American Mineralogist, Volume 86, pages 826–833, 2001

TEM analysis of microbial mediated sedimentation and lithification in modern marine stromatolites

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ABSTRACT

Three sedimentary processes are involved in the growth of living stromatolites at Highborne Cay, Bahamas: (1) trapping of oolitic sands, (2) formation of surface micritic crusts, and (3) formation of fused-grain laminae. The microbial role in each process was investigated by examining the stromatolites using transmission electron microscopy. Species composition and physiological state of the bacteria were discerned by ultrastructure. A well-dispersed population of *Schizothrix gebeleinii* was observed in rapidly accreting surface layers. Their filaments produce copious quantities of amorphous exopolymer and condensed sheath that surround individual, and pairs of cells. Both fresh and degraded sheaths are, however, devoid of carbonate precipitates. This suggests that the primary roles of *S. gebeleinii* are the trapping and binding of unconsolidated sediment and the production of extracellular polymeric secretions. Surface micritic layers are composed primarily of needle-shaped crystals of aragonite. The uppermost surface of the micritic crust is coated with a biofilm comprised primarily of small Gram negative bacteria (i.e., sulfate reducing bacteria) that range in size from 250 to 500 nm in diameter and up to 1 μm in length. Empty cyanobacterial sheaths and occasional Gram positive spores were also observed. Thin sections through resin-casts of ooid microborings in the fused-grain laminae show cells of the endolithic cyanbacterium *Solentia* sp. and evidence of an organic matrix. The micritic crusts and fused-grain layers became lithified laminae that were preserved at depth, although the organisms that formed them were not. This suggests that morphological remains of these organisms (i.e., microfossils) in ancient stromatolites should not be expected.

INTRODUCTION

Stromatolites have been defined as laminated organo sedimentary structures built by the trapping and binding of sediment and/or carbonate precipitation as a result of the activities of microorganisms, primarily cyanobacteria (Walter 1976). To date, there has been little consensus of opinion regarding processes of stromatolite growth and lithification (Grotzinger and Knoll 1999). We were interested to see if in situ analysis by transmission electron microscope (TEM) could provide a better perspective on these processes in modern marine stromatolites and microbial calcification in particular. Modern marine stromatolites have been known in the Bahamas since the early 1930s (Black 1933) and have been the focus of many studies over the past two decades (Monte 1976; Dravis 1983; Dill et al. 1986; Reid et al. 1995; Golubic and Browne 1996). Many of these microbial structures are hard and contain lithified laminae (Dravis 1983; Reid et al. 1995) and thus have the potential to serve as modern analogs of ancient stromatolites. At Exuma Cays, stromatolites are found forming in open ocean water of normal salinity (Dravis 1983; Dill et al. 1986; Reid and Brown 1991; Reid et al. 1995; Steneck et al. 1996, 1997). These domal structures, which may be a few centimeters to over 2 m in height, grade from well laminated stromatolites to unlaminated thrombolites (Feldmann 1996; Reid et al. 1995, 1999; Golubic and Browne 1996; Macintyre et al. 1996). The stromatolite locality at Highborne Cay, which is the site of the present study, is located approximately 50 km southeast of Nassau. The stromatolites there form within a reef complex lying along the windward east coast that is 50 to 100 m wide and about 3 km in length. A more detailed description of the locality can be found in Reid et al. (1995, 1999) and Visscher et al. (1998). The stromatolites are composed primarily of fine-grained carbonate sands that range in size from 125–250 mm and are stabilized by horizontal ribs of lithified laminae giving rise to a classic stromatolitic structure (Reid et al. 1995, 1999).

Three different surface microbial communities have been identified in Highborne Cay stromatolites, and each represents a different growth stage (Reid et al. 2000). The first, Type 1, is dominated by the filamentous cyanobacterium *Schizothrix*

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gebeleinii and characterizes the stromatolite surface during periods of rapid sediment accretion. This community often imparts a caramel color to the sediment. The second, Type 2, is typified by a surface populated by a community of heterotrophic bacteria embedded in amorphous extracellular polymeric secretions (EPS) underlain by filamentous cyanobacteria. This Type 2 community is associated with development of a micritic crust (Reid et al. 2000), which typically gives the surface a white appearance. The third surface community, Type 3, represents further development of the Type 2 community and the colonization of the near subsurface by the endolithic cyanobacterium *Solentia* sp. The cells of *Solentia* sp. impart a deep green color to the sediment. Beneath the surface these communities have different fates. In Type 1 communities, the caramel color bleaches with burial resulting in white unlithified layers. The micritic crust of Type 2 communities is preserved with depth as thin layers of micrite. Type 3 communities maintain their color for several centimeters below the surface, eventually becoming gray-green in color. The continued boring activity of the endolithic cyanobacteria in Type 3 communities and penecontemporaneous cementation within bore holes of the ooids results in a fused-grain layer (Macintyre et al. 2000). The cyclical succession and subsequent burial of these surface communities results over time in the growth and laminated structure of the stromatolite. The lithified laminae (both thin micritic and fused-grain layers) contribute to the overall integrity of the structure (Reid et al. 2000).

In an effort to investigate microbial community structure and lithification in Bahamian stromatolites forming in open marine environments, we examined deposits at Highborne Cay by TEM. We were particularly interested in determining spatial relationships between the microbial communities and carbonate precipitates. TEM has been used to study microbial communities in laminated sediments from hypersaline environments (Stolz 1983, 1984, 1990, 1994, 2000; D'Amelio et al. 1987), and more recently fine-grained continental margin sediments (Ransom et al. 1998). TEM especially lends itself to communities dominated by phototrophic bacteria as these organisms can be readily recognized by their chromatophore membranes and light harvesting structures (Stolz 1991). Other informative ultrastructural details include bacterial cell wall topology (i.e., Gram negative and Gram positive) and intracellular inclusion bodies. This in situ approach, however, has not been widely applied if at all to carbonate sediments. Traditionally, carbonate sediments (Folk 1993; Reid et al. 1995; Seong-Joo et al. 2000) and microbial calcification (Chafetz 1994; Folk and Chafetz 2000; Merz-Preiss 2000) have been examined by scanning electron microscopy. In instances where TEM has been used, the material was decalcified prior to dehydration and embedding (Knorre and Krumbein 2000). Thus this report marks the first in-depth analysis by TEM of the microbial community in a modern marine stromatolite.

METHODS

The samples in this study were collected in June 1997, March and August 1998, and July 1999. They were chemically fixed in the field with 2.5% glutaraldehyde in filter sterilized (0.2 mm) seawater buffer (Stolz 1991). The samples were kept cold and in the dark until processing. Each stromatolite sample was examined under a dissecting microscope and the presence and thickness of each layer was determined. Subsamples of each individual layer were obtained by careful dissection and placed in fresh fixative. Orientation of the samples was maintained throughout the procedure. For this study, samples were oriented perpendicular to the block face so that sections were through the vertical profile. All samples were then rinsed in filter sterilized seawater buffer, post-fixed in 2% osmium tetroxide and stained en bloc with 1% uranyl acetate as described in Stolz (1983). After dehydration with an ethanol series and propylene oxide, the samples were embedded in Spurr's low viscosity embedding medium (Stolz 1983). Thick (~200 nm) and ultrathin (~90 nm) sections were readily obtainable using a diamond knife. Thick sections were stained with toluidine blue (Stolz 1983) and viewed with a Nikon Eclipse 600 light microscope. Thin sections were stained with uranyl acetate (1% w/v) for 20 minutes and lead citrate (1% w/v) for 15 minutes and viewed on a Philips 201 transmission electron microscope at 60 kV. Both light images and TEM negative images were captured using a Diaz CCD color camera, processed with Adobe PhotoShop, and printed with a Codonics dye sublimation printer. All images were digitally enhanced for improved contrast and sharpness.

RESULTS

Trapped oolitic sand laminae

Populations of the cyanobacterium *Schizothrix gebeleinii* dominated stromatolites with a well-developed surface caramel-colored layer (Fig. 1). The filaments in this particular sample (6/97 NS11) were oriented both perpendicular and parallel to the surface (Fig. 1A). Vertically and randomly arranged filaments were also observed in other samples. Both individual filaments as well as bundles of two and occasionally three filaments surrounded by a common thick sheath were readily observable in ultrathin sections (Fig. 1B). Filaments observed in longitudinal section had multiple rows of tightly stacked thylakoids with abundant phycobilisomes and carboxysomes (the organelle involved in carbon fixation) (Fig. 1C), indications of healthy active photoautotrophic organisms (Stolz 1991). The microbial community appeared to be completely enveloped in EPS. The contact between the cyanobacterial amorphous EPS and the ooids was smooth indicating little or no dissolution of the ooid surface (Fig. 1D). Examination by TEM of unlithified white layers 2–3 mm below the surface revealed that they were comprised primarily of empty cyanobacterial sheath and Gram negative bacteria (Fig. 1E). The few cyanobacteria observed were highly vacuolated and had few phycobilisomes and carboxysomes. The compact state and increased density of the sheath material indicated it was semi-hydrated and in the early stages of degradation (Fig. 1E) (Golubic and Hoffman 1976; Golubic and Barghoorn 1977; Stolz 1984). Unlithified white layers from deeper in the stromatolite (below 5 mm) showed sheath material that had degraded to fine fibers (Fig. 1F). Significantly, little or no calcification was seen in these layers.

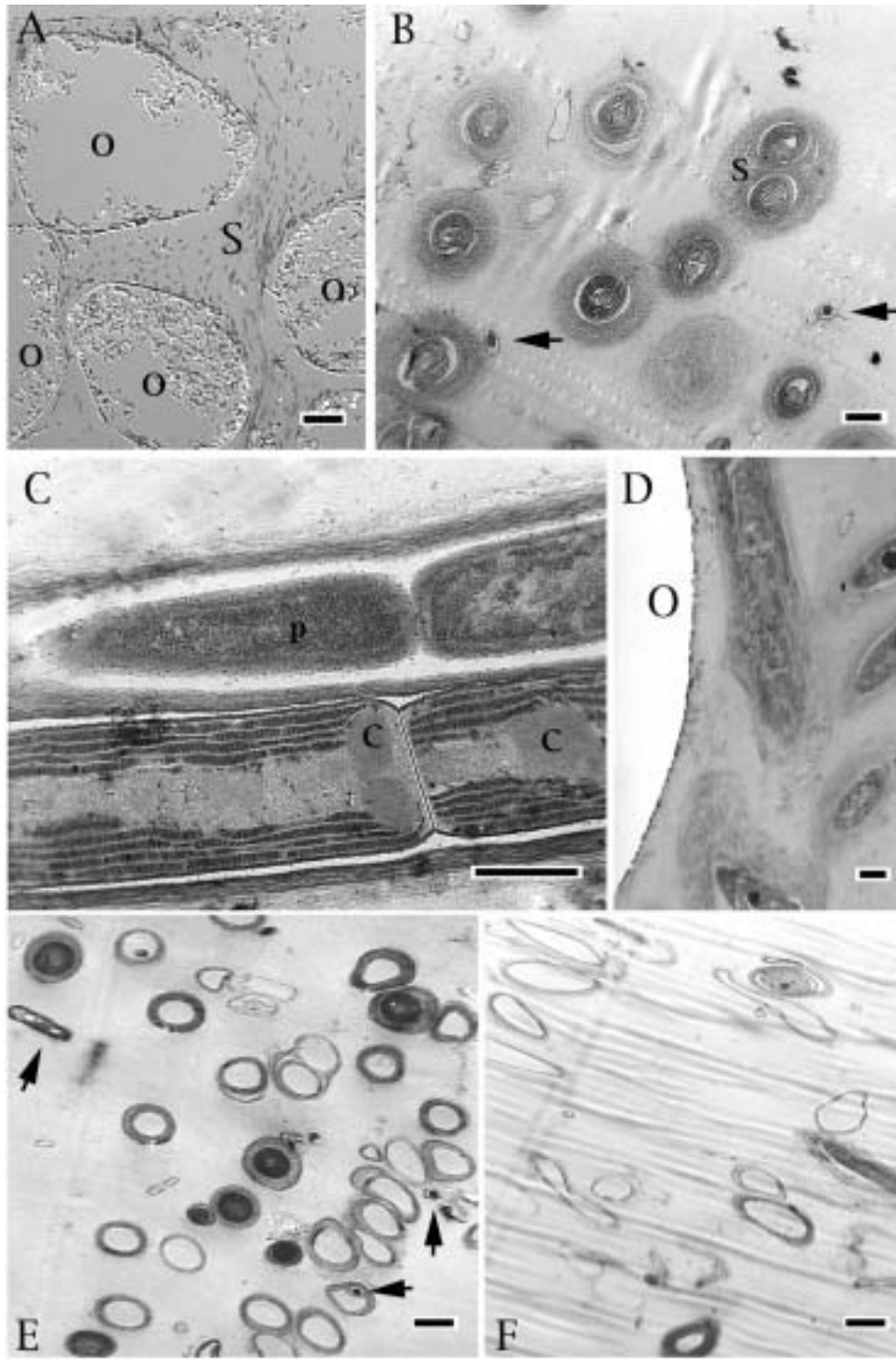

FIGURE 1. The microbial community in laminae comprised primarily of trapped oolitic sand. (**A**) Light micrograph of an oriented thick section through the uppermost 500 μm of the stromatolite (sample 6/97 NS11) showing ooids (o) and abundant filaments of *S. gebeleinii* (S). Toluidene blue stained. (**B**) TEM of the surface layer showing filaments of *S. gebeleinii* in cross section (sample 6/97 NS8n). There are also several Gram negative bacteria encased in sheath (arrows). (**C**) TEM of the surface layer (sample 6/97 NS11) showing filaments of *S. gebeleinii* in longitudinal section with phycobilisomes (p) and carboxysomes (c). (**D**) TEM of the contact between the cyanobacterial EPS (right) and ooid cast (left, o) (sample 6/97 NS8n). (**E**) TEM of the underlying unlithified white layer 2 mm below the surface, revealing mostly empty cyanobacterial sheath in the early stages of degradation and Gram negative bacteria (arrows) (sample 3/98 NS10b). (**F**) TEM of an unlithified white layer 5 mm below the surface (sample 6/97 NS8n) revealing empty sheaths in advanced stages of degradation. Bar in (A) 20 μm, all others 1 μm

Surface micritic crust

Thick sections through the top 500 μm of samples with a well developed surface micritic crust revealed a 20 to 40 μm layer of micrite underlain by a layer of unconsolidated ooids (Fig. 2A). Empty sheaths of *S. gebeleinii* were observed immediately below this surface crust. Transmission electron micrographs of this micritic crust revealed that it was comprised of needle-shaped crystals (Fig. 2B), presumed to be aragonite (based on morphology). Small bacteria, 0.25 to 0.5 μm in diameter and up to 1 μm in length, embedded in a biofilm of amorphous EPS could be seen on the surface of the micritic crust (Fig. 2C). Many of these bacteria were vibrioid-shaped. In some cases microcolonies of vibrioid-shaped bacteria were surrounded by carbonate needles (Fig. 2D), suggesting that bacteria were directly involved in the precipitation process. In addition to the vibrioids, a unique helical filamentous Gram negative bacterium was frequently observed (Fig. 2E) as well as bacterial spores suggesting the presence of Gram positive bacteria (Fig. 2D). Filaments of *S. gebeleinii* were conspicuously rare within the micritic crusts and surface biofilms, but when present lacked phycobilisomes, and were heavily vacuolated (Fig. 2F). Micritic crusts deeper in the stromatolite (i.e., 2 cm below the surface) were also comprised of aragonite needles (Figs. 2G and H). Few bacterial remains were observed within the carbonate matrix of these subsurface layers and little evidence of a biofilm remained.

Fused-grain laminae

An oriented thick section through the surface 500 μm of a Type 3 community (sample 7/99 NS2) is shown in Figure 3A. A micritic surface crust was clearly visible although it was not continuous in this particular section. Silt-sized grains of detrital carbonate were also seen at the surface, appearing as highly refractive material in thick sections observed by differential interference microscopy (Fig. 3A). Cells of *Solentia* sp. were identified within ooids that lay just beneath the micritic crust (Fig. 3). The ooids were well bored and many had lost their spherical shape and had become truncated (Macintyre et al. 2000). Further down section (~200 μm) filaments of *Schizothrix gebeleinii* were also aligned perpendicular to the surface, suggesting that they were trapped in the process of vertical migration.

Although most of the material from the carbonate ooids had fallen out of ultrathin sections, in several instances where the resin was able to penetrate bore holes within the grains, cells of *Solentia* sp. were observed (Fig. 3B). The cells had reticulating thylakoids and abundant electron opaque inclusions. In contrast, the filaments of *S. gebeleinii* were vacuolated and had few thylakoids, suggesting that they were in the early stages of degradation. At depth, the *Solentia* sp.-dominated community could be identified in hand samples by its distinctive gray-green color, hardness (relative to the unlithified laminae), and abundance of fused grains. Examination of this layer at 5 mm by TEM revealed an abundance of cyanobacterial filaments (i.e., *Oscillatoria* sp.) in between grains (Fig. 3C). These filaments had well-developed thylakoids with abundant phycobilisomes suggesting that they were viable. Unfortunately, the resin did not penetrate many of the bored grains that still contained *Solentia* sp., thus ultrastructural details of these cells at this depth were not obtainable. However, in instances where the grains were infilled with resin and remained in the thin sections, evidence of sheath material and bacterial remains could be seen (Fig. 3D).

Discussion and conclusions

Examination of the microbial communities in the stromatolites at Highborne Cay by TEM has provided critical information on community structure, microbial physiology, and microbe-mineral interaction. We determined that the dominant filamentous cyanobacteria in the surface community of Type 1 was *S. gebeleinii* by analysis of thin sections using the dimensions and number of filaments in a common sheath (Golubic and Browne 1996). These filaments had parietally arranged thylakoids, abundant phycobilisomes, and carboxysomes (Figs. 1B and 1C) indicative of healthy, photosynthetically active cells. In contrast, filaments observed below the surface were in progressive stages of degradation, with vacuolated thylakoids that had little or no phycobilisomes (2F). The sheaths had collapsed into conspicuous bands, an indication of localized dehydration and degradation. Even at depths of several centimeters, where vegetative cells were no longer present, little or no carbonate precipitation was observed on the sheaths (1E,F).

TEM also revealed the presence of other less abundant filamentous cyanobacteria (i.e., *Oscillatoria* sp). These were often seen in the fused-grain layer several hundred micrometers below the surface. The presence of thylakoids and phycobilisomes suggests that these organisms are still metabolically active. The continued input of organic carbon to the community by these organisms could enchance lithification as it would provide substrate for the sulfate reducing bacteria (Visscher et al. 1998). The boring endolithic cyanobacterium *Solentia* sp. could also be identified in ultrathin sections. The cells observed were from the surface layer. Examination of subsurface fused-grain layers by light and fluorescence microscopy indicated the presence of chlorophyll. Knowledge of the depth to which healthy *Solentia* sp. cells persist within the mats would allow us to define the extent and duration of microboring activity.

Heterotrophic bacteria were observed in all three communities. The variety of sizes and shapes indicate a diverse population. Interestingly, the diameters of many of the bacteria (0.15–0.3 μm) in the Highborne Cay stromatolites fall within the upper range suggested for nanobacteria (Kajander and Ciftcioglu 1998; Folk 1993). Nanobacteria have been implicated in calcification in kidney stones (Kajander and Ciftcioglu 1998) and carbonate ooids (Folk 1993). Active sulfate reduction has been correlated with the presence of lithified laminae, however the species involved have yet to be identified (Visscher et al. 1998, 2000). Of particular interest is the population of small diameter bacteria associated with the surface micritic crust. Fifty percent of the heterotrophic bacteria observed in the surface crust community pass through a 0.45 μm filter but are retained by a 0.2 μm filter as determined by acridine orange direct counts (P.T. Visscher, unpublished data). The occurrence and abundance of the small diameter vibrioid-shaped bacteria in the surface biofilm of amorphous EPS (Fig. 2D) suggest that they may indeed be the sulfate-reducing bacteria responsible for carbonate precipitation.

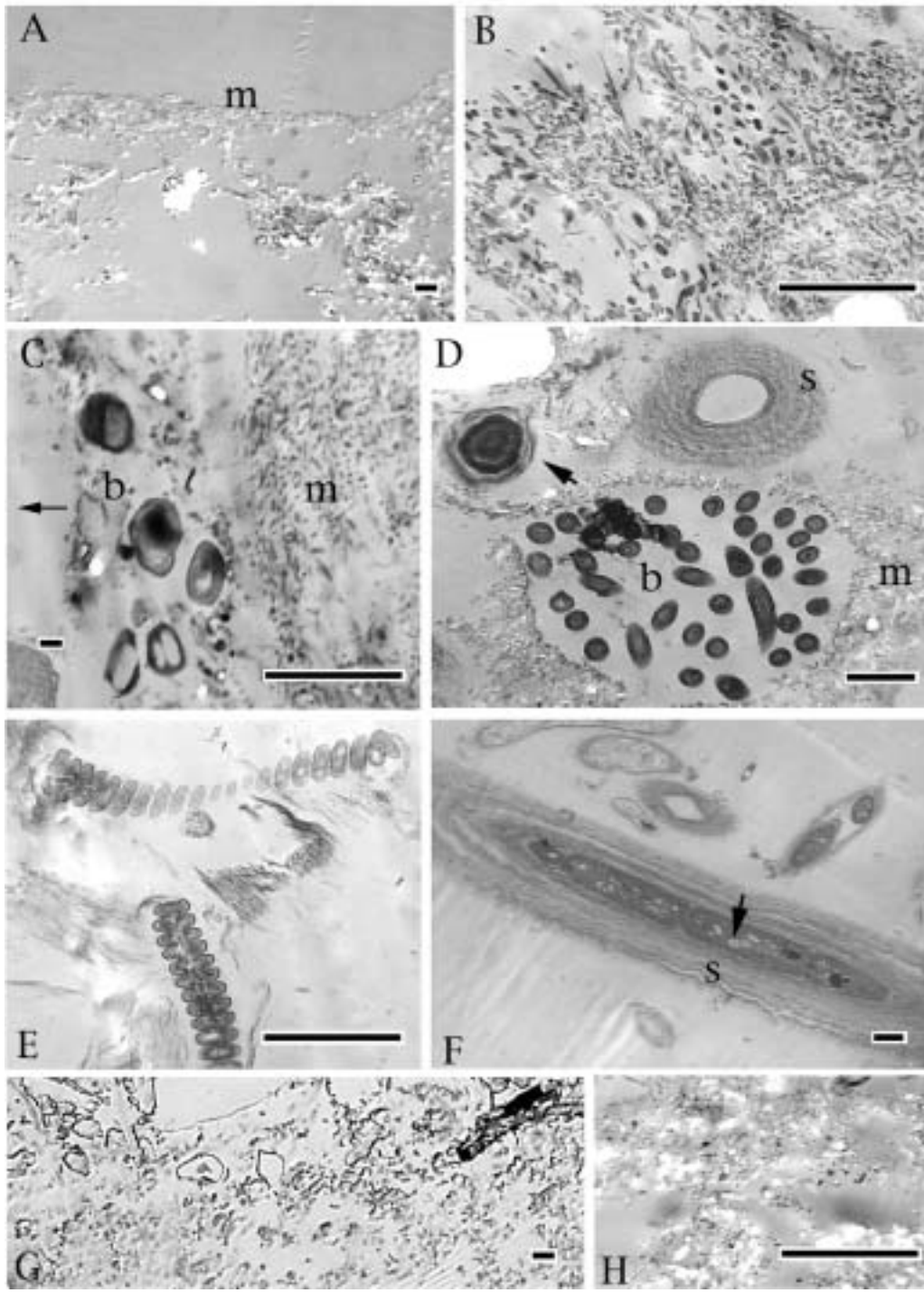

FIGURE 2. The microbial community associated with well-developed surface micritic crust. (**A**) Light micrograph of an oriented section of top 500 μm of a stromatolite (sample 8/98 NS82a) with a well developed micritic surface crust (m) underlain by unconsolidated ooids. Toluidene blue stained. (**B**) TEM of the surface micritic crust revealing needle-shaped carbonate crystals in longitudinal and cross section (8/98 NS82a). (**C**) TEM of the interface between the micritic crust and the surface microbial biofilm (arrow indicates direction of the surface) (8/98 NS82a). Individual bacteria (b) can be seen in the biofilm but not in the micritic layer (m). Inset shows detail of the Gram negative wall. (**D**) TEM of a cluster of bacteria (b) surrounded by micritic deposit (m) (sample 3/98 NS 10a). This section also contains a bacterial spore (arrow) and an empty cyanobacterial sheath (s). (**E**) TEM of an unidentified tightly coiled Gram negative bacterium (sample 7/99 NS2a), (**F**) TEM of a filament of *S. gebeleinii* in an advance stage of degradation from the surface layer (sample 3/98 NS10a). Note the vesiculated thylakoids (arrow), lack of phycobilisomes, and condensed sheath (s). Light micrograph (**G**) and TEM (**H**) of a buried micritic layer that was 2 cm below the surface (3/98 NS10a). Bar in (A) 40 μm, (C) insert 0.2 μm, (G) 20 μm, all others are 1 μm.

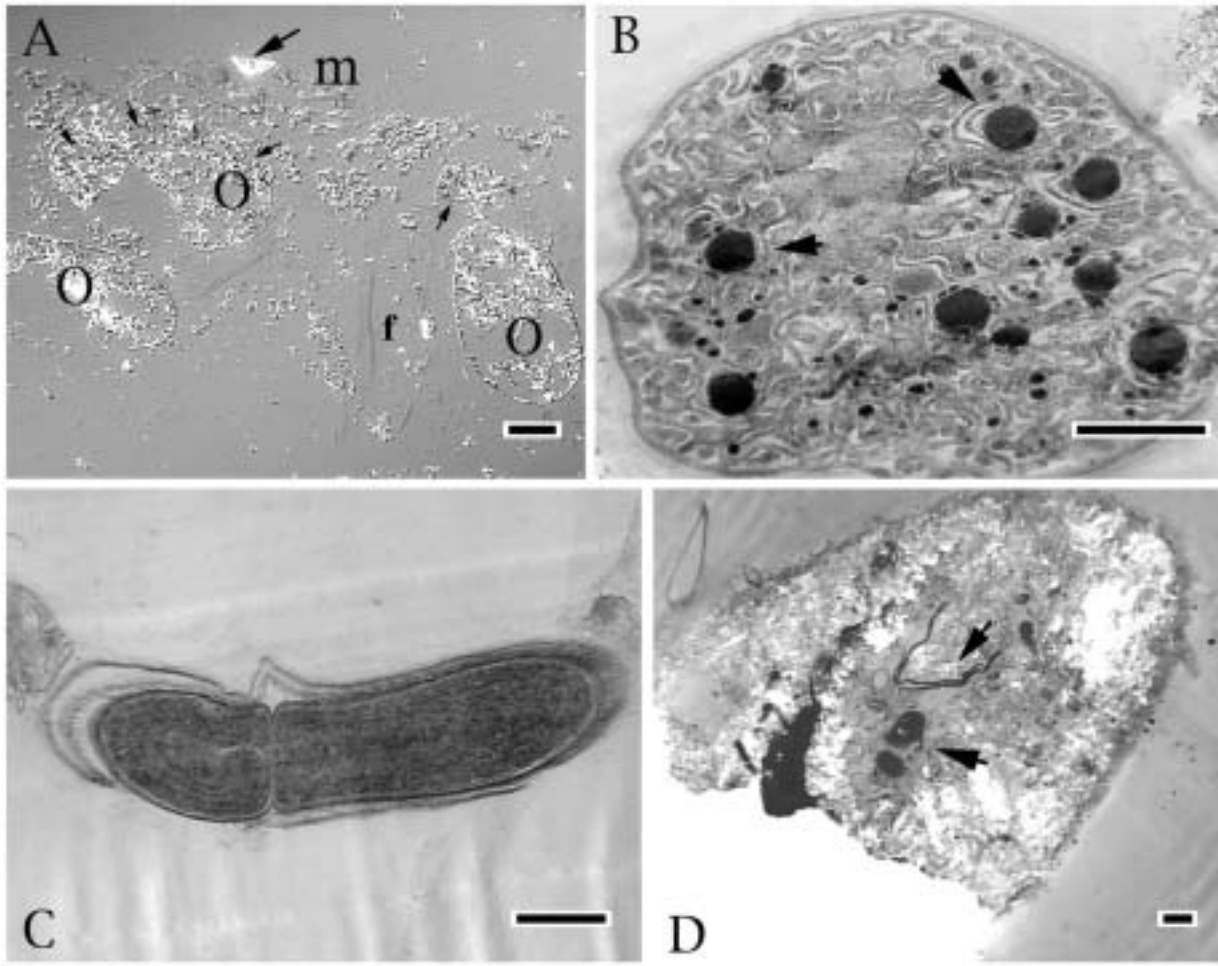

FIGURE 3. The microbial community in the fused-grain laminae. (**A**) Light micrograph of an oriented thick section through uppermost 500 μm of a Type 3 surface community with a well-developed surface micritic crust (m) with clastic carbonate (large arrow), underlying population of *Solentia* sp. (small arrows) and ooids (o) (sample 7/99 NS2). Vertically oriented filaments (f) of *S. gebeleinii* can be seen down section (**B**) TEM of the boring cyanobacterium *Solentia* sp. showing the reticulating thylakoids and electron opaque inclusions (arrows) (sample 8/98 NS82a). (**C**) TEM of an *Oscillatoria*-like cyanobacteria from fused-grain layer 5 mm below surface. Note the presence of well-developed thylakoids and abundance of phycobilisomes (sample 3/98 NS10b). (**D**) TEM cross-section through a resin-infilled ooid showing evidence of sheath material and bacteria (arrows) (sample 3/98 NS10b). Bar in (A) 50 μm, all others are 1 μm.

Our results offer a different view of calcification in modern marine stromatolites than that proposed in previous studies. In their study of modern marine stromatolites at Lee Stocking Island, Bahamas, for example, Seong-Lee et al. (2000) recognized the entrapping of ooid grains by a "cement crust" comprised of densely packed submicron sized aragonite crystals. They attributed the origin of this crust to the activities of *S. gebeleinii*. Their observation of a subsurface micritic crust lying within a well-developed living *Schizothrix* layer led them to conclude that the trapping, binding, and cementing could be attributed to *S. gebeleinii* (Seong-Lee et al. 2000). In contrast, we failed to see calcification in vegetative filaments or sheath in the Type 1 surface community. Furthermore, Decho et al. (in preparation) have found that intact *Schizothrix* sheath actually inhibits calcification. Chafetz (1994) has suggested that lithification in flat laminated microbial mats does not occur at the surface but rather at depth after the cyanobacteria have died. In Highborne Cay stromatolites, however, even at depth the sheaths of *S. gebeleinii* did not show evidence of carbonate precipitates and thus these cyanobacteria are not directly involved in carbonate precipitation. We have concluded that the primary roles of the *Schizothrix* community (i.e., Type 1) in the stromatolites from Highborne Cay are the trapping and binding of unconsolidated sediment and the production of EPS.

The primary cause of calcification in the stromatolites at Highborne Cay is the activity of anaerobic sulfur-metabolizing bacteria (Visscher et al. 1998, 2000). Sulfate reduction and anaerobic sulfur oxidation stimulate carbonate precipitation, whereas aerobic respiration and aerobic sulfur oxidation result in carbonate dissolution in this environment (Visscher et al. 1998, 2000). This implies that anaerobic conditions are necessary for lithification. High rates of sulfate reduction have been

correlated with the thin micritic laminae in the first few centimenters of the stromatolite and in particular those at the surface (Visscher et al. 2000). At depth this activity decreases and becomes more diffuse because organic carbon becomes limiting (Visscher et al. 2000). Bacterial calcification alone, however, does not explain the nature and fabric of micritic laminae in modern marine stromatolites or the genesis of ancient carbonate stromatolites. Stromatolite formation involves the interdependence of both cyanobacteria and heterotrophic communities in a cyclical succession. Although the in situ examination of surface crusts showed that cyanobacteria (e.g., *S. gebeleinii*) are not directly involved in the formation of micritic laminae (Fig. 2), they do provide a stable surface for bacteria to colonize and the photosynthate to feed heterotrophic activity. The thin micritic laminae are formed through the colonization of the surface by heterotrophic bacteria, primarily sulfate reducing bacteria, and the formation of a biofilm (Reid et al. 2000; Visscher et al. 2000). Carbonate precipitation occurs directly underneath and in contact with the biofilm (Fig. 2C). As the micritic crust develops the biofilm coelesces around it often resulting in coated balls of aragonite (Reid et al. 2000). In some cases the bacteria appear to be encased in the precipitate (Fig. 2D). Once a cohesive micritic crust forms, it persists at depth but the biofilm and associated bacteria do not (Figs. 2G and 2H).

Preserved cyanobacterial remains (i.e., sheath) make up the bulk of the microfossils observed in Prephanerozoic rocks (Schopf 1983), yet less than 1% of ancient carbonate stromatolites have fossils associated with them (Grotzinger and Knoll 1999). This lack of preserved remains in fossil stromatolites has been attributed primarily to aggrading neomorphism (Grotzinger and Knoll 1999). Evidence from the present study showing that cyanobacterial filaments and bacterial cells are not calcified and are degraded rapidly in modern marine stromatolites argues that the primary explanation for the lack of preserved fossils in ancient stromatolites may be penecontemporaneous organic degradation, rather than aggrading neomorphism. Furthermore, cyanobacterial sheaths are not directly involved in the formation of micrictic laminae. Rather, stromatolite growth and lithification involves a cyclical succession of three distinct surface communities (Reid et al. 2000). The bacteria responsible for the formation of the micritic laminae are not preserved at depth and therefore, morphological evidence (i.e., microfossil) of their presence in ancient stromatolites should not be expected, except in cases of exceptional preservation.

This study marks the first attempt to obtain information on community structure of a marine carbonate stromatolite using TEM. Future studies will investigate the utility of ultralow viscosity embedding resins to facilitate imaging of intact bored ooids and the preservation of the endolithic cyanobacteria. The current results demonstrate that not only is in situ TEM possible, but also can yield valuable information for species identification and microbe-mineral interactions. Indeed, the submicron sizes of the bacteria involved in lithification processes indicate that this level of resolution is essential.

ACKNOWLEGMENTS

This work was supported in part by NSF grant OCE 9530215 (to R.P.R.) with an ROA supplement (to J.F.S.), OCE 9619314 (P.T.V.), and a grant from the Department of Biology of Colorado College (to T.N.F.). We thank A. Decho for helpful comments and the participants of the Research Initiative on Bahamian Stromatolites (RIBS). This is RIBS contribution 13.

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MANUSCRIPT RECEIVED AUGUST 2, 2000
MANUSCRIPT ACCEPTED FEBRUARY 5, 2001
MANUSCRIPT HANDLED BY GRANT FERRIS