bowser et al., 1985; Lee, 1990; Tendal, 1990). Phagocytotic modes of feeding are believed to mediate endosymbiont acquisition in many unicellular eukaryotes (Taylor, 1983; Jeon, 1995, 1983; Silverstein, 1995). Likewise, the cytoplasmic transmission of endosymbionts from parent to progeny may be facilitated by life cycles that encompass asexual modes of reproduction. Reproduction by asexual multiple fission (agametic or ameiotic), binary fission, plasmotomy or fragmentation and regeneration, have been described in the life cycles of numerous basal taxa as well as the morphologically complex "larger" calcareous foraminiferal species (Richardson, 1993; 1994).

Symbiosis has been shown to be an important mechanism of evolution, not only in the evolution of the eukaryotic cell, but also in the diversification of major eukaryotic clades (Margulis, 1981; 1991; Maynard Smith, 1991; Sogin et al., 1996). The discovery of prokaryotic endosymbionts in what has been considered to be a basal foraminiferal clade (Loeblich and Tappan, 1988; Tappan and Loeblich, 1988), indicates that endosymbiosis may have played a more significant role in the evolution and diversification of foraminiferans than has previously been suspected (West, 1995). Photosynthetic symbioses have been hypothesized to drive the evolution of a number of ecologically specialized crown groups, both planktic and benthic, that dwell in shallow marine waters of the photic zone (Lee et al., 1979; Lee and Hallock, 1987; Hallock, 1985; Lee and Anderson, 1991; Norris, 1992). Living foraminiferans, however, occupy a number of ecologically diverse habitats in the present-day oceans. For example, in the deep sea, foraminiferans have been found not only living in restricted microhabitats within the sediments (Corliss, 1985), but dwelling in proximity to hydrothermal vents (Van Dover et al., 1988; Jonasson and Schröder-Adams, 1996), and encrusting manganese nodules (Mullineaux, 1988; 1989) and phosphate hardgrounds (Resig and Glenn, 1997). Future studies using molecular techniques and examination of ultrastructure may reveal that

the ability of foraminiferans to exploit these habitats depends on their ability to form endosymbiotic associations with diverse prokaryotic and eukaryotic taxa.

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