Geobacillus thermoglucosidasius Endospores Function as Nuclei for the Formation of Single Calcite Crystals

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Geobacillus thermoglucosidasius colonies were placed on an agar hydrogel containing acetate, calcium ions, and magnesium ions, resulting in the formation of single calcite crystals (calcites) within and peripheral to the plating area or parent colony. Microscopic observation of purified calcites placed on the surface of soybean casein digest (SCD) nutrient medium revealed interior crevices from which bacterial colonies originated. Calcites formed on the gel contained [1-¹³C]- and [2-¹³C]acetate, demonstrating that *G. thermoglucosidasius* utilizes carbon derived from acetate for calcite formation. During calcite formation, vegetative cells swam away from the parent colony in the hydrogel. Hard-agar hydrogel inhibited the formation of calcites peripheral to the parent colony. The calcite dissolved completely in 1 M HCl, with production of bubbles, and the remaining endospore-like particles were easily stained with Brilliant green dye. The presence of DNA and protein in calcites was demonstrated by electrophoresis. We propose that endospores initiate the nucleation of calcites. Endospores of *G. thermoglucosidasius* remain alive and encapsulated in calcites.

Biomining is defined as the synthesis of inorganic crystalline or amorphous mineral-like materials by living organisms. Bacteria are capable of forming inorganic crystals either intracellularly (1) or extracellularly (2). Magnetic bacteria migrate along the lines of the earth's magnetic field and synthesize intracellular particles of magnetite that are aligned in a chain-like fashion and enveloped by a membrane (3, 4). The use of bacterial magnetic particles in biomedical applications is increasing because they are easy to handle and to separate from biological samples. In particular, the lipid bilayer surrounding magnetic particles is reportedly amenable to insertion of recombinant transmembrane proteins (5, 6). Calcite (calcium carbonate) precipitation is a well-known example of extracellular bacterial biomineralization. The precipitation of calcium carbonates by *Halobacillus trueperi* (spore-forming, moderately halophilic bacterium) isolated from hypersaline sediments of the Great Salt Lake in Utah has been investigated (7). Scanning electron microscopy indicated that the dominant morphology of *H. trueperi* calcium carbonate is spherulitic calcite with a fibrous radiated interior.

In our own research, we isolated a biomineralizing thermophilic spore-forming bacterium from bark compost that has an optimum growth temperature of 60°C. Phylogenetic analysis of the 16S rRNA gene sequence identified the organism as *Geobacillus thermoglucosidasius* (8). *Geobacillus* spp. are a phenotypically and phylogenetically similar group of thermophilic bacilli (9). To date, we have found that *G. thermoglucosidasius* extracellularly catalyzes the formation of 100-μm spheres and hexagonal crystals at 60°C in hydrogel containing sodium acetate, calcium chloride, and magnesium sulfate (calcite-promoting hydrogel). Microscopic analysis revealed that the minerals have polarizing properties and are thus highly crystallized. Under fluorescence microscopy, excitation occurred at 365 ± 5, 480 ± 20, and 545 ± 15 nm, and broad-spectrum fluorescence was emitted with excitation at 350 nm. The maximum emitted wavelength was 446.9 nm for excitation at 369.6 nm. An X-ray diffraction analysis showed the crystals to be magnesium-calcite in the calcium carbonate group (8). From the observation that calcite was formed on both the

interior and exterior of the *G. thermoglucosidasius* colony, we deduced that endospores constituted the nucleus of the calcites.

The starvation for nitrogen and phosphate sources in calcite-promoting hydrogel promotes sporulation. In this study, we investigated the swimming behavior of vegetative cells in calcite-promoting hydrogel and the effect of hydrogel hardness on calcite formation to test whether the vegetative cells are vehicles for the nonmotile endospores that we believe serve as the foci for calcite nucleation. Glucose is known to repress sporulation in spore-forming Gram-positive bacteria; thus, the effect of glucose on calcite nucleation was also examined. In addition, we investigated whether biological substances such as nucleic acids and proteins are present in calcites to support the hypothesis that *G. thermoglucosidasius* endospores serve as nuclei for calcite formation.

MATERIALS AND METHODS

Stock culture. *Geobacillus thermoglucosidasius* NY05 was inoculated onto soybean casein digest (SCD) agar (Nissui, Japan) plates and incubated aerobically at 60°C for 18 h. Colonies were collected by microbial loop and inoculated onto calcite-promoting hydrogels for assay.

Formation of single calcite crystals (calcites) on hydrogel. Calcite-promoting hydrogels were prepared by adding sodium acetate (25 mM), calcium chloride (7.0 mM), and magnesium sulfate (2.0 mM) to a 1.5% agar (Nacalai Tesque, Japan) solution, bringing the solution to a boil, and allowing it to solidify in 9-cm petri dishes. A wet weight of 10 to 20 mg of *G. thermoglucosidasius* colonies was placed by loop in a circular spot with a diameter of 1.0 cm on calcite-promoting hydrogel plates and incubated at 60°C for 24 to 168 h. To isolate the calcites, the parts of the gel on which calcites formed were excised using a sterilized spatula and solubilized in QX1 buffer (Qiagen, CA) in test tubes at room temperature. Calcites were

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recovered from the bottom of the test tube and washed repeatedly four times with sterilized distilled water and ethanol and then dried at 60°C (8).

Migration of vegetative cells in calcite-promoting hydrogel. A total of 20 mg of fresh *G. thermoglucosidasius* colonies was placed on a 1-cm circle on a calcite-promoting hydrogel plate (parent colony) and incubated at 60°C for 24, 72, 120, and 168 h. The gel, excluding the parent colony, was excised with a sterilized scalpel and placed in contact with the surface of an SCD agar plate, which was then incubated at 60°C for 14 h. The number of vegetative cells migrating away from the parent colony was determined as the number of subcolonies formed on the SCD agar plate. In addition, the number of calcites formed at distances of 0 to 10, 10 to 15, 15 to 20, 20 to 25, and 25 to 30 mm from the center of the parent colony on the calcite-promoting hydrogel was determined after incubation at 60°C for 168 h.

Effect of gel hardness on calcite formation. Calcite-promoting hydrogel dissolved in agar solution (1.0, 1.5, 2.0, 3.0, or 4.0%) was solidified in 9-cm petri dishes. Next, 20 mg of fresh *G. thermoglucosidasius* colonies cultured at 60°C for 24 h on an SCD plate was placed within a 1-cm circle on the surface of each calcite-promoting hydrogel plate, which was then incubated at 60°C. The distance between the edge of the parent colony and the most distant calcite formed was measured with a ruler after 1, 2, 3, 4, and 5 days.

Effect of glucose on calcite nucleation events. A total of 10 to 20 mg of fresh *G. thermoglucosidasius* colonies was placed within a 1-cm circle at the center of calcite-promoting hydrogel plates (parent colony) containing 2 to 10 mM glucose, and the plates were then incubated at 60°C for 96 h. The number of calcites formed per mg of parent colony was determined by counting the number of calcites formed within the parent colony.

Incorporation of isotopically labeled carbon from acetate into calcites. Stable carbon isotope-labeled ($1\text{-}^{13}\text{C}$ or $2\text{-}^{13}\text{C}$) sodium acetate (25 mM) (50 atom% of ^{13}C [Cambridge Isotope Laboratory, Inc.]), calcium chloride (7 mM), and magnesium sulfate (2 mM) were added to liquefied agar solution (1.5%). The solution was brought to a boil, allowed to solidify in 9-cm petri dishes, and employed as calcite-promoting hydrogels for evaluating the uptake of stable carbon isotopes into calcites. A wet weight of 10 to 20 mg of *G. thermoglucosidasius* colonies was placed by loop onto a 1-cm circular spot at the center of each of the calcite-promoting hydrogel plates, which were then incubated at 60°C for 36 h. Calcites were purified according to a previously described method (8). The CO_2 gas produced as a result of the reaction between phosphoric acid and purified calcite was analyzed quantitatively using liquid chromatography-mass spectrometry (LC/MS) (Hitachi RMI-2, Tokyo, Japan) to determine the ratio of ^{13}C atoms to total carbon (^{12}C plus ^{13}C) atoms (atom%) in the calcites (29).

Dissolving calcites. A purified calcite formed peripheral to the parent colony was placed on a glass slide and dissolved in 2 μl of 1 N HCl (10). Residual substances that remained on the glass slide were directly subjected to heat fixation by exposure to a flame, stained with 7.6% Brilliant green (Wako Chemicals, Osaka, Japan) for 10 min, rinsed, and observed under a light microscope.

Extraction of DNA from calcites. Purified calcites (10 mg) formed peripheral to the parent colony were dissolved in 20 μl of 1 M HCl. The particle-like residue was collected by centrifugation and washed 4 times with sterilized distilled water, after which DNA was extracted from the washed residue using a DNA extraction kit (Isoplant; Nippon Gene Co., Ltd., Tokyo, Japan). The extracted DNA was dissolved in 50 μl of TE buffer (pH 7.6), a portion of which was treated with 10 U of DNase I (Roche, Mannheim, Germany) at 25°C for 60 min. The DNA samples were then subjected to electrophoresis on a 1.5% agarose gel to evaluate the integrity of the nucleic acid.

Extraction of protein from calcites, endospores, and vegetative cells. Purified calcites (30 mg) formed peripheral to the parent colony were physically disrupted using a metal spatula, suspended in 10 μl of SDS-PAGE sample buffer, and boiled for 5 min. *G. thermoglucosidasius* cells (20 mg wet weight) cultured on SCD plates at 60°C for 48 h were dissolved in

300 μl of sterilized distilled water and incubated at 37°C for 60 min with 300 μl of lysozyme solution (10 mg/ml) (Wako Chemicals, Osaka, Japan), after which the resulting protoplasts were ruptured by the addition of 30 μl of 10% SDS solution. The resulting endospores were washed with water, dissolved in 10 μl of SDS-PAGE sample buffer, and boiled for 5 min. *G. thermoglucosidasius* cells (20 mg) were dissolved in 10 μl of SDS-PAGE sample buffer and boiled for 5 min to extract total protein from vegetative cells. Proteins extracted from calcites, endospores, and vegetative cells were analyzed on a one-dimensional 12.5% SDS-PAGE gel using conventional methods. Coomassie blue-stained protein bands were excised and washed twice for 10 min each in a mixture of acetonitrile–50 mM NH_4HCO_3 in water (1:1 by volume). The gel pieces were then dried *in vacuo*, and 1 μg of trypsin in 50 to 100 μl of 50 mM NH_4HCO_3 solution was added. The digestion was allowed to proceed overnight at 37°C and was terminated by adding 3 μl of formic acid. Peptide mass fingerprints (PMF) were generated using a Bruker AutoFlexIII matrix-assisted laser desorption ionization–time of flight mass spectrometer (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). Proteins were identified using MASCOT (Matrix Science, London, United Kingdom) search algorithms and a nonredundant database (11).

Morphological observation. Following colloidal gold (50 Å) sputtering, calcites were examined under a scanning electron microscope (SEM) (Hitachi S-4100; Hitachi, Tokyo, Japan) with an accelerating voltage of 5.0 kV. Light microscopic observation of calcite morphology was carried out as described previously (8).

RESULTS

Formation of single calcite crystals (calcites) on calcite-promoting hydrogel. Following inoculation of calcite-promoting hydrogel with *G. thermoglucosidasius* (parent colony) and incubation at 60°C, visible calcites formed both within and to the exterior of the parent colony within 48 h (Fig. 1A). Hexagonal calcites ($141.7 \pm 13.8 \mu\text{m}$) tended to form within the parent colony, whereas spherical or dumbbell-shaped calcites ($114.7 \pm 7.8 \mu\text{m}$) preferentially formed peripheral to the parent colony. The calcites that formed peripheral to the parent colony tended to be smaller than those that formed within the colony.

Germination from calcites. Light microscopic observation of calcites formed peripheral to the parent colony showed that the crystal nucleus was clearly formed (Fig. 1B). Colonies of *G. thermoglucosidasius* were found to grow around purified calcites incubated on the surface of SCD plates at 60°C for 24 h. Stereomicroscopic observation showed calcites embedded in growing colonies. The centrally localized calcites became thin and appeared likely to split open (Fig. 1C). Observation of calcites embedded in growing colonies by SEM following washes with sterilized distilled water and ethanol showed that the calcites appeared to be split in two at the center (Fig. 1D). Traces of crystal nuclei were present at the center of the split calcites. In addition, a clear crevice was noted to form at the center of the calcite (indicated by an arrowhead in Fig. 1E).

Migration of vegetative cells in calcite-promoting hydrogel. The radius of the parent colony did not increase during incubation at 60°C on calcite-promoting hydrogel. Subcolonies of *G. thermoglucosidasius* generated on SCD agar plates following a 24-h incubation on calcite-promoting hydrogel were detected within a 30-mm radius around the center of the parent colony (Fig. 2, top). About 70 subcolonies were generated within a 10-mm radius around the center of the parent colony. These results indicate that individual cells swim through the gel away from the parent colony; the most active cells could swim as far as 25 mm from the edge of the parent colony. The number of individual subcolonies gener-

FIG 1 Microscopic observations of a *G. thermoglucosidarius* parent colony and bacterial calcites generated after incubation at 60°C for 48 h. The direct stereomicroscopic image of calcite-promoting hydrogel shows calcites generated within and on the exterior of the *G. thermoglucosidarius* parent colony (A). (B) Light microscopic image of the nucleus of a single calcite crystal. (C) Direct stereomicroscopic image of a split calcite on SCD nutrient medium (C). (D and E) SEM images of split calcites (D) and a crevice in a single calcite crystal (E).

ated within the 10-mm radius of the parent colony decreased with longer incubation times, and no subcolonies were generated after 168 h of incubation. After incubation for 24 and 168 h, 5 and 70 individual subcolonies, respectively, were generated 25 to 30 mm from the center of the parent colony. This result shows that as incubation time increases so does the distance individual cells can move away from the parent colony.

Placement of a *G. thermoglucosidarius* parent colony on calcite-promoting hydrogel followed by incubation at 60°C for 24 h resulted in formation of tens of calcites exterior to and within the parent colony in a 10-mm radius of the center of the parent colony (Fig. 2, bottom). The number of calcites formed within a 10-mm radius increased to 425 after 72 h of incubation, and this state was stably maintained through 168 h of incubation. Totals of 130, 300, and 310 calcites formed 15 to 20 mm from the center of the parent colony following 72, 120, and 168 h of incubation, respectively. At a distance of 25 to 30 mm from the center of the parent colony, no calcites were formed after 24 h of incubation, but about 20 calcites had formed by 72 h (Fig. 2, bottom). The number of calcites formed on the exterior of the parent colony was shown to be dependent on the incubation time at 60°C and increased with incubation time up to 168 h.

Effect of gel hardness on calcite formation. Distances between calcites and the edge of parent colonies incubated on calcite-promoting hydrogels containing different concentrations of agar were investigated. Incubation for 1.5, 2, and 3 days on calcite-promoting hydrogel containing 1.0% agar resulted in formation of calcites 2.0, 6.0, and 7.0 mm, respectively, from the edge of the *G. thermoglucosidarius* parent colony. On calcite-promoting hy-

FIG 2 Diagrammatic representation of the migration of vegetative cells away from the *G. thermoglucosidarius* parent colony over the course of a 168-h incubation at 60°C (upper). The parent colony was inoculated onto a 1-cm-diameter circle at the center of a calcite-promoting hydrogel plate. Individual dots represent the sites where individual vegetative cells were found at each time point. The number of single calcite crystals formed peripheral to the parent colony is plotted against the distance from the center of the parent colony to the site of single calcite crystal formation (lower). Values are the means $\pm$ standard deviations (SD) from 3 independent experiments, each performed in duplicate.

FIG 3 Inhibition of single calcite crystal formation by addition of glucose. Trials at each concentration were carried out in triplicate, and the experiment was repeated three times. Values are means $\pm$ SD.

drogel containing 1.5% agar, calcite formed 7.0 mm away from the edge of the parent colony at 4 days. The longest distances between the calcite formations and the edge of the parent colony on calcite-promoting hydrogel containing 1.5%, 2.0%, and 3.0% agar were 6.0, 3.5, and 2.0 mm, respectively, for a 3-day incubation. No calcites formed on the exterior of the parent colony on calcite-promoting hydrogel containing 4.0% agar, indicating that it is more difficult to form calcites peripheral to the parent colony on gels of more than 3% agar.

Effect of glucose on calcite formation events. As shown in Fig. 3, calcite-promoting hydrogel lacking glucose supported the formation of 187 calcites per mg of *G. thermoglucosidasius* vegetative cells (parent colony). The number of calcites formed per mg of vegetative cells on hydrogels containing 2.0 and 4.0 mM glucose decreased to 102 and 14, respectively. No calcites were formed on hydrogels containing glucose at concentrations of 6.0 mM or higher, indicating that glucose inhibits *G. thermoglucosidasius*-catalyzed calcite formation.

Incorporation of stable carbon isotopes into calcites. The stable carbon isotope abundance ($^{13}\text{C}/^{12}\text{C}$ plus ^{13}C) in calcites formed with unlabeled sodium acetate was 1.08%, which is close to the natural stable carbon isotope abundance of 1.11% in Pee Dee belemnite standard (13). The stable carbon isotope abundances in calcites formed with $[1-^{13}\text{C}]$ - and $[2-^{13}\text{C}]$ sodium acetate were 43.1% and 37.9%, respectively, indicating that the carboxyl carbon atoms are incorporated into calcites at a higher rate than are methyl group carbons. These data suggested that the carbon in calcites is derived from both the methyl and carboxyl carbons of acetate. A stable carbon isotope abundance less than 50% indicates that ^{12}C is more available for incorporation into calcite than the heavier ^{13}C isotope.

Dissolution of calcites. The dissolving of purified calcite on a glass slide with 2 μl of 1 N HCl was accompanied by the production of carbon dioxide bubbles. A particle-like residue remained on the glass slide after the bubbles dissipated. As shown in Fig. 4, the residual particles were oval, 0.5 to 1.0 μm in size, and were easily stained with Brilliant green, which is frequently used to stain the endospores of Gram-positive bacteria (14). One calcite was found to contain 300 to 400 oval particles. The shapes and sizes of the residual particles obtained by dissolving purified calcite were similar to those of *G. thermoglucosidasius* endospores.

FIG 4 Stereomicroscopic image of a typical single calcite crystal formed peripheral to the parent colony on a calcite-promoting hydrogel (left). The panel to the right shows a light microscopic image of oval particles remaining after the calcite was dissolved with HCl.

Extraction of DNA from calcites. Fragments of DNA extracted from the residue of dissolved calcites were determined to be around 150 to 750 bp in size, based on agarose gel electrophoresis analysis. No bands were detected following treatment with DNase I (Fig. 5A). In addition, analysis of the absorption spectra of the extracts showed an absorption peak at 260 nm attributable to nucleic acids (Fig. 5B). These results indicate that extracts of the oval residual matter contain DNA. The yield of DNA extracted from 10 mg of calcites was 15 μg . Based on these results, we conclude that the DNA in the calcite residue originated from endospores.

Extraction of protein from calcites. An SDS-PAGE analysis of the proteins extracted from calcites showed a band of approxi-

FIG 5 Agarose gel electrophoresis of untreated (lane 1) and DNase I-treated (lane 2) extracts of DNA from single calcite crystals (A). (B) Absorbance spectrum of DNA extracted from single calcite crystals. (C) Coomassie blue-stained SDS-PAGE gel showing proteins extracted from whole vegetative cells (lane 1), endospores (lane 2), and single calcite crystals (lane 3). M, molecular mass markers.

mately 75 kDa (Fig. 5C). The 75-kDa protein band was also present among the proteins extracted from endospores. A prominent 15-kDa band present in the endospore extract was determined to be lysozyme, which was used to lyse the vegetative cells to obtain endospores. Analysis of a digest of the 75-kDa protein using MALDI-TOF MS and subsequent database searching indicated that the protein is closely related to the putative *N*-acetyltransferase YitH (GenBank accession no. CAB12939) (15, 16) derived from *Bacillus subtilis*.

DISCUSSION

Based on the shapes and sizes of the particles remaining after HCl treatment of calcites, we suspected that endospores might be encapsulated in calcites. The calcites were split, and colonies of *G. thermogluco*sidasius were generated from the broken calcites, suggesting that endospores were indeed encapsulated in calcites and that they were still viable. Absorption of nutrients into the internal calcite crystal structure would be possible due to its porous structure (17). The encapsulated endospores could thus have been activated by the nutrient environment, leading to spore germination.

We also demonstrated that individual *G. thermogluco*sidasius vegetative cells are capable of swimming over the gel surface away from the parent colony during incubation at 60°C. Following a 24-h incubation, many swimming cells were detected within a 10-mm radius around the parent colony. Weak calcite formation was observed within this area at this time, but after 72 h of incubation, many calcites began to rapidly form within the 10-mm radius around the parent colony. Migration of individual vegetative cells is linked to the formation of calcites, which are formed peripheral to the parent colony. Thus, swimming individual vegetative cells likely serve as vehicles for endospore migration.

Due to their low concentration of agar, soft gel structures entrap more moisture than hard gel structures (18). Hence, individual vegetative cells were able to swim farther from the edge of parent colony on the soft gel (1% agar), whereas a hard gel (4% agar) hindered individual vegetative cells from swimming away from the parent colony. Calcites formed 7 mm away from the center of the parent colony on gel containing 1% agar. However, no calcite formed to the exterior of the parent colony on gel containing 4.0% agar. These results support the supposition that swimming of individual vegetative cells away from the parent colony is essential for calcite formation on the exterior of the parent colony.

Supplementation of calcite-promoting hydrogel with glucose prevented the formation of calcites by *G. thermogluco*sidasius. In particular, glucose in excess of 6 mM completely inhibited the formation of calcites. Synthesis of the enzymes and permeases involved in the utilization of other carbon-containing compounds is often inhibited in bacteria grown on media containing metabolized carbohydrates (i.e., glucose). Such regulation of gene expression in response to carbon availability is known as catabolite repression (12, 19), and initiation of sporulation is known to be regulated by catabolite repression (20). We confirmed that sporulation of *G. thermogluco*sidasius on calcite-promoting hydrogel is inhibited in the presence of excess glucose (data not shown). These results support the hypothesis that endospores behave as initialized nuclei for calcite formation. Newly generated endospores in calcite-promoting hydrogel may be involved in initialization of calcite formation.

Half of the carbon atoms in calcites formed in the presence of [1-¹³C]- or [2-¹³C]acetate were replaced with the stable carbon isotopes, indicating that calcites derive carbon from acetate. The carbon in the calcite crystalline lattice was shown to consist of acetate-derived carbon. In bacterial metabolism, acetate is utilized in the glyoxylic acid cycle to synthesize oxaloacetic acid, which is subsequently converted to carbon dioxide through the tricarboxylic acid cycle (20). Hence, the carbon atoms in the carbon dioxide produced on dissolution of calcite are derived from acetate. *G. thermogluco*sidasius endospores utilize carbon dioxide derived from acetate metabolism by vegetative cells along with calcium ions contained in the gel to form the calcite lattice.

The presence of DNA in the particle-like residue produced by dissolution of calcites with HCl suggested that the particle-like structures were organisms. We attributed the relatively short length of the extracted DNA fragments (150 to 1,000 bp) to exposure of the particle-like structures in the residue to 1.0 N HCl prior to DNA extraction. We also confirmed that DNA extracted from vegetative cells following exposure to 1.0 N HCl was also 150 to 1,000 bp in length.

The predominant protein extracted from calcites had a molecular mass of 75 kDa. The band pattern of proteins extracted from calcites was similar to that of the proteins extracted from *G. thermogluco*sidasius endospores. Homology searching using MASCOT PMF indicated that the calcite-specific proteins are related to putative *N*-acetyltransferase YitH (GenBank accession no. CAB12939) (15, 16, 21), which is a member of the acetyltransferase family in *B. subtilis*. The enzyme *N*-acetyltransferase catalyzes the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to glucosamine 6-phosphate, the precursor of *N*-acetylglucosamine, a constituent of the peptidoglycan cell wall of Gram-positive bacteria (21, 22). The thick peptidoglycan shell of endospores is known as the spore cortex and aids in survival during dormancy (23). The expression of *N*-acetyltransferase thus plays an important role in the heat and chemical resistance of bacterial endospores. The presence of this protein in calcites is further evidence that endospores are present in calcites.

Bacillus sp. spores are noted for their longevity and high degree of resistance to environmental extremes (24, 25). Many of the molecular factors responsible for the resistance of spores have been identified within the spore core. A major factor implicated in spore resistance to environmental extremes is the small molecule pyridine-2,6-dicarboxylic acid (dipicolic acid [DPA]). The Ca²⁺ chelate of DPA is a minor constituent in the core of dormant spores (26). Endospores are known to concentrate calcium ions on the cell surface prior to intracellular uptake (27). We propose that *G. thermogluco*sidasius endospores also concentrate Ca²⁺, creating the supersaturation conditions necessary for calcite nucleation. Endospores have a strong calcium-concentrating effect and thus cooperate to induce an oriented calcite nucleation. It is assumed that 200 to 300 individual endospores with concentrated Ca²⁺ on their surface aggregate and act as nuclei for calcite formation. Based on these nuclei, calcites grow larger with the incorporation of Ca²⁺, Mg²⁺, and carbon dioxide produced via acetate metabolism in the respiration of vegetative cells. The inhibition of calcite formation by glucose can be attributed to inhibition of sporulation, because sporulation is linked with the nuclei for calcite orientation. We propose that the carbon dioxide (carbonic acid ion) produced as a result of the metabolism of acetate and newly generated endospores is essential for the formation of cal-

cites by *G. thermoglucosidasius*. There are several reports (7) suggesting that the bacterial cell surface can serve as a nucleation site during the calcification process. Since the cell wall of the bacteria is negatively charged, the bacteria adsorb Ca^{2+} ions from the environment to deposit on their cell surface. The Ca^{2+} ions subsequently react with the CO_3^{2-} ions, leading to the precipitation of calcite at the cell surface that serves as a nucleation site.

Lian et al. reported calcite formation by *Bacillus megaterium* (28). They showed that the calcite crystallization process appears to be influenced by the physical presence of bacterial cell surfaces and extrapolymeric substance. The bacterial cell surface would serve as the nucleation site for calcite formation.

That microorganisms play an important role in mineral precipitation in natural habitats is widely accepted. Bacteria are believed to have a particular effect on carbonate precipitation, which may involve different mechanisms in different environments. As shown in Fig. 1, calcite formation was initiated under oligotrophic conditions in which acetate and Ca^{2+} were present (8) along with trace amounts of Mg^{2+} . Endospores are not normally formed during active vegetative cell growth. Differentiation to endospore formation occurs under conditions of nutrient limitation. *G. thermoglucosidasius* cells (parent colony) placed on calcite-promoting hydrogel would normally form endospores because the nutrient supply in the hydrogel is limited. When *G. thermoglucosidasius* endospores are exposed to conditions unsuitable for vegetative growth in the natural environment, they catalyze the formation of calcites and become encapsulated in the calcite crystals, where they survive in a cryptobiotic state until conditions improve. It is thought that encapsulation of *G. thermoglucosidasius* endospores in calcite enables the endospores to survive until favorable oligotrophic conditions are restored. Calcite formation may therefore be one of the means that *G. thermoglucosidasius* uses to survive in extreme environments.

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