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A laboratory investigation of cyanobacterial extracellular polymeric secretions (EPS) in influencing CaCO₃ polymorphism

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Abstract

Bahamian stromatolites are well-laminated structures, consisting of lithified layers alternating between unlithified layers containing fine-grained carbonate ooids. The lithified layers consist of abundant aragonite needles embedded within a matrix of extracellular polymeric secretions (EPS) by cyanobacteria, *Schizothrix* sp. Laboratory investigations were conducted using EPS extracted from natural stromatolites and laboratory isolates of *Schizothrix* sp., to chemically characterize EPS, and determine in vitro how EPS may influence CaCO₃ polymorphism. EPS mainly consisted of acidic polysaccharides and proteins. Biochemical analyses indicated that contents of uronic acids and carbohydrates in EPS from lithified layers decreased when compared with unlithified layer EPS, while the protein content remained relatively constant. CaCO₃ nucleation experiments demonstrated that EPS from the lithified layer, induced aragonite crystal formation in vitro, as confirmed by scanning electron microscopy and Fourier transform infrared (FT-IR) spectroscopy. In contrast, EPS from the unlithified layer or laboratory-cultured *Schizothrix* sp. induced calcite crystal formation. These laboratory results suggest the possibility that the biochemical composition, specifically small proteins, of EPS influences the resulting mineralogy of CaCO₃. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Marine stromatolites, found on the margins of Exuma Sound, Bahamas are the only known

examples of stromatolites presently forming in open marine environment of normal seawater salinity [1]. The surfaces of Highborne Cay stromatolites are well laminated, consist of alternating lithified and unlithified layers. The lithified layers typically contain an abundance of amorphous extracellular polymeric secretions (EPS), a metabolically diverse community of heterotrophic microorganisms [2,3] and aragonite needles. Near

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the surfaces of stromatolites, unlithified layers are typically laden with copious EPS produced by cyanobacteria [2]. The cyanobacterium, *Schizothrix* sp., is a dominant species that secretes EPS in this system [1]. Aragonite precipitates, approximately 1 μm in length, form spherical aggregates 2–5 μm in diameter and are embedded in the EPS matrix [1]. Bacteria are abundant and commonly observed at the edges of the aragonite spherules. Unlithified layers alternate between lithified layers, and are sedimentary structures composed mainly of fine-grained, well-sorted carbonate ooids, i.e., sand, approximately 125–250 μm . The EPS matrix is a key structuring component of microbial biofilms [4,5]. Many EPS are widely produced by microbes for attachment and protection, and serve important roles in providing nucleation sites and facilitating sediment trapping [6]. EPS predominantly contain acidic polysaccharides, mainly uronic acids, and proteins [4,7].

It has been suggested that the negative charges of uronic acids play important roles in the various functions mentioned above [4]. Aragonite precipitation in marine stromatolites is considered to be a biologically induced mineralization and a by-product of several microbial processes [1]. The mechanism of induction for aragonite precipitation in marine stromatolites may be related to specific interactions of microbial photosynthesis, respiration and sulfate reduction. Magnesium is known to induce aragonite formation in seawater and in vitro at ratios of $\text{Mg}/\text{Ca} > 4$, but has never been demonstrated in vivo [8]. Therefore, factors influencing CaCO_3 polymorphism in marine stromatolite are not clearly understood.

In the present study we have focused on aragonite precipitation by EPS under in vitro conditions. In this study, EPS isolated from both “lithified” and “unlithified” layers of natural marine stromatolites, and from laboratory cultured stromatolite forming cyanobacterium, *Schizothrix* sp. mats were used to examine specific influences on aragonite precipitation. CaCO_3 nucleation experiments were conducted to determine if EPS influences aragonite precipitation in vitro.

2. Experimental procedure

2.1. EPS isolation

Samples of intertidal marine stromatolites were collected from Highborne Cay, Bahamas (76°49'W, 24°43'N). Samples were returned to the laboratory, and EPS were scraped off from both lithified and unlithified layers of the samples using a sharp, pointed scalpel under a dissecting microscope. Only the upper 2 mm of mat were used to isolate natural mat layers for EPS. The “lithified” and “unlithified” layers were carefully separated under a dissecting microscope. The isolated layers, consisting of cells, sediment, EPS, and precipitate fragments were placed in 1.5 ml Eppendorf tubes and suspended in 0.05 mM EDTA solution, heated at 40°C, and stirred for 30 min.

The cyanobacteria, *Schizothrix* sp., was isolated from marine stromatolites at Highborne Cay, Bahamas. This species was grown in CHU-10 medium [9] consisting of 0.004 M $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$, 0.006 M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.014 M K_2HPO_4 , 0.025 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 M Na_2CO_3 , 0.012 M Fe-EDTA, 3.7×10^{-8} M B_{12} , 4×10^{-7} M biotin, and 5.9×10^{-7} M thiamine in seawater of 32 ppt salinity on a light:dark cycle of 12:12 h, at approximately 100 $\mu\text{Einsteins}$. Bahamian sediment, consisting of well-sorted CaCO_3 ooids (mean grain size 100–250 μm), was collected from the study site, sterilized, cleaned in sodium hypochlorite and rinsed thoroughly in distilled H_2O . The sediment was added to culture flasks to a depth approximately 0.5 cm as a substratum for growth. The cultures were grown for several weeks until a firm mat of cyanobacteria was present on the surface of the sediment.

For isolation of EPS from laboratory cultured *Schizothrix* sp., culture medium was discarded, then each of the cultures was suspended, stirred, and heated to 40°C for at least 30 min to strip EPS from the cyanobacteria. Then, the separated suspensions from both natural and laboratory samples were centrifuged at 12000 rpm for 15 min to shear remaining EPS from cells. To remove small-molecular weight (MW) components, the supernatant was dialyzed (MW cutoff 14,000 Da)

in de-ionized water with constant stirring for 48 h. The dialyzed solution was lyophilized and stored at -70°C .

2.2. Chemical analysis of EPS

The phenol-sulfuric acid spectrophotometric method was used to measure carbohydrate content as hexamine [10]. Uronic acid content was measured using carbazole in 80% sulfuric acid with borate ions added [11]. Protein content was measured according to Bradford's method using BioRad Protein Assay Kit [12]. All samples were measured in triplicate.

2.3. CaCO_3 nucleation experiment

EPS from lithified and unlithified layers, and laboratory-cultured *Schizothrix* sp. were used for this experiment. EPS were fixed using 4% formaldehyde on to solid agarose beads [13]. Then, the EPS attached to agarose beads were placed in solutions containing 10 mM CaCl_2 , 5 mM NaHCO_3 , and 30 mM MgCl_2 , and incubated for 5 days [14,15] in scintillation vials. Agarose beads, containing no EPS coating, were used as controls. At the end of the experiment, agarose beads were filtered on a $0.45\ \mu\text{m}$ membrane filter. The filter was washed with dilute $\text{NH}_4(\text{OH})$ (pH 8), air-dried, mounted on a specimen holder, sputtered with gold, and examined with an Hitachi Delta 2500 scanning electron microscope (SEM). The type of crystal formed on agarose beads was investigated using a KeveX energy dispersive X-ray spectrometer (EDAX) attached to the SEM. Attenuated total reflectance (ATR) Fourier transform infrared (FT-IR) spectra were obtained using NEXUS 670 FT-IR (Thermo-Nicolet, Inc.), equipped with a germanium internal reflection element (Spectral-Tech. Inc.).

3. Results

Table 1 shows the contents of carbohydrate, uronic acids and protein in EPS both from lithified and unlithified layers, and laboratory-produced EPS. Carbohydrate and uronic acid content

Table 1

Contents (w/w) of carbohydrate, uronic acids and protein in EPS from lithified and unlithified layers and laboratory cultured *Schizothrix* sp. biomat in marine stromatolites, Hayborne Cay, Bahamas

	Carbohydrate (%)	Uronic acids (%)	Protein (%)
Lithified layer	0.9	4.3	2.5
Unlithified layer	17.24	15.27	2.8
<i>Schizothrix</i> sp.	22.4	19.34	1.9

(17.24% and 15.27%, respectively) in the unlithified layer EPS were higher than those (0.9% and 4.3%, respectively) in lithified layer EPS. However, carbohydrate and uronic acids contents (22.4% and 19.34%) in laboratory-produced EPS were similar to those EPS collected from the unlithified layers. Protein contents from lithified layers, unlithified layers and laboratory produced EPS were 2.5%, 2.8% and 1.9%, respectively.

After 5 days of incubation, CaCO_3 minerals were present on the surface of EPS-coated agarose beads (Fig. 1). In controls, lacking EPS, CaCO_3 minerals were not observed (data not shown). The presence of Ca in the precipitates was confirmed using EDAX analyses. Crystals formed on the lithified layer EPS-coated agarose beads were hexagonal, and spindle-shaped (Fig. 1C). FT-IR spectra revealed peaks splitting near $700\ \text{cm}^{-1}$ (711 and $699\ \text{cm}^{-1}$), that are characteristic of the aragonite structure (Fig. 2C) [16] and confirmed that the CaCO_3 crystals formed on the agarose beads were aragonite. In contrast, the shape of crystals formed on the unlithified layer (Fig. 1A) and laboratory cultured *Schizothrix* sp. EPS (Fig. 1B) were rhombohedral and FT-IR analysis confirmed that those crystals formed on agarose beads were calcite (Fig. 2A and B).

4. Discussion

Aragonite needles are commonly found within the EPS matrix in the surface layers of stromatolites [1]. Similar aragonite needle formation, found in *Halimeda cylindracea*, is known to be associated

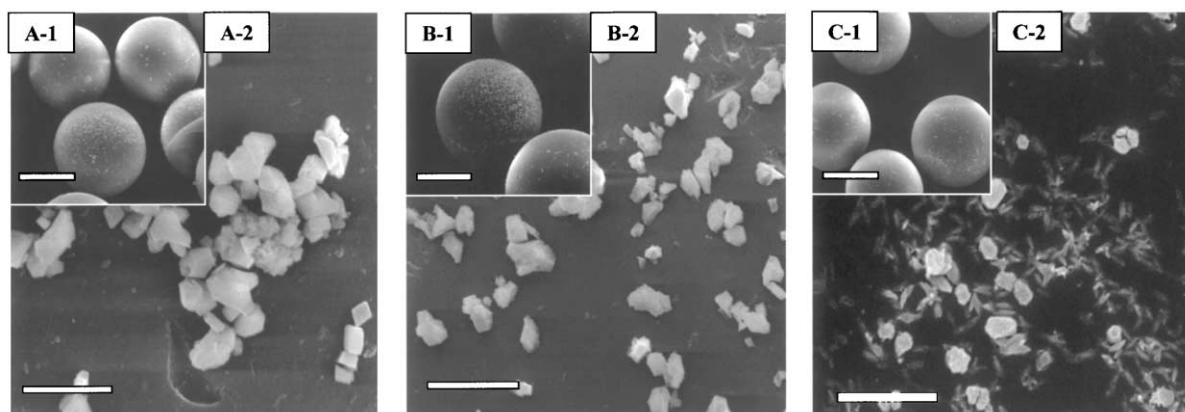


Fig. 1. CaCO_3 crystals formed on agarose beads coated with different EPS. (A) Unlithified layer EPS. Scale bars: 500 μm (A-1), 0.5 μm (A-2), (B) *Schizothrix* sp. EPS. Scale bars: 500 μm (B-1), 0.5 μm (B-2). (C) Lithified layer EPS. Scale bars: 500 μm (C-1), 0.5 μm (C-2).

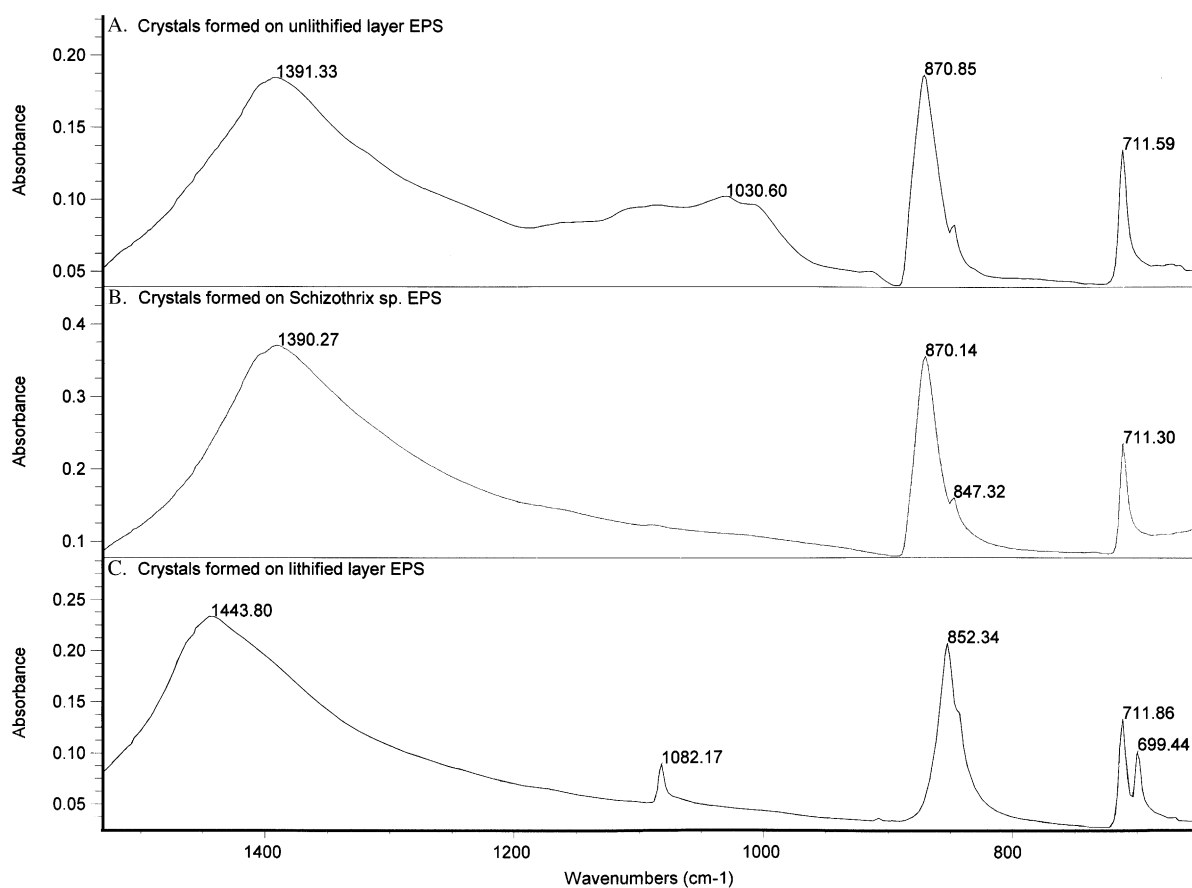


Fig. 2. Fourier transformed infrared (FT-IR) spectrum of calcium carbonate crystals formed on agarose beads which coated with unlithified layer EPS: calcite (A), *Schizothrix* sp. EPS: calcite (B) and lithified layer EPS: aragonite (C).

with organic material [17]. Nakahara and Bevelander [18] found that organics were coating *Halimeda incrassata* aragonite needles. Biochemical analysis of organic materials found in *Halimeda* [19] showed that they are largely polysaccharides with some proteins which are very similar to the composition of EPS found in marine stromatolite. However, the roles of those organics in nucleation and in determining the polymorphism of CaCO_3 are not well understood.

In this study, FT-IR spectra confirmed that the crystals that formed on lithified layer EPS coated agarose beads were aragonite, but crystals formed on unlithified layer and laboratory cultured *Schizothrix* sp. EPS coated agarose beads were calcite. Biochemical analysis showed that contents of uronic acids and carbohydrates in EPS from lithified layers were decreased when compared to unlithified layer EPS, but protein content remained relatively constant. Previously, we have shown [20], using pH drift assays with chemically modified EPS, that protein fractions are more important in CaCO_3 precipitation than uronic acids in the lithified layer. In contrast, both protein and carboxylated polysaccharides are involved in the inhibition of CaCO_3 precipitation in unlithified layer [20]. These results suggest that as microbial degradation of EPS proceeds, specific protein fractions in EPS may become increasingly exposed.

The results of the present study showed that EPS, which differed in composition, induced the precipitation of different CaCO_3 crystal types in vitro. Previously, it was proposed that organic components in biominerals may determine the type of CaCO_3 [21]. Wada et al. [22] reported that acidic polysaccharides isolated from calcareous algae induced Mg-rich calcite precipitation in a double-diffusion experiment. Acid polysaccharides inhibited the growth of aragonite crystals by sorption onto the surfaces of the crystals [22]. Also, Albeck et al. [23] suggested that the polysaccharides contained in glycoproteins may modulate calcite crystal growth in vitro. Since unlithified layer and laboratory-cultured *Schizothrix* sp. EPS contained greater relative amounts (Table 1) of acidic polysaccharides than the lithified layer EPS, calcite, rather than aragonite

formation was selectively induced by those EPS in the nucleation experiment.

It has been demonstrated that acidic proteins isolated from aragonitic biominerals induce aragonite formation [24,25]. Levi et al. [26] reported that the specific amino acid sequence $(\text{Asp-Leu})_n$ in protein isolated from aragonitic biominerals was capable of inducing aragonite formation. In the present study, amino acid compositions of acidic proteins in EPS from marine stromatolites [20] showed similarity, being specifically enriched in aspartic acid and glutamic acid, when compared with key amino acids (aspartic acid and glutamic acid) found in the organic matrices associated with other biominerals [27–30]. Changes in stereochemical structure, resulting from attachment of acidic proteins to a solid surface (e.g. chitin), have been suggested to be an important requirement for CaCO_3 nucleation and CaCO_3 polymorphism [26,31–33]. Although our results are preliminary, the data suggest that biochemical composition in EPS influence the precipitation, composition, and CaCO_3 polymorphism in the lithified layers of marine stromatolites.

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