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Carbonate biomineralization induced by soil bacterium *Bacillus megaterium*

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Abstract

Biogenic carbonates spawned from microbial activities are common occurrences in soils. Here, we investigate the carbonate biomineralization mediated by the bacterium *Bacillus megaterium*, a dominant strain separated from a loess profile in China. Upon completing bacterial cultivation, the ensuring products are centrifuged, and the resultant supernatant and the concentrated bacterial sludge as well as the un-separated culture are added separately into a Ca-CO_3 containing solution for crystallization experiments. Results of XRD and SEM analysis indicate that calcite is the dominant mineral phase formed when the bacteria are present. When the supernatant alone is used, however, a significant portion of vaterite is also precipitated. Experimental results further reveal that the bacteria have a strong tendency to colonize the center area of the calcite $\{1\,0\,\bar{1}\,4\}$ faces. Observed crystal morphology suggests that the bacterial colony may promote the growth normal to each individual $\{1\,0\,\bar{1}\,4\}$ face of calcite when the cell concentration is high, but may retard it or even cause dissolution of the immediate substrate surfaces when the concentration is low. SEM images taken at earlier stages of the crystallization experiments demonstrate the nucleation of calcite on the bacterial cell walls but do not show obvious morphological changes on the nanometer- to submicron-sized nuclei. $\delta^{13}C$ measurements unveil that the crystals grown in the presence of bacteria are further enriched in the heavy carbon isotope, implying that the bacterial metabolism may not be the carbon sources for the mineralization. Based upon these findings, we propose a mechanism for the *B. megaterium* mediated calcite mineralization and conclude that the whole process involves epi- and inter-cellular growth in the local microenvironments whose conditions may be controlled by cell sequestration and proton pumping during bacterial respiration.

1. Introduction

Microorganisms and microbially mediated mineralization processes are active in almost every environment on earth (see Ehrlich, 1990; Thompson et al., 1990; Westbroek, 1991; Chafetz and Buczynski, 1992; Mandernack et al., 1995; Banfield and Hamers, 1997; Shen et al., 2001; López-García et al., 2005) and possibly in extraterrestrial systems as well (McKay et al., 1996; Russell et al., 1999). For obvious reasons, the interplay of microbes and carbonate minerals at near-surface conditions is particularly extensive. Microbes from soils and aqueous media have been frequently reported to induce the precipitation of calcium carbonate mineral phases in both natural and laboratory settings. Because of this, microbial activity is regarded as an important player in the formation of carbonate sediments and soil carbonate deposits (Krumbein and Giele, 1979; Morita, 1980; Chafetz and Folk, 1984; Chafetz, 1986; Rivadeneyra et al., 1993, 1994, 1998; Peckmann et al., 1999). In marine environments, widespread carbonate precipitation can occur through biogenic processes that lead to the development of calcareous skeletons or protective shells in organisms such as foraminifera (Mann, 1988, 2001). Due to the significant roles marine microbes have played and continue to play in providing model systems for biomimetic materials and regulating global CO_2 levels, most studies on carbonate biomineralization in

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the past two decades have been focused on understanding the development of calcification in marine species (Novitsky, 1981; Weiner, 1986; Lowenstam and Weiner, 1989; Vincent, 1990; Mann, 2001).

The significance of microbially mediated carbonate precipitation in terrestrial environments may be equally as pronounced. According to Boquet et al. (1973), calcite formation by soil bacteria may be a general phenomenon. As such, the study of carbonate precipitation in soils may bear an even greater importance as it explores the terrestrial carbon sink through deciphering the pedogenic "trapping" of CO_2 in the form of authigenic carbonate (Lal et al., 1999), in addition to expanding our knowledge basis in soil formation. Although traditional views often ascribed the pedogenic carbonate mineralization to inorganic processes (Gile et al., 1966; Rabenhorst et al., 1991), more and more researchers (Monger et al., 1991; Bruand and Duval, 1999; Chen et al., 2005) are now depicting a strong involvement of microbial activities and leaning toward biogenic origins for these minerals.

A good many studies have investigated carbonate mineralization induced by microbes (see a recent review by Wright and Oren, 2005), including that by soil bacteria (Monger et al., 1991; Stocks-Fischer et al., 1999; Rivadeneyra et al., 1997, 2000; Cacchio et al., 2003). However, the precise roles of bacteria and bacterial activities in the process of carbonate crystallization remain largely elusive but seem to fall categorically into three different yet related tracks. First, mineralization occurs as a by-product of microbial metabolism (Knorre and Krumbein, 2000) that involves either autotrophic pathways inducing local CO_2 depletion or heterotrophic pathways leading to an increase of CO_3^{2-} in the media (Castanier et al., 1999, 2000). In these passive routes, reactions such as enzymatic hydrolysis of urea, dissimilatory reduction of NO_3^- and SO_4^{3-}, and ammonification of amino acids cause a pH rise that, in turn, shifts the biocarbonate–carbonate equilibrium to produce more CO_3^{2-} and, ultimately, to precipitate $CaCO_3$ if free Ca^{2+} is present. An alternative hypothesis proposes that the $CaCO_3$ precipitation is controlled by intracellular calcium metabolism (Anderson et al., 1992; McConnaughey and Whelan, 1997; Hammes and erstraete, 2002), instead of by changing CO_3^{2-} concentration. Second, carbonate nucleation takes place on cell walls due to ion exchange through the cell membrane (i.e. an active process, Castanier et al., 2000) following some still poorly known mechanisms (Greenfield, 1963; Del Moral et al., 1987; Rivadeneyra et al., 1994; Castanier et al., 1999, 2000), or due to promotion by the negatively charged specific cell wall functional groups that adsorb divalent cations such as Ca^{2+} (Schultze-Lam et al., 1996; Rivadeneyra et al., 1998). The third possibility involves the extracellular macromolecules (Braissant et al., 2003) because these biopolymers are shown to be able to trap calcium ions or serve as growth modifiers to control crystallization. For example, it is suggested that cyanobacteria's extracellular polymeric secretions may influence the polymorphic development of $CaCO_3$ during mineralization (Kawaguchi and Decho, 2002). Collectively, current understanding appears to agree upon the viewpoint that bacterially mediated carbonate crystallization is not regulated genetically and the resultant minerals do not have specific biological functions, suggesting that induced biomineralization (Lowenstam, 1981; Mann, 2001) is still in dominant play herein. However, the existence of multiple possible conduits for biological involvement highlights the high level complexity of the biomineralization process in question.

Another unsolved issue regarding bacterially mediated carbonate biomineralization concerns the occurrence of the wide variety of crystal morphology. Although abiotic calcium carbonates are known to have a plethora of crystal habits governed by environmental conditions, kinetics, as well as the presence of impurities such as Mg (Folk, 1974; Given and Wilkinson, 1985; Chafetz et al., 1991), most biomineralic carbonates exhibit highly unique morphologies not commonly seen on their inorganic counterparts. For marine organisms, it is generally understood that the distinct morphologies are produced genetically to serve specific biological functions such as structural supports, lenses, and gravity sensors (see Mann, 2001). For bacteria, however, the variety in crystal morphology and the reasons for it are not so clearly known. Buczynski and Chafetz (1991), after studying the calcite formed in the presence of two carbonate forming bacteria, documented that the morphology of single crystals tend to be dominated by a brush shape, but that of the bundled ones varies between two end habits of rod and sphere with dumbbell the most common middle form. Whereas the authors in this report did not offer explanations for their observations, a more recent study (Braissant et al., 2003) proposed that morphological transition may be related to the types of amino acids as well as the quantity of extracellular polymeric substances (EPS) in the growth media. The latter study, in addition, also suggested the importance of bacterial outer structure compositions in inducing the morphological variations.

In this study, we investigate how the bacterium *Bacillus megaterium* isolated from a loess profile in China mediates carbonate mineralization. Although found in a variety of environments, *B. megaterium* is generally considered a soil microbe (Vary, 1994) and has been shown to precipitate carbonate minerals (Cacchio et al., 2003). The loess–paleosol sequences in the Loess Plateau of China are regarded as one of the best continental archives of paleoclimatic and paleoenvironmental records in the Late Cenozoic era. Carbonate minerals, chiefly calcite of both detrital and pedogenic in origin, are seen throughout the sequences and contribute approximately 7–30% to the overall mineralogical composition. A recent TEM study (Chen et al., 2005) on the morphologies and compositions of the nano-calcite crystals found in the loess–paleosol sequences suggested that the mineral might be biogenic in origin. Together, these understandings indicate that it is broadly significant to decipher how microbes mediate carbonate

biomineralization in the loess. Currently little is known about the roles bacteria may have played in the formation of pedogenic loess carbonate. Here, we collected fresh and undisturbed soil samples from which the *B. megaterium* was isolated. We then conducted carbonate mineralization experiments in the presence of the bacteria and their metabolic products. Our goal is to assess the effect of *B. megaterium* on carbonate mineralization and eventually to gain a greater understanding of the formation of pedogenic carbonates in loess.

2. Experimental procedures

2.1. Bacteria cultivation and identification

Thirty bacterial isolates were obtained from the soil samples of the loess layers in a loess–paleosol sequence in Gansu Province located in northwestern China, and nine dominant ones were cultivated for crystallization essay experiments to test the response of carbonate minerals to the bacterial presence. The strain DZ011 was chosen for further study because (1) it grew quickly and produced plentiful slime around the cells and (2) the crystallization behavior of carbonates in the presence of this strain (i.e. delayed crystallization and higher counts of non-rhombohedral crystal morphology) differed substantially from that in the control experiments. The selected strain was used to inoculate a sterilized (20 min in autoclave at 121 °C) liquid culture made by mixing beef extract (3 g), proteose peptone (10 g), and NaCl (5 g) into 1000 ml of distilled water. The final pH of the culture was around 7.0–7.2. Upon inoculation, the culture was incubated for 36 h at 37 °C (optimal growth temperature for DZ011 determined from preliminary experiments) in a rotating shaker at a rotation rate of 160 rpm. The final reading in the optical density (OD) of the culture was around 1.2, indicating the late exponential phase of cell growth.

The bacterial genomic DNA of the strain DZ011 was extracted using a commercially available extraction kit (Genomic DNA Purification Kit 713, Shenergy Biocolor Biotech Ltd., Shanghai). The PCR (polymerase chain reaction) amplification of the 16S rDNA was carried out using a forward primer (5′-AGAGTTTGATCCTGGTCAGAA C-3′) and a reverse primer (5′-TACGGCTACCTTGTTA CGACTT-3′) by a thermal cycler (MJ Research PTC-100). The PCR conditions are: pre-denaturation, 94 °C for 5 min; denaturation, 95 °C for 1 min; annealing, 55 °C for 1 min; extension: 72 °C for 2 min except the last cycle that had 5 min. A total of 30 cycles were completed for the PCR amplification. The PCR products were purified and sequenced commercially. The resultant sequence was submitted to the GenBank at www.ncbi.nlm.nih.gov (Accession No. DQ408589) and analyzed following the method given by Altschul et al. (1997). A positive identification of the strain DZ011 was given in *B. megaterium* (endospore-forming aerobe or facultative anaerobe). A Gram stain test confirmed that the organism was Gram positive. TEM (Hitachi H600A-II) images of the individual cells showed a clear rod-shaped morphology with a dimension of approximately 2–3 × 0.5–1 μm (Fig. 1).

Fig. 1. TEM image of individual cells of *Bacillus megaterium*.

2.1.1. Crystal growth and analysis

At the end of the incubation period, a portion of the final liquid culture containing *B. megaterium* was centrifuged at 10,000 rpm for 15 min to concentrate the bacterial cells. The resultant supernatant was separated and further filtered using a 0.22 μm filter paper to eliminate any remaining scraps of broken cell membranes. The remaining un-centrifuged liquid culture, the filtered supernatant, and the concentrated bacteria sludge were then stored in a refrigerator at 4–5 °C for future use.

Growth solutions for crystallization experiments were made by adding one of the three above made bacterial cultivation products to a calcium and carbonate containing inorganic salt solution. The salt solution was prepared by mixing equal amounts (2×10^{-3} M) of reagent grade $NaHCO_3$ and $Ca(NO_3)_2{\cdot}4H_2O$ into distilled deionized water, subsequently acidified by using HCl to adjust the initial pH to approximately 3. This solution is undersaturated with respect to any calcium carbonate minerals with a maximum saturation index (defined as the ratio of ionic activity product to solubility product) to be less than 10^{-7} (calculated using MINTEQ, Allison et al., 1991). Three growth solutions, each containing 25 ml of the salt solution and 1.5 ml of a biological additive, were made and named Super (for containing the supernatant), Mix (for containing the unseparated liquid culture), and Bac (for containing the concentrated bacteria sludge), respectively.

Crystal growth experiments were conducted using an ammonium carbonate free-drift method (see Jiménez-López et al., 2001; Becker et al., 2003). The general setup of the reaction chamber is shown in Fig. 2. Crystallization reactions take place in a closed container, which in this study is a 10-liter desiccator. A permeable horizontal separator divides the internal space of the container into two compartments. Ammonia diffuses from the lower section into the upper space where the reaction occurs. In this study, a number of Petri dishes containing different experimental solutions were placed on the separator. Each experimental run was accompanied by a control experiment that used only the salt solution. Three experimental runs, each containing two to three repeats (i.e. Petri dishes), were performed for every biological additive. The final pH values of the growth solutions were approximately 8.0–8.5. All experimental procedures were performed under a laminar flow hood to avoid contamination from air-born particles.

We first conducted a series of testing runs to determine the optimal conditions for producing the largest possible crystals. We did so by varying the exposure and the amount of $(NH_4)_2CO_3$ used while monitoring the crystals formed in the Petri dishes using optical microscopy (OM) periodically up to 7 days. We found that ~10 g of ammonium bicarbonate powder stored in two 50 ml open beakers sitting on the bottom of the desiccator produced the best results. In the experimental runs, we placed a number of $\sim 0.5 \times 0.5$ cm glass covers in each Petri dish. One glass cover was taken out for OM and Scanning Electron Microscopy (SEM, Leo, 1530VP and Joe, JSM-5610LV) examination every 2 h in the first 10-h period and every 8 h after that up to 4 days. This procedure was repeated until we saw no further changes in crystal sizes and morphologies. In the end, the crystals precipitated on the bottom of each Petri dish were scraped off using a blade and collected for MS (Mass Spectrometry, ThermoFinnigan, MAT 253) and XRD (X-ray Diffractometry, Bede, D1) analysis to determine the isotopic and polymorphic composition.

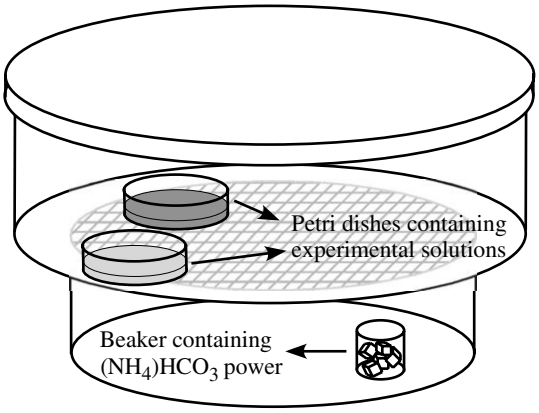

Fig. 2. Schematics of the experimental setup.

3. Experimental results

Visible crystals were detected by optical microscopy within 6–8 h in the control experiments and in the Super solutions, but took up to 22–24 h to be seen when the bacteria were present. The optimal sizes of the crystals formed in the experimental solutions were about 50–80 μm in diameter, which contrasted to the size of approximately 30 μm in the control experiments. As expected, the crystals formed in the control experiments (Fig. 3) were exclusively calcite as indicated by the polyhedron morphology and confirmed by the XRD analysis (not shown here). The XRD results further revealed that calcite was the dominant polymorph in the two bacteria-bearing solutions Mix and Bac; however in the Super experiments where the growth solutions contained only the metabolic products of the *B. megaterium*, a significant amount of vaterite was found along with calcite (Fig. 4). Furthermore, the crystal morphology and texture differed in each of the three types of experimental solutions. It appears that, in reference to the cleavage rhombohedron (i.e. the $\{1\,0\,\bar{1}\,4\}$ form) commonly observed in the control experiments, the morphological and textural changes became more and more prominent with an increasing presence of bacteria; that is, the least changes were induced by the addition of the supernatant, the most by the concentrated bacterial sludge, and an intermediate amount by of the unseparated culture.

3.1. Crystals formed in super solutions

The calcite crystals seen in the solutions containing the supernatant largely maintained the same rhombohedral morphology as those formed in the control experiments. What was unique was that some calcite grains appeared as twins (Fig. 5-A1) or even polycrystals (Fig. 5-A2). Moreover, the corner and edges connecting the positive faces [i.e. $\{1\,0\,\bar{1}\,4\}$, $\{\bar{1}\,1\,0\,4\}$, and $\{0\,\bar{1}\,1\,4\}$] and the negative faces [i.e. $\{0\,\bar{1}\,1\,4\}$] and the negative faces [i.e. $\{0\,1\,\bar{1}\,\bar{4}\}$, $\{\bar{1}\,0\,1\,\bar{4}\}$, and $\{1\,\bar{1}\,0\,\bar{4}\}$] of these crystals were roughened and often rounded, indicating an attempted development of the prismatic faces. In addition, higher magnification of SEM images showed that the individual facets were not smooth and often composed of coarse steps (Fig. 5-B1 and B2).

A more interesting observation in the Super solutions was the formation of spherulitic vaterite (Fig. 6). SEM and OM examination showed that the amount of vaterite in three separate experiments ranged from ~10% to ~60%. At first glance, these vaterite grains, seemingly containing a hollow core, appeared to be made of co-axially spun strings (Fig. 6-A1, B1, C, and D). However, higher magnification of the core region (Fig. 6-A2 and B2) revealed that the sperulites were actually formed by oriented growth of individual leaves around the void core. The spheroids seem to be composed of six cloves and hence have a 6-fold axis (Fig. 6-C and D). Most of the time, the vaterite spherulites were larger than the coexisting calcite rhombohedron (Fig. 6-C).

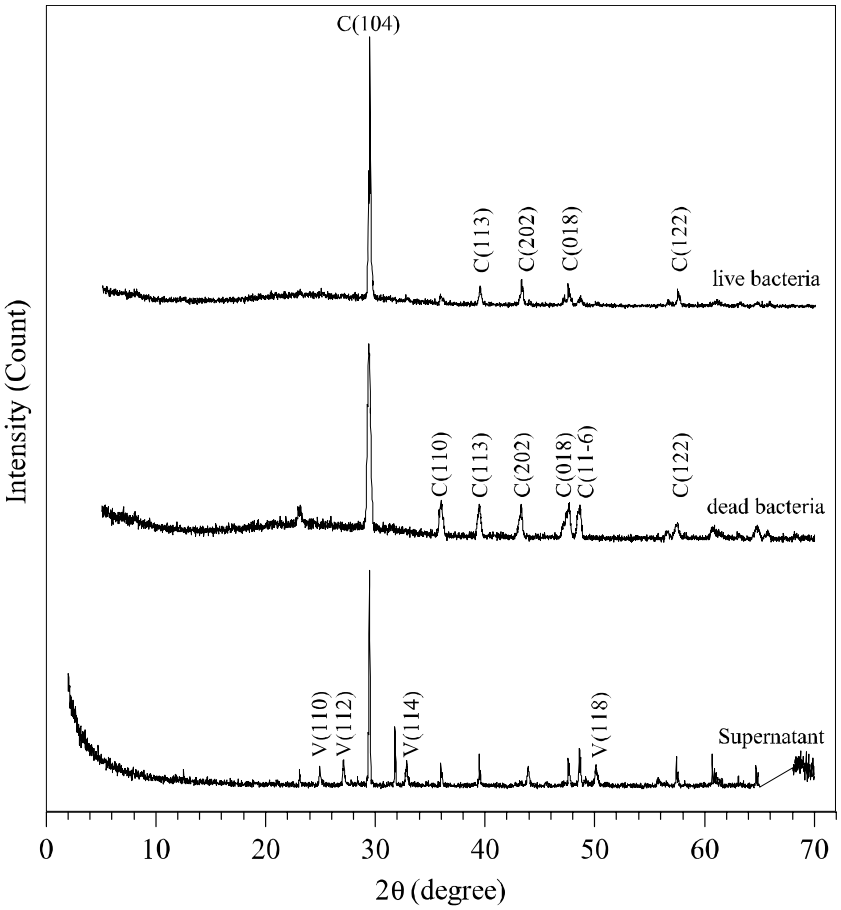

Fig. 3. XRD measurements of the crystals harvested from growth solutions containing *B. megaterium* or its metabolic products. Numbers in the parentheses indicate the Miller indices, whereas C and V denote calcite and vaterite.

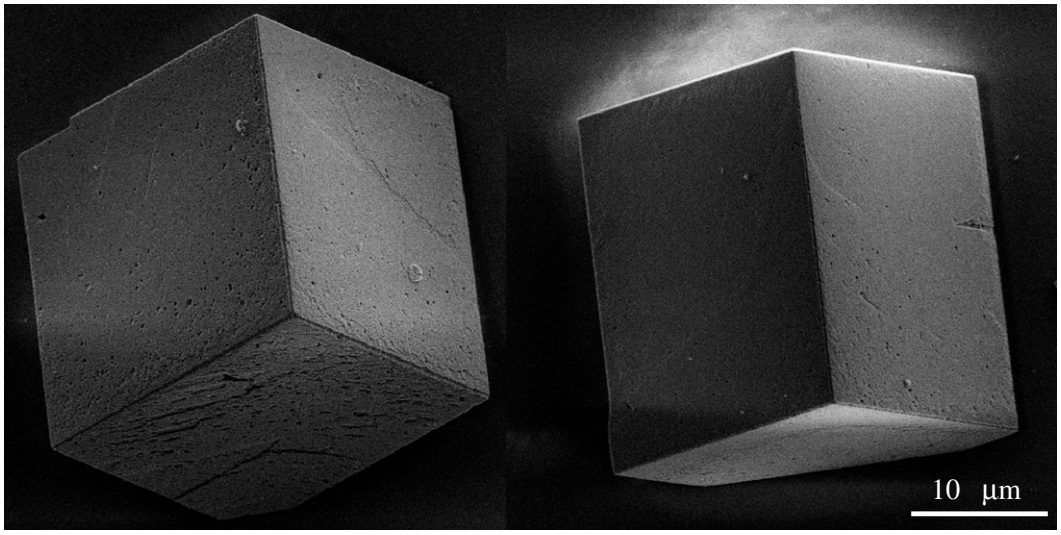

Fig. 4. Polyhedral calcite crystals grown in the control experiments. Shown here are two grains in the $\{1\,0\,\bar{1}\,4\}$ rhombohedral form.

3.2. Crystals formed in mix solutions

The mineral found in the growth solutions that contained the unseparated culture was predominantly calcite (Fig. 3) with well-maintained rhombohedral morphology. Compared to what was found in the supernatant solutions, these calcite crystals did not show obvious signs of the development of the prismatic faces. Nonetheless, these crystals were unique in the sense that the individual grains were often decorated by large holes that grew in the center of each $\{1\,0\,\bar{1}\,4\}$ face (Fig. 7), in addition to the rougher and more porous overall appearance of the crystal surfaces. It was common

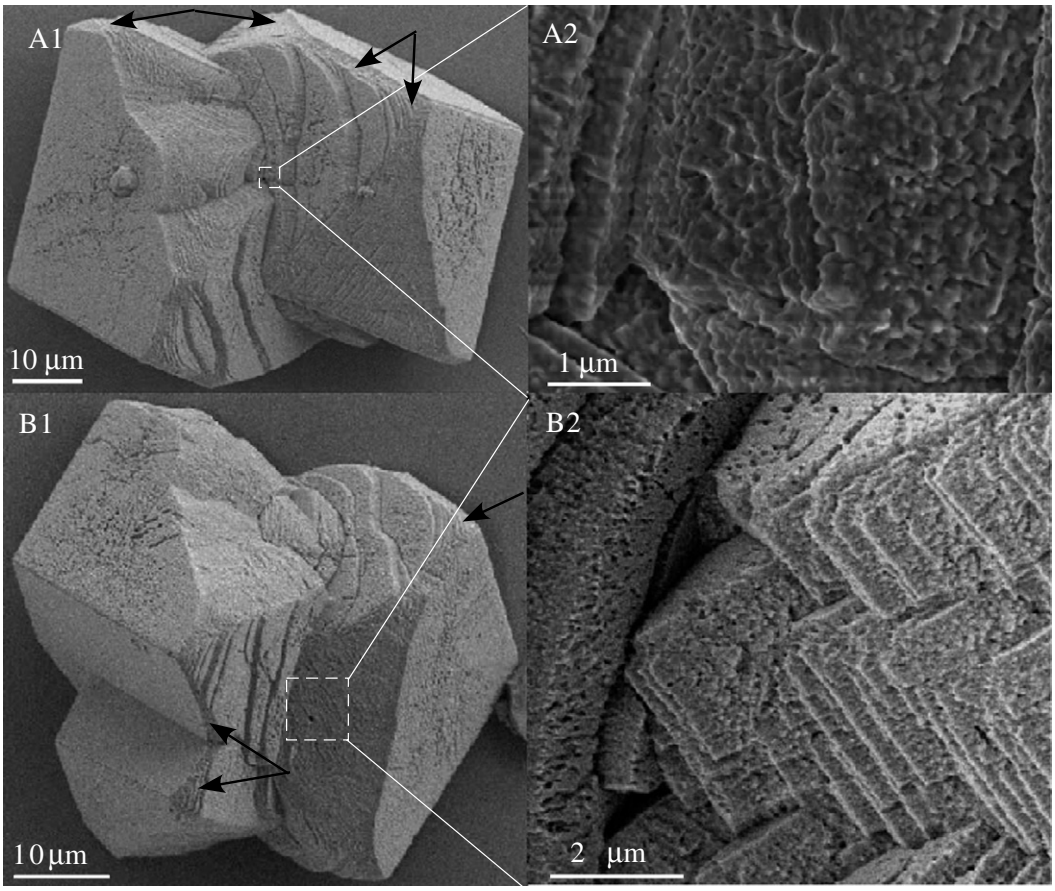

Fig. 5. Twin- (A1) and poly-crystals (B1) of calcite found in the Super experiments. Close-up views (A2 and B2) show the roughness of the individual facets. Notice the rounded edges and corners such as those denoted by the black arrows on the crystals.

to see the association of bacterial colony with the central depression on the crystal faces (Fig. 8). On well-developed rhombohedral calcite grains such as those shown in Fig. 7 (A1 and B1), the corners appeared to be over-extended, as indicated by the curved edges, resembling the skeletal crystals seen by Chafetz et al. (1991, and references therein) and suggesting that the corners grew at a higher rate.

3.3. Crystals formed in Bac solutions

Finally, when a high concentration of bacterial cells was used, significant changes in shape and texture were observed on the calcite crystals formed wherein. First, the rhombohedral morphology seemed no longer in control, and the overall appearance of the crystals may be described as resembling that of a three-dimensional cross (Fig. 9-A, B, C1, and D1). It looked that the 3D crosses were formed as if the six faces on the rhombohedral nuclei began to extend solely in the direction normal to each surface. In most cases, the cross was largely symmetric, indicating that the rates of extension in different directions were comparable to each other. Second, in contrast to the crystals with relatively dense, featureless textures seen in the Super and Mix experiments, the calcite formed in the Bac solutions showed a clear development of textural characteristics. This was marked by a fibrous pattern parallel to the extension. Because of this texture, the terminal faces perpendicular to the extension directions looked extremely porous. Individual cells of the bacterium and sometimes amalgamation of seemingly dead cells could be seen on the crystals, particularly on the fibrous surfaces (Fig. 9-C2, D2, and E2). In a few occasions, individual faces on the crystals were covered by an aggregate of large quantity of bacterial cells that resembled a bio-film (Fig. 10).

4. Discussion

Given the unique morphology and texture of the calcite crystals as well as the formation of vaterite observed in our experiments, it is safe to conclude that *B. megaterium* strongly affects carbonate crystallization. This understanding alone is of little surprise because a variety of bacteria have been shown to have the ability to mediate or control carbonate mineral formation (e.g. Rivadeneyra et al., 1991, 1993, 1994, 1998; Douglas and Beveridge, 1998; Peckmann et al., 1999; Warren et al., 2001; Cacchio et al., 2003; Hammes et al., 2003). However, the results from the current study point to several important issues that may shed light on the routes through which bacteria exert effect on biomineralization.

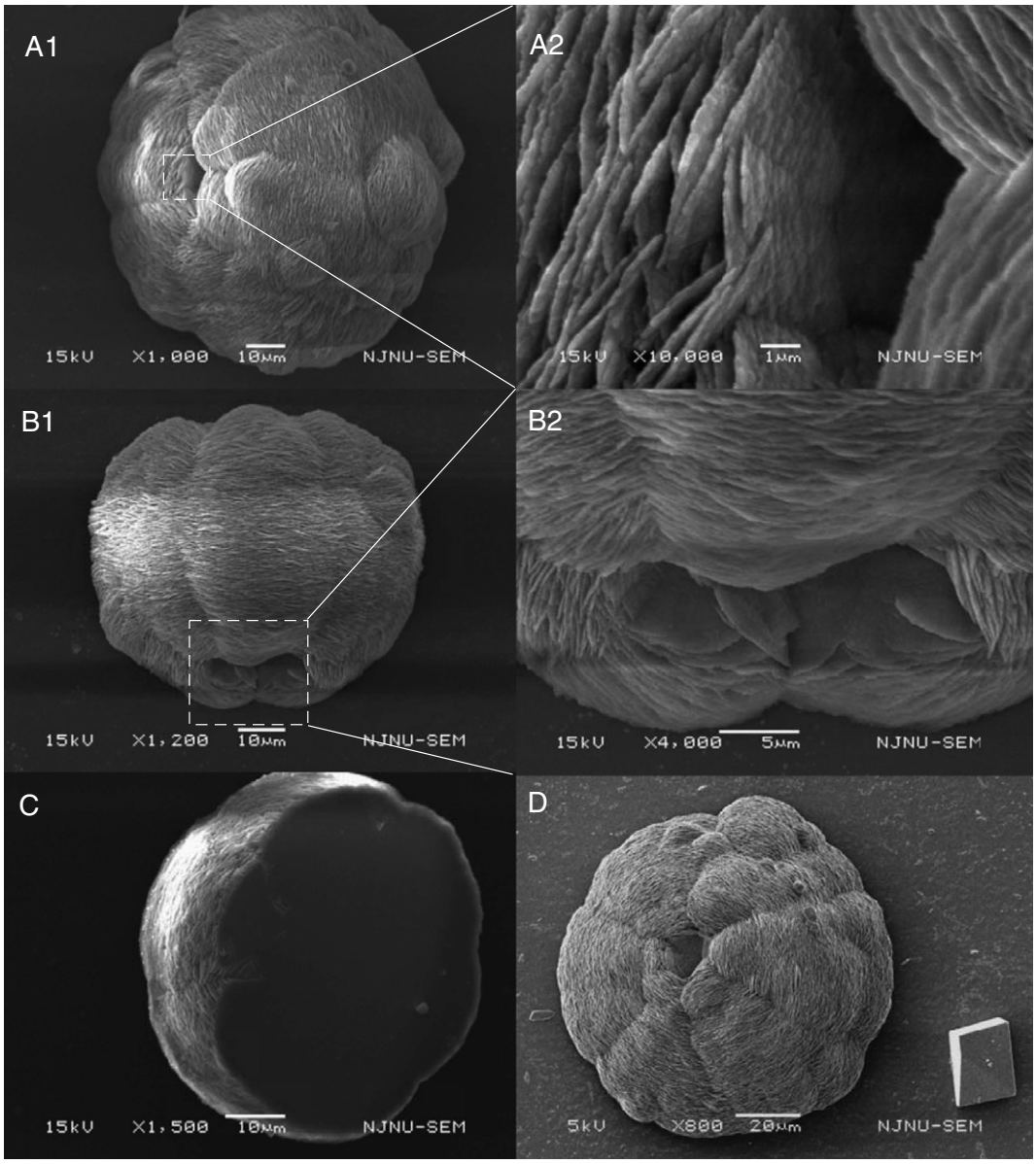

Fig. 6. Spherulitical vaterite grains (A1, B1, C, and D) and their cores (A2 and B2) seen in the Super experiments. Notice the 6-fold symmetry on the spheroid and the relatively larger size of vaterite in comparison to calcite (D).

4.1. Bacteria metabolism and carbon source for crystallization

Our experimental observations show that the "apparent" induction time for crystallization (the time needed to detect crystals by optical microscopy) is significantly longer in the presence of *B. megaterium* (22–24 *vs.* 6–8 h when the bacteria are absent). Because the initial conditions of all experimental solutions, including those for the control experiments, are very similar, it is reasonable to suggest that the presence of bacteria is responsible for the discrepancy. Possible explanations for the delayed crystallization may be (1) minerals form as a direct product of bacterial metabolism rather than solution chemistry change, and (2) bacterial metabolic processes or products affect the solution chemistry in the course of the experiments.

To further understand the relationship between microbial activities and carbonate crystallization, we measured the ^{13}C isotopic compositions of the crystals grown in different solutions in three experimental runs (Table 1). The data acquired from runs I and II are very consistent with each other and exhibit a clear trend of light isotope depletion in the crystals formed in the presence of bacteria. The data from run III, however, are more ambiguous and deviate somewhat from those in runs I and II with no obvious reasons. Nonetheless, all measurements seem in agreement with each other in that (1) as expected, the experimentally grown crystals are enriched in ^{13}C in comparison to the reagent $NaHCO_3$ used in this study; (2) there is a further enrichment of heavy isotope in the crystals harvested from the experimental solutions relative to those from the control experiments; and (3) the largest

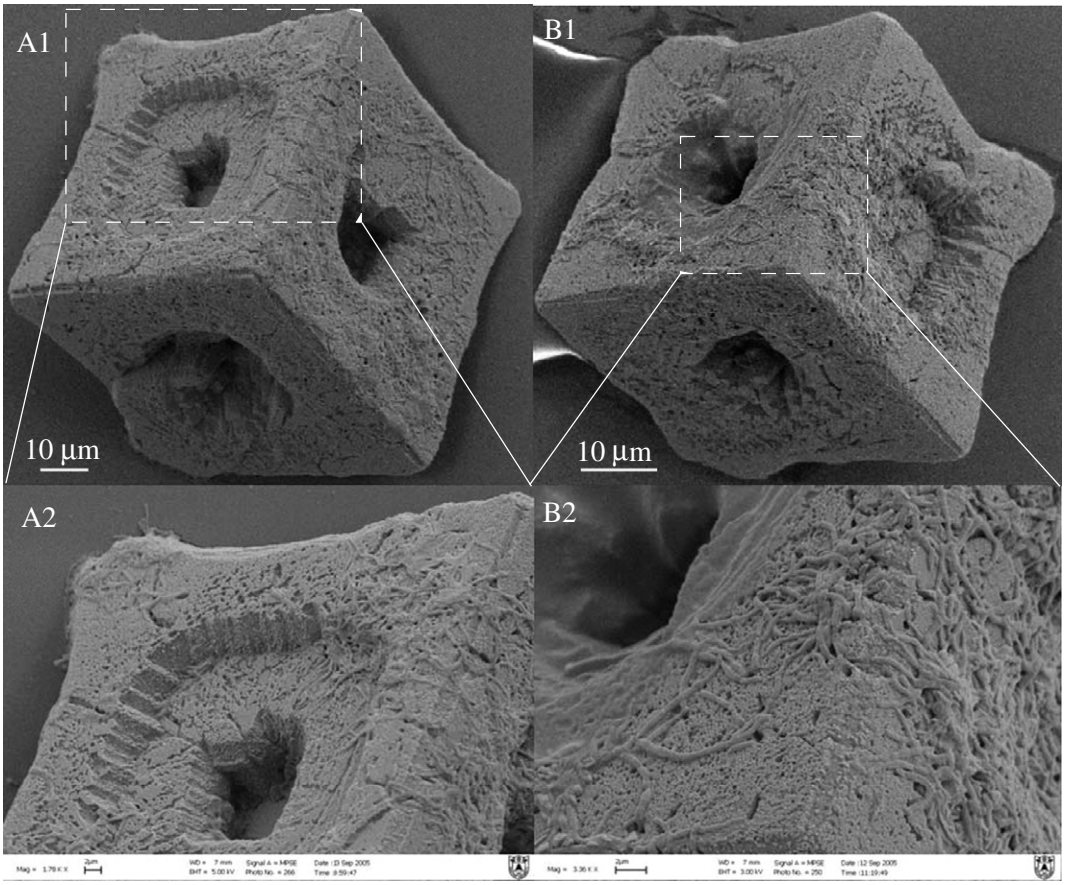

Fig. 7. Rhombohedral calcite grains (A1 and B1) grown in the Mix experiments. Close-up views (A2 and B2) reveal the presence of bacterial cells on the crystal surfaces.

depletion of the light isotope occurs when the concentration of the bacteria is the highest. Given the complexity of microbial participation and our lack of understanding in the biomineralization processes, one may not be able to pinpoint the cause of the bacterially related ^{13}C enrichment. However, it appears that the significance of these results is 2-fold. First, they suggest that, in all likelihood, the carbons for carbonate crystallization were not derived from the metabolic pathways. This is because metabolic processes usually have a preference for selecting light isotopes. If the metabolism provided C for the crystallization, the resultant crystals should have more negative $\delta^{13}C$ values, contradicting the experimental results. Note that one cannot completely exclude the direct involvement of metabolic activities in the mineralization process all together based upon these isotope results because the organism can still control the mineral formation through intracellular calcium metabolism, as suggested by previous studies (Anderson et al., 1992; McConnaughey and Whelan, 1997; Hammes and erstraete, 2002). Second, they seem to imply that the bacteria may have respired on the inorganic carbon in the growth solutions to result in reduced carbonate concentrations and an enrichment of ^{13}C. Most *Bacillus* species, as versatile chemoheterotrophs as they are, need a variety of simple organic compounds (sugars, amino acids and organic acids) for respiration. Some species may be able to use more complex biological polymers such as lipids, polysaccharides, and nucleic acids because these organisms produce a range of extracellular hydrolytic enzymes that can degrade large organic molecules. We, however, did not find any literature reports directly documenting an instance of *Bacillus* living on inorganic C species such as HCO_3^- and CO_3^{2-}. On the other hand, *B. megaterium* may be unique in that, according to *Bergey's manual of systematic bacteriology*, it can multiply without organic growth factors on ammonium salts or with nitrate and glucose as sole sources of N and C (Sneath, 1986). Thus, we speculate that, given the special condition that there was an abundant presence of ammonium ions in the growth solutions, the particular strain of *B. megaterium* used here lived on the carbon-bearing species HCO_3^- and CO_3^{2-} in this study. In doing so, the bacteria's respiration activities reduced the concentrations of C-containing aqueous species while leading to a further enrichment of ^{13}C in the solution. This explains why the "apparent" nucleation induction time was longer in the presence of the bacteria: it took more time to produce CO_3^{2-} by NH_3 shifting the equilibrium $HCO_3^- \Longleftrightarrow CO_3^{2-} + H^+$. This also implies that the crystals formed in the bacteria-containing solution should be enriched in ^{13}C because the bacteria preferentially consumed the light isotope.

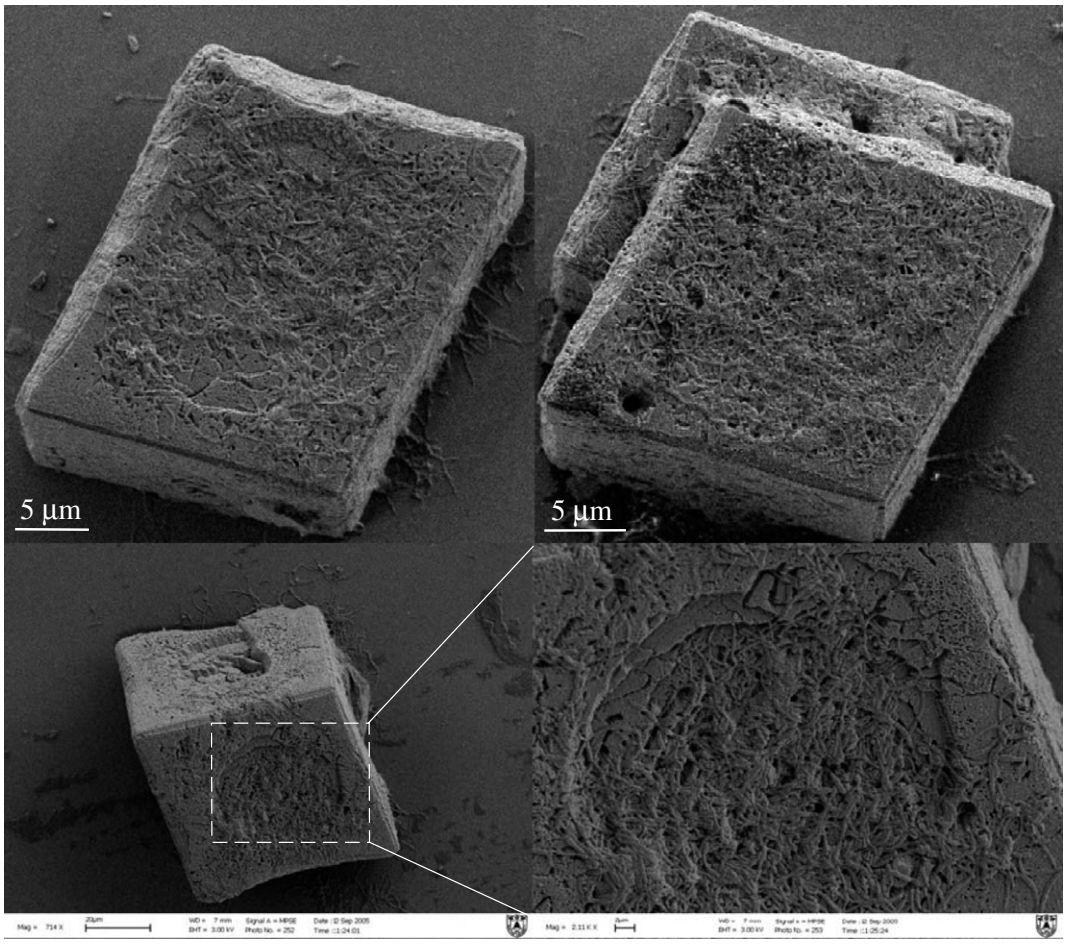

Fig. 8. Bacterial colonies found on the {1 0 $\bar{1}$ 4} faces of calcite in the Mix experiments. Notice the development of depression in the center area of the crystal surfaces and the center-bound tendency of the colonization.

It is possible that the bacteria lived on the residual organic carbons from the culture medium because we did not rinse the cells prior to the crystallization experiments. In this case, the respiration process can produce organic acids to counterbalance the pH increase associated with ammonia diffusion and, hence, delay the crystallization. However, it is not immediately clear how this scenario can lead to the enrichment of heavy C isotope in the crystals.

An interesting question arising from the observed enrichment of ^{13}C in the crystal phases is whether a depletion of light isotope can serve as a signature of past life presence, as an enrichment of light isotope does. Because mineralization associated with low life, particularly with bacteria, is predominantly *induced* biomineralization (see Mann, 2001), the minerals produced thereby reflect the environmental changes, including isotopic fractionation, that result from life's activities but not the biological process itself. Hence, an accumulation of biologically unwanted substances in the environments, as in the case of heavy isotope enrichment in the calcite in our experiments, should indicate the occurrence of biological activities.

4.2. The occurrence of bacteria–crystal interactions

Crystallization is conventionally divided into two phases, namely, nucleation and post-nucleation growth. As such, any external influence on crystal morphology can take place at either or both stages. Whereas oriented growth on Langmuir films (see Berman et al., 1995; Archibald et al., 1996) is an example of biological or organic controls during nucleation, the morphological changes in the presence of additives such as amino acids (see Mann et al., 1990) and the spatial control resultant from crystallization within confined boundaries (i.e. supramolecular preorganization, see Mann, 2001) are due largely to organic–inorganic interactions during growth. For bacteria-mediated mineralization, a question that rises naturally is at which stage bacteria–mineral interactions play the critical role in controlling the overall crystallization processes. The results presented herein seem to suggest that the bacterial effect takes place predominantly at the post-nucleation growth stage, at least for *B. megaterium*. This conclusion is arrived on the basis of two observations. First, the crystals with a three-dimensional cross shape seen in the Bac solutions appear to have a core upon which six limbs extend

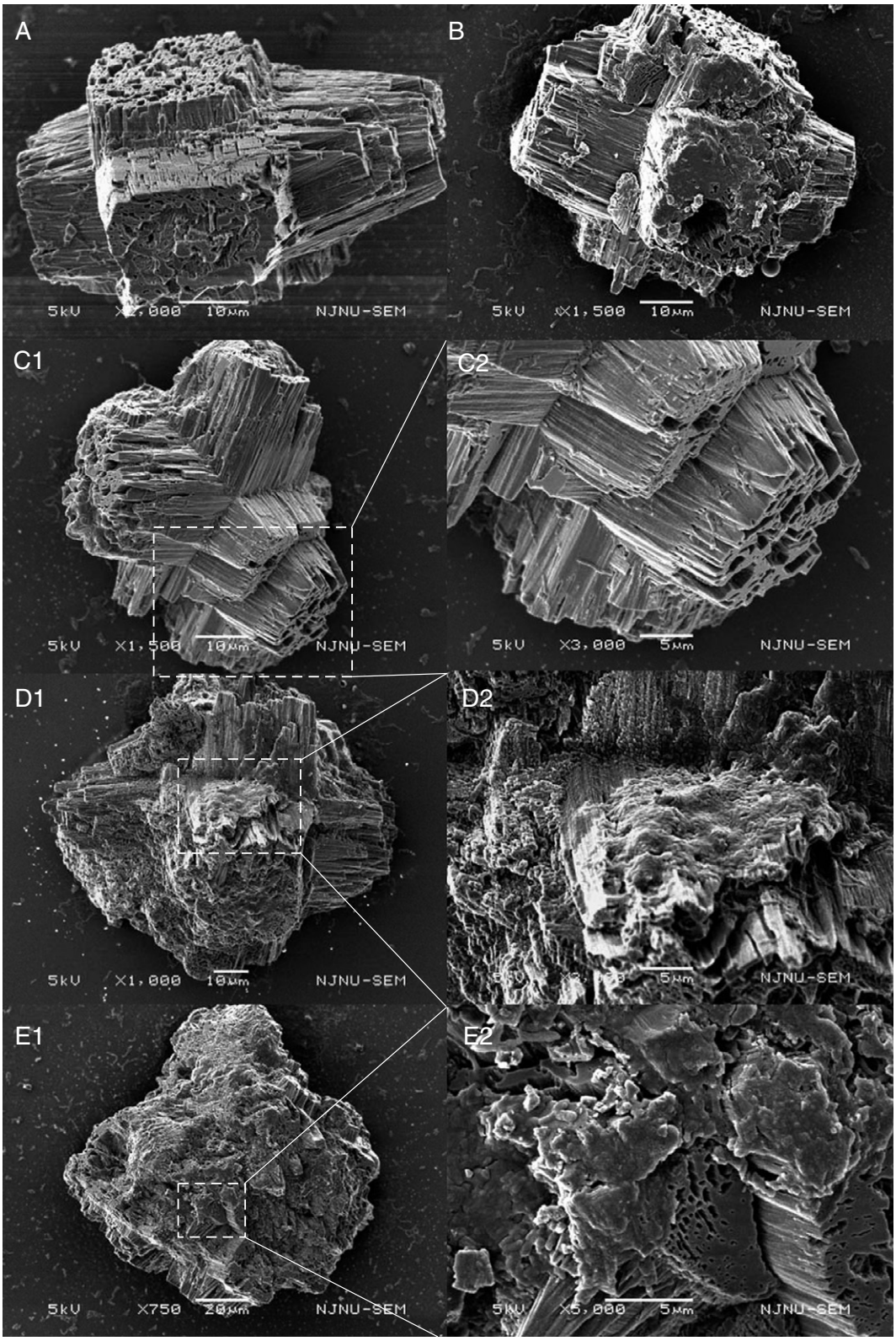

Fig. 9. Calcite grains seen in the Bac experiments showing the three dimensional cross- shaped crystal morphology (A, B, C1, and D1) and the locations of the bacterial growth (C1, D1, E1, and E2). Notice the porous and fibrous textures of the crystals.

vertically. The profile view (Fig. 11-A) of the crystal shows that the core and the extensions have different textures. While the extensions are fibrous and porous without the appearance of single crystals, the core seems to maintain the rhombohedral morphology of calcite with no significant textural indications for biological effects. Second,

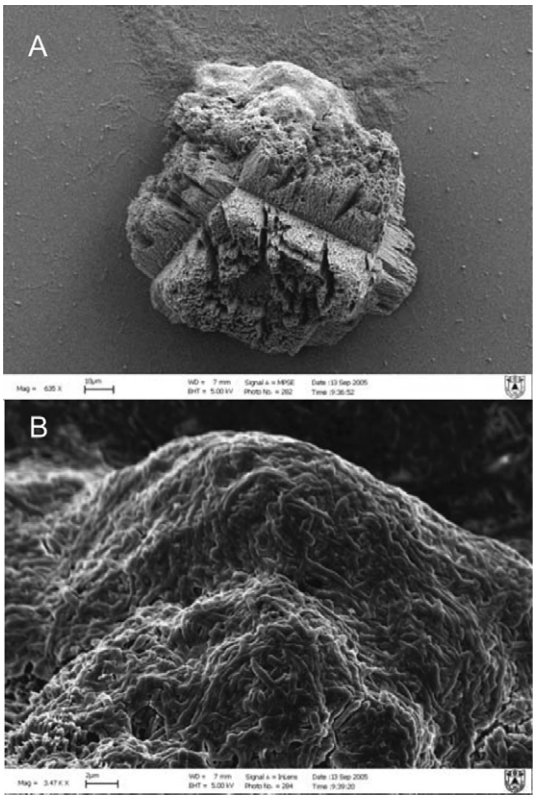

Fig. 10. Bacterial colony (A) and its blow-up view (B) on a calcite grain grown in the Mix experiments.

Table 1
Measurements of ^{13}C contents in the crystals

	$\delta^{13}C$ (‰, PDB)		
	Run I	Run II	Run III
$NaHCO_3$ (reagent)	−17.09	−17.11	−17.12
Control	−11.80	−11.90	−17.08
Supper	−11.87	−11.67	−12.50
Mix	−10.32	−10.29	−14.28
Bac	−8.03	−8.12	−9.27

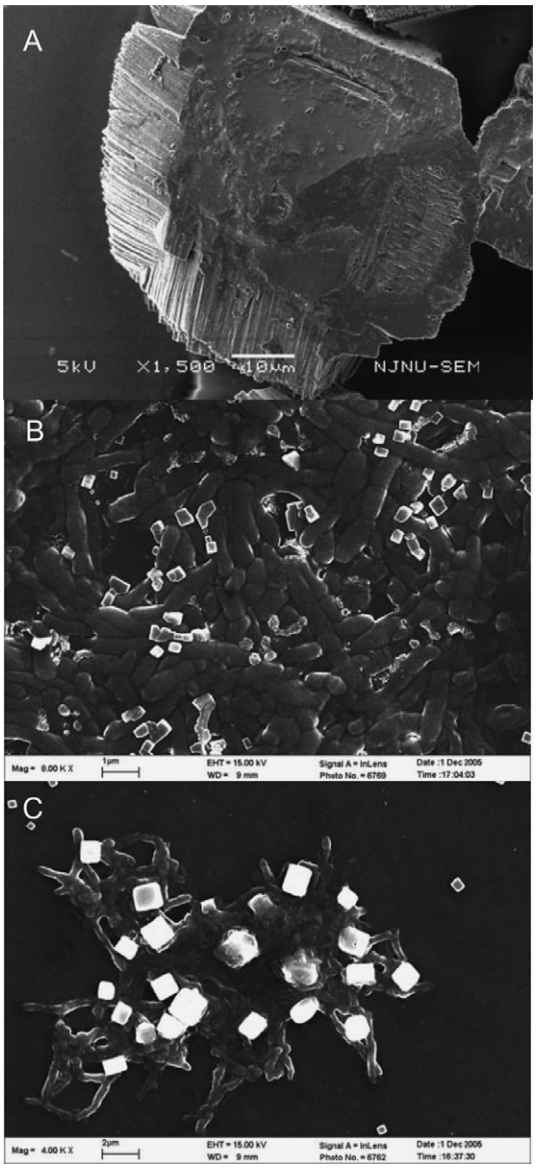

Fig. 11. SEM images showing the cross section of a calcite crystal found in the Bac experiments (A) and the rhombohedral morphology of the calcite nuclei formed 2–6 h in the growth experiments on (B) living and (C) dead bacterial cells. Note the textural difference between the core and the edge area in the crystal shown in (A).

despite the unique morphology of the crystals grown in the presence of *B. megaterium*, the nanometer to submicron nuclei formed on the bacterial cell walls at earlier stages (2–6 h after the addition of the bacteria) of the experiments consist of predominantly simple rhombohedral calcite (Fig. 11-B). These observations suggest that the bacteria-crystal interactions, particularly the biological impact on crystal morphology, may be fairly limited at the early stage of the mineralization process but become more pronounced when the crystals are sizable.

One possible explanation for this delayed bacterial action may be that it takes time for the bacteria to colonize growing surfaces. Even if the nucleation takes place on the cells, as suggested by many previous studies (see Schultze-Lam et al., 1996) and shown by Fig. 11-B, the bacterial effect in the beginning may be limited only to the crystal surfaces in contact with the substrate cell walls. This is because it is less likely for newly growing faces on the nuclei to be immediately adjacent to any bacterial cells during crystallization. Consequentially, the bacteria-crystal interactions are less prominent for small crystals. During the continuous growth of the nuclei, the bacteria can gradually establish close contact with these new crystal faces by developing colonies on them.

The argument of bacterial colony formation on crystal faces seems to be consistent with the experimental observations. A common feature on the crystals formed in the presence of bacteria is the occurrence of larger holes (the Mix experiments, Figs. 7 and 8) and sub-micrometer pores (the Bac experiments, Fig. 9). It seems that such void spaces have been occupied by the bacteria at some time in the course of crystallization. Several SEM images show that the cells have either closely cuddled into the pores (Fig. 9-C2 and D2) or have completely colonized the holes

(Fig. 8). This may indicate the occurrence of a mutually beneficial relationship between the bacteria and the calcite: the crystals provide substrate surfaces for the bacteria to develop colonies, and the bacterial colonization either promotes or retards the further growth of the crystal faces.

4.3. Formation of vaterite in supernatant solutions

A surprising observation in the growth solutions that contain only the supernatant without a presence of bacterial cells is the formation of vaterite. Although non-calcite polymorphs aragonite (Rivadeneyra et al., 2000; Kawaguchi and Decho, 2002; Cacchio et al., 2003) and vaterite (Braissant et al., 2003; Hammes et al., 2003) are reported to form through microbial processes, calcite is by far the dominant carbonate phase related to bacterial activities (Stocks-Fischer et al., 1999). We do not have a quantitative explanation for the formation of vaterite seen in the present study. However, we hypothesize that this may be a kinetics related phenomenon. In the presence of bacteria, if the nucleation of calcite on the cell walls is kinetically favored over that of vaterite, the resultant crystals should be dominated by calcite, even if the solutions are supersaturated with respect to both minerals. The rhombohedral nuclei attached to the bacterial surfaces seen by SEM at earlier stages of crystallization experiments (Fig. 11-B and C) suggest that calcite may indeed be the preferred nucleation product on the cell surfaces of *B. megaterium*, living or dead. On the other hand, numerous previous studies (e.g. Agarwal et al., 2003; Lakshminarayanan et al., 2003) have demonstrated that vaterite can form in the presence of macro organic molecules such as PVA (poly vinyl-alcohol) and PAA (poly acrylic-acid) that are rich in hydroxyl and carboxyl groups. It is highly possible that hydroxyl and carboxyl rich compounds are available in the supernatant solutions because *B. megaterium* can produce plenty of EPS, which is known to contain compounds rich in hydroxyl and carboxyl functional groups (see Omoike and Chorover, 2004). When the bacteria are absent, calcite nucleation may be tardy due to a lack of substrate. In contrast, the formation of vaterite facilitated by the hydroxyl and carboxyl rich compounds may take place at a relatively faster pace. Such a reverse in nucleation kinetics between calcite and vaterite may ultimately lead to the formation of vaterite in the supernatant solutions. An interesting note is that enzymatic precipitation experiments performed using urea and $CaCl_2$ (Sondi and Matijevic, 2001) revealed the occurrence of vaterite with an amorphous precursor, and the vaterite quickly gave rise to calcite upon aging. The vaterite found in our experiments seems fairly stable, as the XRD measurements (Fig. 4) were performed days after we completed the crystallization experiments.

4.4. Proposed model for *B. megaterium* mediated calcite biomineralization

To sum up the experimental observations and the above discussion, we propose the following model for *B. megaterium* mediated calcite biomineralization. First, negatively charged functional groups on the bacterial cell walls attract Ca^{2+} to induce a local supersaturation so that calcite nucleation takes place on the cell surfaces. This is possible because, albeit different in the source of charge occurrence among different strains, bacterial cell walls are usually electronegative (Schultze-Lam et al., 1996). For Gram-positive bacteria, the electronegativity is derived primarily from the secondary polymers teichoic acids that contribute extra negative charges from carboxyls and phosphates (Hogg, 2005) to the existing peptidoglycan framework, and both groups bind strongly with Ca^{2-}. Although we did not rinse the cells prior to the crystallization experiments, Ca^{2-} adsorption on the cell surfaces may still be possible because any pre-sorbed ions (presumably from the culture media) should have been washed off once the cells were immersed into the experimental solutions. This stage may be best described as epicellular growth (Mann, 2001) in which the crystallographic controls dominate and the calcite crystals maintain the rhombohedral morphology. Second, bacterial colonization begins to develop on the newly grown crystal faces in addition to the ones in contact with the substrate cell surfaces. The development of bacterial colonization on these crystal faces now establishes two types of substrate for further growth: one is the colony itself in the center area and the other the remaining edge portions of the $\{1\,0\,\bar{1}\,4\}$ faces. The relative kinetics of the subsequent growth of the two regions will decide the ultimate morphology of the crystals. If the concentration of bacteria is high to result in a bacterial scaffold that bears a large area of cell walls, epicellular and intercellular growth originated from the colony will outpace the homo-epitaxial growth in the surrounding area. This happens because the local environment within the colony may have a higher saturation state than the bulk solutions. It was suggested that the aqueous microenvironments associated with microbes are different from those around larger organisms, and one of the differences is that ionic solutes tend to concentrate in the hydrodynamic boundary layers surrounding the bacterial cells due to bacterial sequestration (Thompson et al., 1990; Schultze-Lam et al., 1992, 1996). Faster growth of the colonized section leads to continuous colonization in the newly grown area that brings about further extension of the central portion of the $\{0\,\bar{1}\,4\}$ faces. However, owing to the colony's center-bound tendency, the growing protrusion becomes thinner and thinner in the course of growth, as demonstrated in Fig. 9-A and C1. Eventually the cytolysis after the death of the bacteria frees up the space occupied by the cell wall scaffold to reveal pores in the crystals. In the case of low bacterial concentration, it is not obvious as to why the vertical growth relinquishes its dominance. We hypothesize that the higher saturation resulting from cell sequestration in the microenvironments where bacterial population is low may not be enough to overcome the effect of protons pumped out from the cells during respiration. Urrutia et al. (1992) showed that bacteria such as *B. subtilis* pump

out protons through cell walls during respiration. These protons will presumably occupy the negatively charged cell surface sites to result in a lower capacity for cation binding, or lower the pH of the local environments; both of them are against further crystallization. Hence, the epicellular growth on the cell wall in this case may be disadvantageous compared to the homo-epitaxial growth in the area not colonized by the bacteria. As a result, faster growth will take place around the edges and corners of the $\{1\,0\,\bar{1}\,4\}$ faces, leaving a depression in the central region. The resultant crystals will thus have holes in the faces with over-stretched corners, as shown by the crystals in Figs. 7 and 8.

5. Conclusion remarks

Bacillus megaterium promoted carbonate crystallization may be characterized as a biologically induced biomineralization process since epi- and inter-cellular activities seem to play the dominant roles. The entire crystallization process appears to be heavily influenced by the physical presence of bacterial cell surfaces and metabolic products or EPS. For nucleation, the effect of bacteria manifests itself in the form of cell walls serving as substrate surfaces; during growth, the crystal surfaces become the site of bacterial colonization. The mutual benefit, as well as the homo-epitaxial growth in the area not colonized by the bacteria, determines the overall morphology of the resultant biogenic calcite crystals. When the bacterial cells are excluded from the metabolic products, the lack of substrate surfaces may slow down the nucleation for calcite; meanwhile, vaterite crystallization promoted by the products of bacterial metabolism or EPS comes into play. The combined effect affords vaterite to be an important component in the resultant carbonate mineral assembly formed therein.

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