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POTENTIAL ROLES OF EXTRACELLULAR POLYMERIC SECRETIONS (EPS) IN REGULATING CALCIFICATION. A STUDY OF MARINE STROMATOLITES, BAHAMAS

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ABSTRACT

Bahamian stromatolites produce a variety of forms of calcium carbonate precipitates. Many of these occur in close association with cyanobacterial biofilms and their extracellular polymeric secretions (EPS). EDTA-soluble fractions of EPS were isolated from the lithified layers of stromatolites. Laboratory investigations were conducted to examine biochemical characteristics and the role of EPS in $CaCO_3$ precipitation. EPS consisted of carbohydrates, proteins, nucleic acids and lipids. SDS-PAGE showed that the lithified layer EPS have high molecular weight acidic polysaccharide and small proteins. EDTA-soluble EPS from the lithified layers inhibited $CaCO_3$ precipitation *in vitro*, as determined using pH drift assays examining the pH decrease that occurs in response to $CaCO_3$ precipitation. Also, $CaCO_3$ induction experiments as confirmed by scanning electron microscopy demonstrated that EDTA-soluble EPS from the lithified layer induced $CaCO_3$ crystal formation *in vitro* when attached to a solid surface. Our data suggest that cyanobacterial EPS may have divergent roles in the regulation of calcification in stromatolites. A major role of freshly secreted cyanobacterial EPS may be to "inhibit" calcification via binding of Ca^{2+} ions by acidic functional groups. Geochemical gradients generated by photosynthetic activities are concomitant with precipitation in supersaturated environments; therefore, cyanobacteria must actively maintain fresh EPS in proximity to cells in order to resist precipitation from occurring. A second role of EPS occurs in facilitating precipitation. Environmentally altered EPS, i.e., partially degraded or cross-linked, when attached to a surface, may facilitate precipitation. Soluble forms of EPS may inhibit $CaCO_3$ precipitation, but when attached to an insoluble substrate, EPS may act as a nucleation site for $CaCO_3$ precipitation. Our results suggest that while microbial activities are creating appropriate geochemical microenvironments for $CaCO_3$ precipitation, EPS may serve more important roles in the regulation of calcification within marine stromatolites than previously thought.

INTRODUCTION

Lithified laminated sedimentary structures known as stromatolites are formed by complex interactions of microbes, sediments, and the environment.

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These structures represent the oldest macroscopic evidence of life on earth (Walter 1983; Riding 1994). As a potential source of information on interactions of the biosphere, atmosphere, hydrosphere, and lithosphere for 3.5 billion years, stromatolites have been the focus of much research. A long-standing controversy concerning stromatolite formation, is the origin of micritic carbonates in ancient and present-day stromatolites (Grotzinger & Knoll 1999).

Modern subtidal and intertidal marine stromatolites are found in the Exuma Cays, Bahamas (Reid & Browne 1991). The surfaces of these stromatolites consist of alternating lithified and unlithified layers. Lithified layers are millimeter-scale spaced laminations that indicate periodic episodes of lithification, i.e., $CaCO_3$ precipitation. 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 mm. Near the surfaces of stromatolites, unlithified layers are typically laden with copious extracellular polymeric secretions (EPS) produced by cyanobacteria (Visscher *et al.* 1998; Decho *et al.* submitted). The cyanobacterium, *Schizothrix* sp., is a dominant species that secretes EPS in this system (Reid *et al.*, 2000)

The EPS matrix is a key structuring component of microbial biofilms (Decho 1990). Therefore, EPS may influence a number of biological and geochemical processes that are critical to stromatolite formation: (1) precipitation of $CaCO_3$ (Pentecost & Riding 1986; Defarge *et al.* 1994); (2) buffering of Ca^{2+}, perhaps preventing precipitation of $CaCO_3$ (Arp *et al.* 1999a); (3) physical stabilization of microbial cells and calcium carbonate ooids against the high-energy environments, e.g., waves, tidal currents, in which these structures commonly occur (Reid & Browne 1991); and (4) provision of chemically protective microenvironment for cells (Decho 1990; Arp *et al.* 1999b).

Many EPS contain predominantly acidic polysaccharides, mainly uronic acids, and proteins (Decho 1990; Trichet & Defarge 1994). It is believed that the negative charges of uronic acids play important roles in the various functions mentioned above (Decho, 1990).

In the present study we focused on the inhibition and promotion of $CaCO_3$ precipitation by EPS in marine stromatolites. EPS were isolated from both "lithified" layers of marine stromatolites to study its functions in $CaCO_3$ precipitation. A range of $CaCO_3$ inhibition assays and $CaCO_3$ induction experiments were conducted to determine if EPS regulate $CaCO_3$ precipitation *in vitro*.

MATERIALS AND METHODS

EPS isolation

Samples of marine stromatolites were collected at Highborne Cay, Bahamas (76° 49' W, 24° 43' N). EPS were scraped off from the lithified layers of the samples using a sharp pointed scalpel under a dissecting microscope. Lithified layers formed a hard white layer approximately 30 - 100 mm in thickness. The isolated layers, consisting of cells, $CaCO_3$ precipitate, and EPS were placed in 1.5 ml eppendorf tubes and suspended in 0.05m*M* EDTA solution, heated at 40°C, and stirred for 30 min. Then, the suspensions from samples were centrifuged at 12000 r.p.m. for 15 min in order to shear remaining EPS from cells. To remove small-molecular weight (MW) impurities, the supernatant was dialyzed (MW cutoff 14,000) in de-ionized water with constant stirring for 48 h. The dialyzed solution was then lyophilized and stored at -70°C. Lyophilized fractions are EDTA-soluble EPS.

Antibody production and Alexa Fluor 488 labeling

Polyclonal antibodies were raised in a New Zealand White male rabbit against the lithified layer EPS. Rabbits were initially injected with 100 mg of the antigen suspended in Freund's complete adjuvant. Over a four-month period, three additional injections were performed in similar fashion using Freund's incomplete adjuvant. Two weeks from the third injection, the rabbit was killed under anesthesia and sera were separated from blood, and stored at -70°C until use. The antibody was labeled with fluorescent dye using Alexa Fluor 488 Protein Labeling Kit (excitation=488 nm; emission=519 nm; Molecular Probes, Eugene, OR) for immunohistochemistry.

Scanning electron microscopy and transmission electron microscopy

A piece of marine stromatolites sample (0.5 cm x 1 cm x 0.5 cm) containing the lithified layer was fixed in 30% formaldehyde, washed with filtered sea water and critical point dried. Then, the sample was mounted on a specimen holder, sputtered with gold, and examined with an Hitachi Delta 2500 scanning electron microscope (SEM).

For Transmission electron microscopy (TEM), pieces of marine stromatolite sample (0.5 cm x 1 cm x 0.5 cm) were fixed in 2% paraformalaldehyde (PFA) and 0.5% glutaraldehyde in 0.1M sodium cacodylate sea water buffer (pH 7.4) for 1 hr. The samples were then washed in two changes, of 0.1M sodium cacodylate sea water buffer (pH 7.4), and dehydrated through a series of ethanols. They were then placed in Epon 812 embedding media at room temparature for 24 hrs. The specimens were embedded in BEEM capsules in fresh Epon 812 and incubated at 60°C for 24 hrs. Ultra thin sections were cut with a diamond knife and mounted on carbon-coated copper grids. The sections were observed with JEOL 100C tranmission electron microscope at 80kV.

Confocal laser scanning microscopy

Fluorescent probes were used to image the microspatial arrangements of cyanobacteria, heterotrophic bacteria, ooids, and EPS in the lithified top layer of natural stromatolites. The following fluorescent probes were used: Propidium Iodide to label cyanobacteria and heterotrophic bacterial cells; and Alexa Fluor 488 labeled antibody made against the lithified layer EPS (anti-LIEPS) for EPS; and autofluorescence to image ooids and $CaCO_3$ precipitates.

Freshly collected samples were sectioned using sterile razor blades. Immediately after collection, the sections of stromatolite were preserved in EM-grade 3% formaldehyde in seawater. The sections were incubated in different fluorescent probes for 1 h (Decho & Kawaguchi 1999; Kawaguchi & Decho 2000). After incubations, excess unbound probes were washed 3 times with 20 ml sterile-filtered seawater for 10 min. Excess water was gently removed using pipets. After placing samples on a glass slide, a cover slip was mounted on samples on glass slides with a mounting

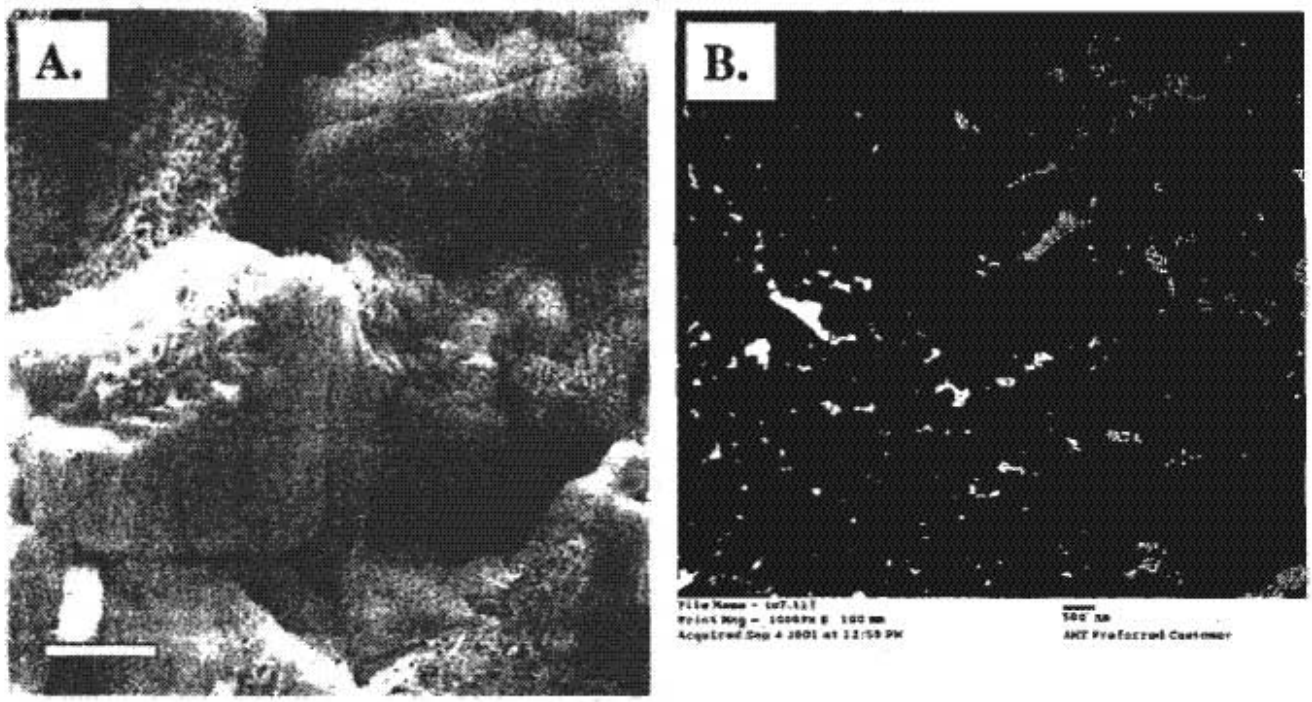

Figure 1. *The image of the lithified layer of marine stromatolites taken by the scanning electron microscope (A: Scale=10 um) and the image of the cross section of the lithified layer taken by the transmission electron microscope (B: Scale=500 nm). Aragonite needles (1mm length) appeared to be embedded in EPS at the top lithified layer.*

solution. Samples were viewed under MRC 1024MP confocal and two-photon laser scanning microscopy system (Bio-Rad Laboratories, Hercules, CA, USA) equipped with an Eclipse TE 300 compound-inverted microscope (Nikon, Tokyo, Japan). A single photon (krypton-argon) laser 488 nm line was used for imaging Alexa 488 anti-LIEPS labeled EPS, and propidium iodide labeled bacterial cells. Autofluorescence was used for imaging carbonate sand grains, i.e., ooids, lithified layer.

Determination of carbohydrate, uronic acid, and protein in EPS

The phenol sulfuric acid spectrophotometric method was used to measure carbohydrate content as hexamine (Dubois *s*. 1953). Uronic acid content was measured using carbazole in 80% sulfuric acid with borate ions added (Taylor & Buchanan-Smith 1992). Protein content was measured according to Bradford's method using BioRad Protein Assay Kit (Bradford 1976). All samples were measured in triplicate.

Analysis of amino acid composition

Amino acid analyses of EPS were conducted at Commonwealth Biotechnologies, Inc. (Richmond, VA). EPS samples from the lithified layers of stromatolites were hydrolyzed in 6N HCl for 24 h at 110°C under nitrogen and analyzed using a Hewlett-Packard Amino-Quant amino acid analyzer. Cysteine and triptophan were not determined. Hydroxyproline and hydroxylysine were also analyzed.

SDS-polyacrylamide gel electrophoresis

7.5% SDS-polyacrlamide gel electrophoresis (SDS-PAGE) on EPS from the lithified layers was carried out. The Mini-PROTEAN II (Bio-Rad, CA) system was used following the methods of Laemmli (1970). Silver staining (Merril *et al.* 1981) and Alcian Blue were applied to visualize protein and carbohydrate, respectively.

$CaCO_3$ inhibition assay

The precipitation experiment of $CaCO_3$ in vitro was carried out as described by Wheeler *et al.* (1981). The rate of $CaCO_3$ precipitation was determined by recording the decrease in pH of a solution containing 3 ml of 20 m*M* $NaHCO_3$ (pH 8.7) and 0.3 ml of H_2O to which 3 ml of 20 m*M* $CaCl_2$ (pH 8.7) was added. The decrease in pH due to $CaCO_3$ precipitation results from the following reaction:

$$Ca^{2+} + HCO_3^{2-} \text{ D } CaCO_3 + H^+ \quad (1)$$

To determine the effect of EPS on the precipitation of $CaCO_3$, 0.2 ml of an EPS isolated from the lithified layer were added instead of H_2O.

$CaCO_3$ induction experiment

EPS from the lithified layers were lyophilized and attached to solid agarose beads using 4% formaldehyde (Gunthorpe *et al.* 1990). The EPS attached to agarose beads were placed in solutions containing 10 m*M* $CaCl_2$, 5 m*M* $NaHCO_3$, and 30 m*M* $MgCl_2$, and incubated for 5 days (Crenshaw, 1991; Greenfield *et al.* 1984). Agarose beads, containing no EPS coating, were used as controls. At the end of the experiment, agarose beads were filtered on a 0.45 mm membrane filter. The filter was washed with dilute $NH_4(OH)$(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 and with Nicolet NEXUS 670 Fourier transform infrared (FT-IR) spectrometer.

RESULTS

Scanning electron microscopy and transmission electron microscopy

Fig. 1A showed the surface of the lithified marine stromatolite. Aragonite needles (1μm length) are emdedded in EPS matrix. Fig. 1B showed that cross section of aragonite needles at the top lithified layer. Aragonite precipitates sometimes form spherical aggregates 2 to 5μm in diameter.

Confocal and two-photon laser scanning microscopy

Confocal imaging of lithified layer showed a close association of cyanobacterial sheaths, heterotrophic bacteria, and $CaCO_3$ minerals (Fig. 2A). Alexa-488 labeled anti-LIEPS was localized between $CaCO_3$

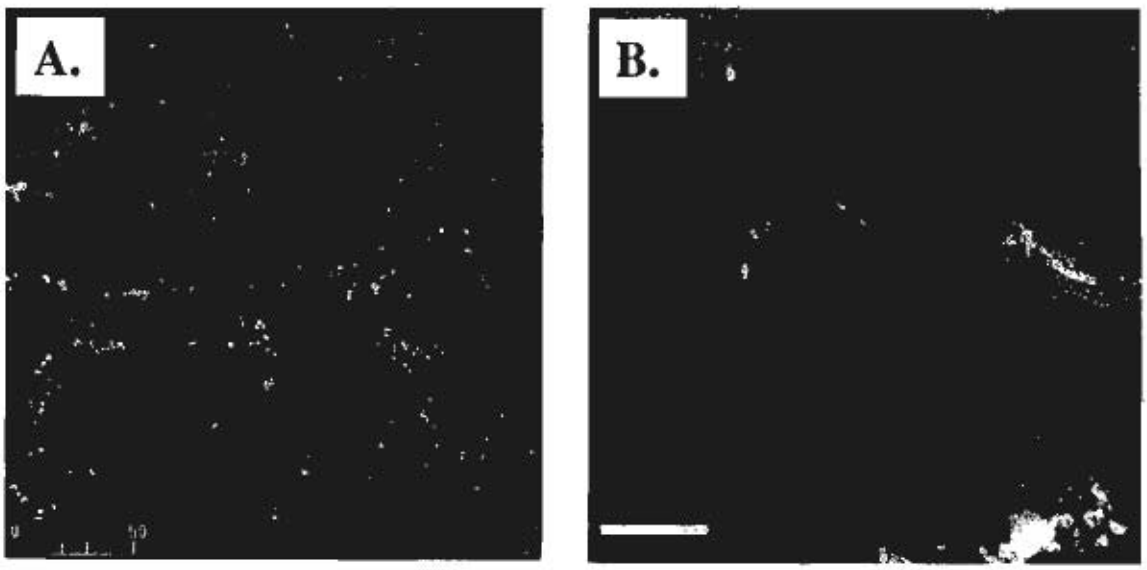

Figure 2. *Confocal imaging of the cross section of the lithified layer of marine stromatolites. (A) $CaCO_3$ precipitates (blue) formed among EPS (green) and cyanobacteria, Schizothrix sp. sheath (blank) in the lithified layer. Cyanobacterial sheathes are not calcified. Scale=50 mm. (B) High magnification image of $CaCO_3$ precipitates in the lithified layer. Alexa 488 anti-LIEPS labeled EPS (green) and Schizothrix sp. sheaths (blank tubes) are found among $CaCO_3$ precipitates (blue). Scale=10 mm.*

minerals indicating the presence of EPS among $CaCO_3$ precipitates (Fig. 2B). However, cyanobacterial sheaths were not calcified (Fig. 2A).

Carbohydrate as hexamine, uronic acids and protein content

Table 1 shows the contents of carbohydrate, uronic acids and protein in EPS from the lithified layers EPS. Carbohydrate and uronic acid contents in the lithified layer were 0.9 and 4.3%, respectively. Protein contents from the lithified layer was 2.5%.

Amino acid composition in EPS

Amino acid composition in the EPS from the lithified layer was enriched in aspartic acid and glutamic acid (Table 2). Aspartic acid and glutamic acid comprised up to 30% of total residues. Hydroxyproline was not found in all samples, which indicates the absence of collagen.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE protein profiles of the lithified layer EPS, stained by Alcian Blue, showed a single discrete band with a high molecular-weight (MW) of 300 kDa (Fig. 3). Also, silver staining revealed that low MW proteins (65kD, 60kD, 56kD, 50kD, 45kD, and 38kD) existed in the lithified layers (Fig. 3). These results indicated that the lithified EPS contained a high MW acidic polysaccharide and small proteins.

$CaCO_3$ inhibition assay

Various concentrations of EPS (100 - 500 mg) isolated from the lithified layers inhibited $CaCO_3$ precipitation (Fig. 4A). The inhibitory effect was concentration dependent.

CaCO3 induction

After 5 days of incubation, $CaCO_3$ minerals were formed on the surface of EPS-coated agarose beads (Fig. 4B). In controls, lacking EPS, $CaCO_3$ minerals were not observed. The presence of Ca in the precipitates was confirmed by EDAX analyses. Crystals formed on the agarose beads were hexagonal, and spindle-shaped (Fig. 4B). The FT-IR spectrum revealed peak splitting at around 700 cm^{-1} (711 and 699 cm^{-1}), which is characteristic of the aragonite structure (Balmain *et al.* 1999). FT-IR analysis thus confirmed that the $CaCO_3$ crystals formed on the agarose beads were aragonite.

DISCUSSION

The following mechanisms have been proposed for calcification processes within stromatolites: (1) abiotic processes of submarine cementation (Logan1961; Dill *et al.* 1986); (2) calcification of cyanobacterial sheaths related to photosynthetic activities (Monty 1976); (3) heterotrophic bacterial decomposition of cyanobacterial sheaths in subsurface layers (Chafetz & Buczynski 1992; Bartley 1996); (4) involvement of endolithic cyanobacteria for micritized grains (Macintyre *et al.* 2000; Reid *et al.* 2000); and (5) cyanobacterial photosynthesis, sulfate reduction, and anaerobic sulfide oxidation within lithified layers (Visscher *et al.* 1998; Reid *et al.* 2000). Among those mechanisms, bacterial metabolic activities (5) have been attributed to lithification of surface mats in the marine stromatolites. Hetrotrophic bacteria may facilitate $CaCO_3$ precipitation by increasing the PCO_2 and by active excretion of Ca^{2+} ions through bacterial degradation of the EPS (Visscher *et al.* 2000). Also, Visscher & colleagues (2000) showed a micrometer-scale correlation of sulfate-reducing bacterial (SRB) activities and micritic laminae formation. Although the highly alkaline conditions formed by photosynthetic microbial activities may create geochemical conditions for calcification in marine stromatolites, $CaCO_3$ precipitation may not occur due to the presence of inhibitors and/or a lack of a nucleating surface (Wilbur 1980). Therefore, the role of EPS in calcification has to be elucidated.

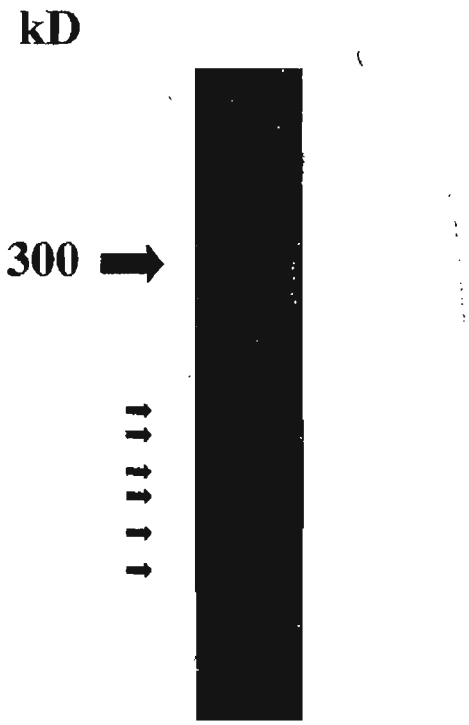

Figure 3. *7.5% SDS-polyacrylamide gel electrophorests profiles by double stainings (silver stain and Alcian blue) of the lithified layer EPS. High molecular weight band (300 kD) appeared as acid polysaccharide. Six low molecular weight protein bands (65kD, 60kD, 56kD, 50kD, 45kD, and 38kD) appeared.*

In the present study, we demonstrated that EDTA-soluble EPS isolated from the lithified layer inhibited $CaCO_3$ precipitation in vitro when in solution, but induced $CaCO_3$ precipitation when they attached to a solid surface. Aragonite needles are commonly found within the EPS matrix in the surface layers of stromatolites (Reid *et al.* 2000). Our results showed that EPS isolated from lithified layers induced $CaCO_3$ crystals in vitro. FT-IR spectra confirmed that the crystals that formed on agarose beads are aragonite. It has been proposed that organic matrix in biominerals determine the type of $CaCO_3$ (Watabe & Wilbur 1960). Wada *et al.* (1993) also 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 on to the surfaces of the crystals (Wada *et al.* 1993). Therefore, EPS from the lithified layer may play an important role in the determination of crystal mineralogy in stromatolites. Yet, further study will be necessary to determine the effect of EPS on crystal polymorphism.

Our results are consistent with the immobilized-matrix/crystal nucleation (IC) hypothesis proposed for the mechanism of $CaCO_3$ precipitation in other biominerals (Crenshaw *et al.* 1988; Crenshaw 1991; Wheeler & Sikes 1989). The IC hypothesis proposes that the EDTA-soluble matrix isolated from biominerals when free in solution inhibits in vitro $CaCO_3$ precipitation, but when attached to an insoluble matrix framework may promote precipitation (Campbell *et al.* 1989; Crenshaw *et al.* 1991; Linde *et al.* 1989; Wheeler *et al.* 1988; Wheeler & Sikes 1984). Our results showed that the lithified layer EPS attached to the solid surface provided a nucleation site for $CaCO_3$ precipitation. We also found that contents of uronic acids and carbohydrates in EPS from lithified layers

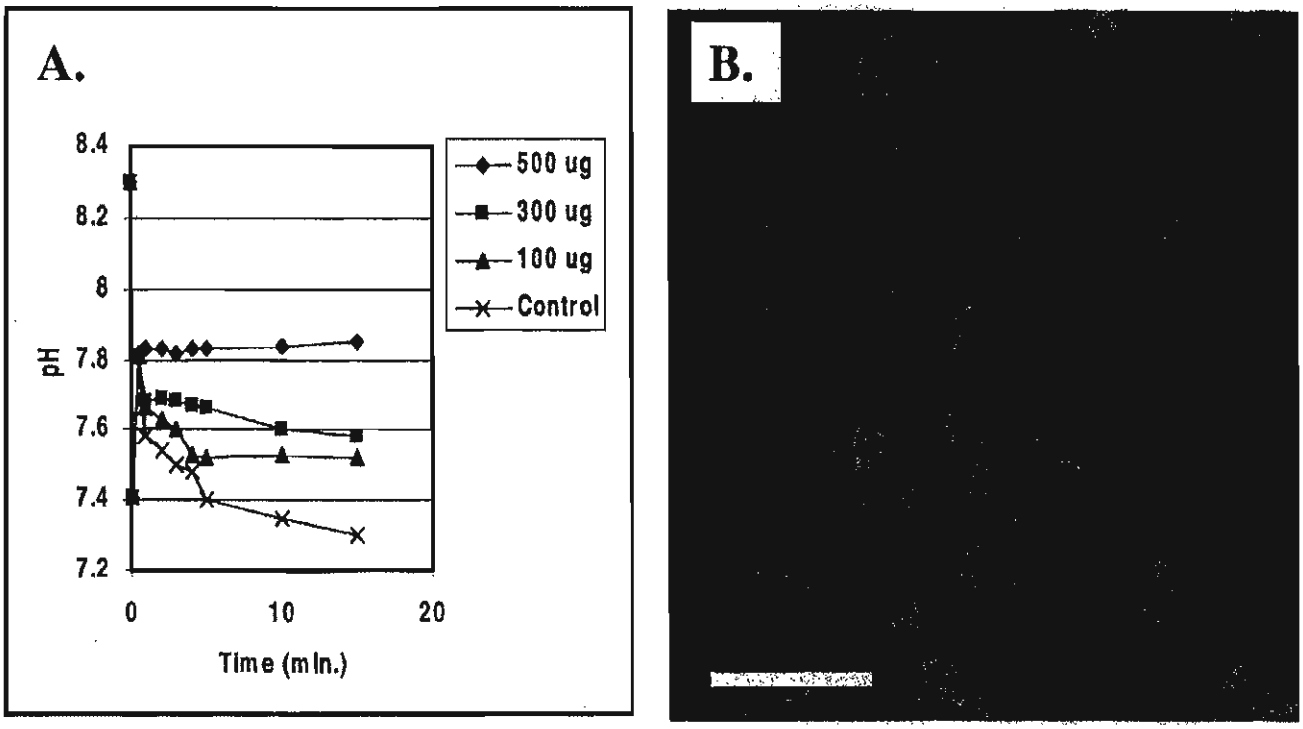

Figure 4. *Effect of the lithified layer EPS on $CaCO_3$ precipitation with various concentrations (100, 300 and 500 mg) using pH drift assay. H_2O was added as a control (A). Calcium carbonate crystals formed on agarose beads which coated with the lithified layer EPS (B). Scale=10 mm.*

decreased compared to unlithified layer EPS (Kawaguchi & Decho, submitted), but protein content remained relatively constant. This result suggests that as microbial degradation of EPS proceeds, protein fractions in EPS become increasingly exposed. Previous researchers reported that high sulfate reduction activities correlated with bands of $CaCO_3$ precipitation in stromatolites (Visscher *et al.* 1998). It has been suggested that cyanobacterial photosynthesis, sulfate reduction, and anaerobic sulfide oxidation, together, are responsible for $CaCO_3$ precipitation by creating highly alkaline conditions (Visscher *et al.* 1998, 2000).

Results of confocal imaging showed that EPS appeared to be closely associated with $CaCO_3$ precipitation. Immuno-localization of the lithified layer EPS confirmed that the presence of this EPS among $CaCO_3$ minerals. Therefore, when heterotrophic bacteria such as sulfate-reducing bacteria degrade carbohydrate in EPS, labile acidic proteins may remain after bacterial degradation, attached to a solid surface such as polymerized insoluble EPS. This EPS remnant may act as a nucleator to induce $CaCO_3$ precipitation.

Although our results are preliminary, our data suggested that EDTA-soluble EPS might inhibit $CaCO_3$ precipitation when in solution. Once attached to an insoluble substrate, EPS may provide the nucleation site for $CaCO_3$ precipitation. Therefore, EPS may accomplish divergent roles in $CaCO_3$ precipitation by providing a nucleation site in the lithified layer of marine stromatolites, and inhibiting $CaCO_3$ precipitation in the unlithified layer. The conspicuous lack of bacterial microfossils in association with the laminae of many Precambrian stromatolites may be due to precipitation events that occured within the amorphous EPS matrix, rather than in close association with cells (Reid *et al.* 2000). Therefore, EPS may play more important role in calcification in marine stromatolite than previously thought.

Table 1.
Contents (w/w) of carbohydrate, uronic acids and protein in EPS from the lithified layer in marine stromatolites, Highborne Cay, Bahamas.

	Carbohydrate (%)	Uronic acids (%)	Protein (%)
Lithified layer	**0.9**	**4.3**	**2.5**

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Table 2.
Amino acid composition of lithified layer EPS of the marine stromatolites, Highborne Cay, Bahamas (residues per 1000). Trp and Cys were not determined.

	Lithified Layer
As(x)	**166**
Gl(x)	**160**
Ser	**57**
His	**0**
Gly	**60**
Thr	**66**
Ala	**74**
Arg	**61**
Tyr	**30**
Cys	**ND**
Val	**67**
Met	**0**
Trp	**ND**
Phe	**63**
Ile	**56**
Leu	**92**
Lys	**29**
Pro	**37**

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