Nucleation of Celestite and Strontianite on a Cyanobacterial S-Layer

SUSANNE SCHULTZE-LAM* AND TERRY J. BEVERIDGE
Department of Microbiology, College of Biological Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Received 21 September 1993/Accepted 15 November 1993

Synechococcus strain GL24 is a unicellular cyanobacterium that was isolated from Fayetteville Green Lake, New York, a meromictic lake which has high Ca\(^{2+}\) and SO\(_4^{2-}\) concentrations. Epiclean mineralization of Synechococcus cells in the lake is the mechanism by which extensive calcitic bioherms (or microbial reefs) have been formed on the lake's shore and a marl sediment has been built on the lake bottom. Previous studies have shown that calcium carbonate (calcite) formation on the Synechococcus surface is dependent upon an alkaline pH, which is produced in the cellular microenvironment by the cells as their activity increases with seasonal warming of the lake water. At the circumneutral pH of bulk lake water, calcium sulfate (gypsum) is formed.

In this study, we show that Synechococcus mediates a similar sulfate-to-carbonate transformation when Sr\(^{2+}\) is the major divalent cation present, forming celestite and strontianite. In experimental systems to which equimolar amounts of Ca\(^{2+}\) and Sr\(^{2+}\), Ca\(^{2+}\) or Sr\(^{2+}\) and Mg\(^{2+}\), or all three ions together were added to artificial lake water, Ca\(^{2+}\) and Sr\(^{2+}\) were incorporated equally into mineral formation to form CaSr(CO\(_3\))\(_2\). No Mg\(^{2+}\)-containing carbonates were formed when either or both of the other two ions were present. Mineral formation takes place on a hexagonally arranged proteinaceous template (an S-layer) which forms the outermost surface of the Synechococcus cell. Our results provide evidence that the S-layer exhibits selectivity with respect to the ions bound and subsequently incorporated into carbonate minerals and that celestite and strontianite, previously thought to be purely evaporitic minerals, can be biogenically formed.

The involvement of cyanobacteria in the formation of carbonate minerals has been extensively studied. However, most studies focused on calcium carbonates in marine systems (1). Recently, it has been shown that cyanobacteria can mediate the formation of calcite in freshwater environments and, indeed, are essential to the process (5, 7). In waters which contain abundant Ca ions and dissolved CO\(_2\), present predominantly as HCO\(_3^-\) in alkaline waters, cyanobacteria provide nucleation sites for mineral formation to commence. The use of HCO\(_3^-\) as a carbon source and the subsequent release of OH\(^-\) (8, 12) lead to a rise in pH of the microenvironment surrounding each cell. As has been previously described (11, 14, 15), this alkalinization determines the mineral phase which develops on the cell surface. The formation of carbonate minerals is highly pH dependent, with carbonate phases being deposited at alkaline pH (pH > 8). At lower pH levels, other mineral phases tend to be formed, depending on the ion composition of the aqueous solution (10). In Fayetteville Green Lake, New York, CaSO\(_4\)-2H\(_2\)O (gypsum) forms initially on the Synechococcus cell surface, but as the cells become more active, the dominant mineral phase becomes calcite (14, 15). The use of bicarbonate (HCO\(_3^-\)) as a carbon source by the cells and the accompanying release of OH\(^-\) lead to an alkalinization of the microenvironment around each cell (8, 12).

Synechococcus strain GL24 cells are surrounded by an S-layer, a surface structure made up entirely of numerous identical protein molecules joined together to form a symmetrical pattern (11). The S-layer of Synechococcus strain GL24 has a hexagonal monomer arrangement and provides regularly spaced, chemically identical nucleation sites for mineral growth. The ability of this S-layer to mediate calcium mineral formation has been previously established (11). Mineral formation begins within the large holes of the array when Ca\(^{2+}\) binds to negatively charged sites on the S-protein and is joined by SO\(_4^{2-}\), initiating the formation of a mineral aggregate. By providing nucleation sites, the S-layer allows initial energy barriers to be overcome and mineral formation to commence in an environment in which the geochemistry is controlled by the metabolic activities of the Synechococcus cells (11). Eventually, the S-layer becomes encrusted with mineral and is shed so that cells have a patchy appearance with respect to the location of mineralized portions of their surface.

Although the involvement of cyanobacteria in the formation of calcium carbonate has been well established, microbial involvement in the formation of other carbonate minerals has not been extensively studied. Large deposits of strontianite (SrCO\(_3\)), magnesite (MgCO\(_3\)), and other carbonate minerals do exist, but they are held to be formed by abiogenic mechanisms such as evaporation (9, 13). In this study, we were interested in determining whether the Synechococcus S-layer could promote mineral formation by using other alkali earth cations, namely, Sr\(^{2+}\) and Mg\(^{2+}\).

MATERIALS AND METHODS

Mineralization studies. An artificial lake water medium (GL medium) from which alkali earth cations (Ca\(^{2+}\), Mg\(^{2+}\), and Sr\(^{2+}\)) were omitted was devised on the basis of the ionic concentrations in Fayetteville Green Lake water as given by Brunskill and Ludlam (4). This medium contained K\(_2\)HPO\(_4\)....
(0.3 mM), FeSO₄·7H₂O (0.02 mM), NaMoO₄ (0.0016 mM), Na₂SiO₃ (0.047 mM), NaHCO₃ (3.2 mM), HBO₂ (0.01 mM), Na₂EDTA (0.027 mM), and Na₂SO₄ (10 mM). Divalent cations (Ca²⁺, Sr²⁺, and Mg²⁺) were added as nitrate salts (10 mM) to create the different experimental systems. Sulfate levels mimicked those found in natural lake water, in which the concentration of this anion is approximately equal to that of calcium (10 mM). The medium was filter sterilized with 0.2-μm-pore-size sterile nylon filters (Millipore Corp.) to avoid carbonate precipitate formation by heating and added to sterile glass test tubes which had been acid washed and rinsed five times with ultrapure water (deionized to 18 MΩ·cm⁻¹).

For all experimental systems (GL plus Sr, GL plus Mg, and natural lake water, which is dominated by Ca), nine tubes, each containing 9 ml of the respective medium, were inoculated with *Synechococcus* cells from a 1-month-old culture to give a final cell count (by light microscopy) of 10⁶ cells per ml. A tenth tube was left uninoculated to serve as an abiotic mineralization control. The experimental cultures were incubated at room temperature (22°C) under Grolux fluorescent lights, which provided an intensity of 20 microeinsteins·m⁻²·s⁻¹. At each sampling time, one tube for each system was sacrificed. Samples were taken at 4, 8, 24, 48, 96, 144, 192, 240, and 288 h after inoculation. The cells from 2 ml of culture were pooled and used to prepare whole mounts for analytical electron microscopy by placing drops of the cell suspension on Formvar- and carbon-coated, 200-mesh copper grids. The remainder of the cell suspension was used for pH measurements. Similar systems with either Ca²⁺, Sr²⁺, or Mg²⁺, or combinations of these (Ca²⁺ plus Sr²⁺ or Mg²⁺, Sr²⁺ plus Mg²⁺, and all three together, to a total concentration of 10 mM), were set up to assess whether certain ions were more readily incorporated in mineral formation.

**EDS and SAED.** Grids were viewed in a Philips EM400T transmission electron microscope equipped with a Link LZ-5 light element detector and a Link exl. multichannel analyzer operating at 100 keV with a cold trap in place to obtain elemental analysis of the minerals. Energy-dispersive X-ray spectra (EDS) spectra were taken with a beam current of 0.1 μA and a spot size of 400 or 200 nm. Typical counting times were 100 s (live time). Camera lengths for selected area electron diffraction (SAED) were 575 to 1,650 nm, depending on the spacing of spots in the diffraction patterns.

**Inductively coupled plasma spectroscopy.** Cell-free supernatants from cell suspensions used in the mineralization studies were acidified with nitric acid (final concentration, 2%) in order to maintain cations in soluble form. Levels of alkali earth cations were determined with a Leco Plasmaray spectrometer equipped with a photodiode array detector.

**RESULTS**

**pH changes.** In all experimental systems, there was a rapid rise in pH during the first 48 h after inoculation (Fig. 1). In the Mg system and in natural lake water, the highest pH was reached at approximately 144 h after inoculation, and thereafter pH gradually decreased. In contrast, pH levels in the Sr system declined markedly after reaching a peak at 48 h after inoculation. After the initial decline, pH neither increased nor decreased appreciably for the rest of the study period in the Sr system. No pH changes occurred in the uninoculated controls.

**Mineral formation on Synechococcus cells.** In the Sr²⁺ system, substantial epicellular mineral formation was noted within hours of inoculation and followed a distinct morphogenic sequence (Fig. 2). No mineralization was observed in the uninoculated controls. The mineral precipitates initially appeared as small patches of mineralized S-layer (Fig. 2A) on the cell surface. The mineralized patches on the cells grew in three dimensions until the entire cell was enclosed (Fig. 2B). SAED of precipitates similar to those on the cell in Fig. 2B gave crystal lattice dimensional (d) spacings of 3.43 (0.34 nm), 3.27 (0.33 nm), and 2.12 Å (0.21 nm), which closely correspond to those of celestite (SrSO₄). Spacings of 2.84 (0.28 nm) and 3.45 Å (0.35 nm), indicating the presence of strontianite (SrCO₃), were also observed. Evidently both mineral types were present on the cells. No shedding of Sr-mineralized S-layer was seen. EDS indicated that, as mineralization proceeded (with an increase in pH), a relative increase in the strength of the Sr to Mg ratio was noted (Fig. 3A and B) accompanied the transformation from celestite to strontianite (SrCO₃). When the Sr/Mg ratio in minerals formed on the cells was plotted against time, a steady increase was observed (Fig. 4). This confirmed that, with time, the proportion of strontianite to celestite increased in the Sr-amended artificial lake water system. The decrease in this ratio at the very end of the experimental period may be correlated with a loss of viability seen in the cells, with a concomitant release of protons and a loss of alkalizing activity (Fig. 1).

Throughout the study, few minerals formed on the cells in the Mg system. The cells frequently appeared to be "peppered" with small-grained precipitates composed mainly of Mg and Sr.

**Measurement of cation levels in the experimental systems.** The trends in concentration of the major cations in each system (Fig. 5) correlated well with the observations of mineralized cells by electron microscopy. The initial decrease in Ca²⁺ levels in the natural lake water system was a consequence of the binding of this ion to surface-localized charged groups, principally those of the S-layer. By 24 h after inoculation, Ca²⁺ levels had risen to a level that was thereafter maintained for the rest of the study period. This was likely a reflection of the shedding of mineralized S-layer. Since supernatants were measured, the Ca²⁺ which was incorporated in the minerals on the shed material was included in the measurement. The pattern for Mg²⁺ was similar to that for Ca²⁺. While little mineral formation was seen in the Mg system, the cells were able to bind Mg²⁺ ions and shed S-layer. In contrast, Sr levels decreased steadily throughout the early part of the study.
FIG. 2. Images of unstained cells from the Sr system showing the initial and advanced stages of strontium mineral formation on *Synechococcus* cells. Mineralization follows a morphogenetic sequence in which mineral grains form within the S-layer fabric and spread to cover the entire cell surface. In the initial stages (A), the S-layer pattern is visible, although patches are smaller and the pattern is less clear than in the Ca system. Eventually (B), the cells become entirely encrusted in Sr mineral aggregates, resulting from their inability to shed mineralized S-layer. Bars = 200 nm.

period. Cells were unable to shed mineralized S-layer in this system, so that not only naked cell surface but also the minerals present upon it provided nuclei for additional Sr mineral formation.

**Ion competition experiments.** Mineralization studies in artificial lake water to which more than one major divalent cation was added showed that the S-layer preferentially adsorbed Sr$^{2+}$ and Ca$^{2+}$ (equally) for mineral formation, with a consis-
FIG. 3. EDS of mineralized patches of S-layer from cells in the Sr system. Spectrum A is of a patch in the initial stages of mineral formation similar to those on the cell in Fig. 2A. At this stage, S is present and stoichiometric analyses confirm that the mineral present is celestite (SrSO₄). In advanced stages, when a “knob” precipitate from a cell like the one in Fig. 2B is analyzed (B), the Sr signal is much stronger and S is no longer present. Stoichiometric analysis and SAED show that the dominant mineral type is SrCO₃ (strontianite). In both spectra, the P signal is due to underlying cell material and the Cu signal originates from the copper specimen grid. Trace amounts of Ca and K (unlabelled peaks at 3.4 and 3.7 keV, respectively) are also present but decrease in relative proportion to Sr as mineralization advances.

DISCUSSION

Previous studies (11) have shown that the hexagonal S-layer from Synechococcus strain GL24 (Fig. 6) can nucleate gypsum (CaSO₄·2H₂O) and calcite (CaCO₃) formation. The dominant mineral type present is influenced by the cyanobacterially controlled pH and cation concentration of the cellular microenvironment so that carbonate formation is related to the bicarbonate uptake activity of the cells (14). Within a few

FIG. 4. Graphical depiction of the changing ratio of Sr to S throughout the 12-day experimental period. Each point represents an average ratio derived from five different stoichiometric determinations of elemental proportions detected by EDS. The high Sr/S ratio seen toward the end of the study period indicates a change in dominant mineral type from celestite (SrSO₄) to strontianite (SrCO₃).

or Ca²⁺ plus Sr²⁺ plus Mg²⁺ was tested, a 1:1 (Ca,Sr)CO₃ was formed.

FIG. 5. Changes in divalent ion concentration of the bulk fluid phase of the artificial lake water systems and of natural lake water throughout the experimental period. ●, Sr; ●, Mg; ■, lake water.
hours of inoculation, gypsum microcrystals begin to form within the large diamond-shaped holes (Fig. 6B) of the S-layer lattice structure so that its pattern can be seen in unstained preparations of cells from lake water (Fig. 7). Eventually, encrustation by mineral precipitates becomes substantial enough to obscure the S-layer pattern. While the cells are still active, the mineralized S-layer is shed so that cells in suspension are accompanied by sloughed off S-layer, which can continue to nucleate mineral formation.

In our artificial lake water systems, Sr mineral formation underwent a similar progression, with the S-layer pattern being visible in the early stages but eventually becoming obscured by the growing load of precipitates. However, the S-layer pattern as outlined by Sr minerals was less clear than with gypsum or calcite, and mineral growth occurred as rounded aggregates rather than large patches which cover extensive portions of the cell surface.

The variations in growth habit of the minerals on the cells...
are likely a reflection of the differing physical characteristics of the ions themselves. Ca$^{2+}$ and Sr$^{2+}$ have very similar hydrated ionic radii and charge character (6) and should behave as structural analogs, having the ability to interact similarly (but not identically) with any given binding site on the cell surface and with anions present in their aqueous environment. The small radius of Mg$^{2+}$ and its avidity for water molecules differentiate this ion from the other two. Therefore, although Mg-containing precipitates did form on the Synechococcus cells, the S-layer pattern could not be visualized and mineral formation was easily inhibited by Ca$^{2+}$ and/or Sr$^{2+}$.

Cells in the Sr system were unable to shed mineral-encrusted S-layer. This was reflected by the trends in cation depletion of the supernatants from the experimental systems (Fig. 5). The steady decline in Sr$^{2+}$ levels for most of the 12-day study period was an indication that Sr$^{2+}$ was being immobilized on the cell surface through adsorption and incorporation into mineral aggregates. The synthesis of new S-layer needed to replace that which had become heavily mineralized was likely inhibited by the interference of Sr$^{2+}$ with photosynthesis in the Synechococcus cells. Ca$^{2+}$ is an essential component of photosystem II, in which it is involved in primary photosynthetic processes at the reaction center (2, 3). In the Sr artificial lake water system, Ca$^{2+}$ was omitted from the medium. Trace amounts of Ca$^{2+}$ were likely present as contaminants in some of the salts used to prepare the medium but at levels insufficient to support active photosynthesis in the presence of high Sr$^{2+}$ levels. As a result, Sr$^{2+}$, as a structural analog of Ca$^{2+}$, was able to interfere with photosynthesis to a degree sufficient to compromise viability. This led to a decrease or elimination of alkalization by the cells, which, coupled with an increased release of H$^+$, led to a discernible acidification of the medium.

In the mixed-cation systems, cells were equally capable of incorporating Ca and Sr into carbonate mineral formation. When both were present in equimolar amounts, a 1:1 (Ca,Sr) CO$_3$ was formed. In the initial stages, the S-layer pattern was visible and appeared similar to that on cells in natural lake water, although slightly less clear. In addition, despite the high levels of Sr present (5 mM), the cells remained photosynthetically competent and were able to shed encrusted surface layers. Therefore, it appears that, as long as some Ca$^{2+}$ is available, cells in high-Sr environments can persist as a viable population while initiating the formation of celestite and strontianite.

A key finding of this study is that it is possible for celestite and strontianite to be formed biogenically and that a bacterial S-layer can serve as their template. There are also important implications for the geochemistry of natural freshwater environments. Noncalcitic carbonates are generally believed to be deposited evaporitically, and no gypsum- or strontium-containing carbonate minerals are believed to form in lacustrine environments (9, 16). Nevertheless, gypsum, celestite, and strontianite are formed by Synechococcus and, of these, gypsum formation has clearly been demonstrated to form in the lake water itself (14, 15). Few geochemical studies of carbonate mineral formation in freshwater systems include an analysis of Sr$^{2+}$ concentration or partitioning, yet extensive deposits of celestite and strontianite do exist (13). It is to be expected that Sr-containing minerals can also be formed by the same biogenic mechanism as Ca-containing minerals, given the proper geochemical environment, and it may be that the role of cyanobacteria in the formation of these minerals will prove to be a crucial and substantial one.
ACKNOWLEDGMENTS

This research was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) operating grant to T.J.B. and was conducted at the NSERC-Guelph Regional Stem Facility, which is partially supported by an infrastructure grant from NSERC. S.S.-L. was supported by a graduate scholarship from NSERC.

We thank G. Spiers of the Department of Land Resource Science, University of Guelph, for assistance with inductively coupled plasma spectroscopy.

REFERENCES