Calcite precipitation induced by bacteria and bacterially produced carbonic anhydrase

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Two types of experimental systems with and without *Bacillus* sp. which could produce extracellular carbonic anhydrase (CA) were studied to determine the effects of bacteria and bacterially produced CA in calcite precipitation. The results showed that the precipitation rate of Ca^{2+} was significantly faster in the experimental system with the bacteria than that without the bacteria. The X-ray diffractometry and Field Emission Scanning Electron Microscopy (FESEM) results showed that calcite was the dominant mineral phase, and FESEM analysis indicated that there were some bacterial imprints on the surface of calcite crystals in the experimental system with the bacteria. These results suggested that bacteria themselves could serve as nucleation sites for calcite precipitation. On the other hand, the precipitation rate of Ca^{2+} in the absence of CA inhibitor was faster than that in the presence of CA inhibitor for both experimental systems, which implied that bacterially produced CA may promote calcite precipitation as an activator.

Keywords: Bacteria, calcite precipitation, carbonic anhydrase, nucleation.

CALCITE precipitation plays an important role in many geological processes, including early diagenesis of marine sediments[1], hydrochemical evolution of karst streams[2], formation of travertine and speleothem[3,4], and the relevant global carbon cycle[5]. The importance of selective cementation has been widely recognized in civil engineering, which is also related to calcite precipitation. Based on the importance of the above processes, studies on calcite precipitation have attracted much attention[6–8].

It has been known that microorganisms play an important role in promoting calcite precipitation. Although there is no unified understanding of the formation mechanisms by microorganisms, quite a few researchers proposed that the following conditions will induce deposition. (1) The depositing particles are captured or adhered to by microbial mat or biofilm[9,10]. (2) Extracellular polymeric substances (EPS) absorb Ca^{2+} continually so that carbonate microcrystals form on the surface of the biofilm and result in calcification[9]. (3) In the environment with higher content of dissolved inorganic carbon (DIC), EPS degradation will release the Ca^{2+} ions that are chelated inside it, so that the supersaturation degree of Ca^{2+} in the environment will be continuously enhanced, resulting in promoting the precipitation of calcium carbonate ($CaCO_3$)[11,12]. (4) Some bacteria can induce precipitation of $CaCO_3$ extracellularly through such processes as photosynthesis, ammonification, denitrification, sulphate reduction and anaerobic sulphide oxidation[13,14]. (5) Degradation of urea by urea-decomposing bacteria increases pH and alkalinity of the environment, leading to $CaCO_3$ precipitation[15].

However, studies on the above-mentioned biogenic mechanisms have not considered microbial carbonic anhydrase (CA, EC4.2.1.1). CA is a zinc-containing enzyme that can dramatically catalyse the reversible hydration of CO_2 ($CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$)[16]. It is thus reasonable to expect that CA might accelerate calcite dissolution or precipitation under appropriate conditions. At present, quite a few studies have been done on the role of bovine CA in calcite dissolution[17,18], and our previous research has demonstrated the significant driving effect of microbial CA on limestone dissolution[19,20]. However, there has been no adequate study on the role of CA from organisms in calcite precipitation. In order to further clarify the contribution of CA from organisms to calcite precipitation, it is necessary to study the role of CA from microorganisms, which are ubiquitous in natural eco-environment, in calcite precipitation. In this article, the effects of bacteria and bacterially produced CA in calcite precipitation were studied through two types of experimental systems with and without bacteria.

Materials and methods

Microbial strain and cultivation

A *Bacillus* sp. which was screened and isolated from a karst soil in Southwest China was used in this study. It was chosen because it can produce and secrete extracellu-

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lar CA, and displays a capacity of crystallization behaviour of carbonates in the experimental system. The strain was used to inoculate with 10% inoculation amount into a sterilized liquid culture made by mixing beef extract (5 g), proteose peptone (10 g), NaCl (5 g) and zinc sulphate (10 μM) into 1000 ml distilled water. The final pH of the culture was around 7.2. Then the culture was incubated for 24 h at 30°C in a rotating shaker at a rotation rate of 120 rpm. The final reading in the optical density (OD_{600}) of the five-fold diluted culture was around 0.5.

Preparation of crude enzyme liquid of microbial CA

At the end of the incubation period, a portion of the final liquid culture containing the bacteria was centrifuged at 7000 rpm and 4°C for 10 min to remove the bacterial cells. The resultant supernatant was separated and further filtered using a 0.22 μm filter membrane to eliminate any remaining scraps of broken cell membranes, and then a crude enzyme solution containing extracellular CA was obtained. The CA activity of this crude enzyme solution was 0.907 U ml^{-1}.

Experimental systems

Two types of experimental systems were designed; with and without the bacteria. For the experimental system with the bacteria, two test groups were designed as groups A and B. Acid-washed 250 ml flasks containing 100 ml of the uncentrifuged liquid culture containing the bacteria were used in each group. The bacterial culture group was named group A. The group supplemented with CA-specific inhibitor acetazolamide (AZ) to inhibit extracellular CA produced by the bacteria, was named the bacterial culture + CA inhibitor group or group B for convenience. The final concentration of AZ in the solution was 1.17×10^{-6} mol l^{-1}, which was sufficient for the inhibition of bacterial CA. For the experimental system without the bacteria, two test groups were designed as groups C and D. Acid-washed 250 ml flasks containing 100 ml of the crude enzyme solution were used in each group. The culture solution group was named as group C. The group which was supplemented with AZ (final concentration was 1.17×10^{-6} mol l^{-1}) to inhibit CA activity in the solution was named the culture solution + CA inhibitor group or group D for convenience. Two control groups were designed as groups E and F for the two types of experimental systems. Acid-washed 250 ml flasks containing 100 ml of sterilized liquid medium were used in group E, which was named the medium control group and acid-washed 250 ml flasks containing 100 ml distilled water were used in group F, which was named the water control group. All groups were supplemented with 25.2 mM (final concentration) of $NaHCO_3$ and $CaCl_2$ at the beginning of experiments respectively. The flasks for each group were then put on a rotating shaker at a rotation rate of 120 rpm at 30°C. Separate flasks of samples were made up for analysis at sampling periods of every 1 h from 0 to 3 h and every 3 h after 3 h. After each sampling period, the samples were centrifuged and the supernatant was analysed for Ca^{2+} concentration. The experiments continued for 24 h. After the experiments, the crystals precipitated at the bottom of each flask in each group were collected through filtration, washed and dried for analysis of morphology and mineralogical composition. The entire experiment was repeated three times.

Analytic methods

Ca^{2+} concentration was measured by ethylenediaminetetraacetic acid (EDTA) titration[21]. CA activity was determined from the rate of CO_2 hydration by following the change of pH traced on a chart recorder, as described earlier[19]. The precipitated crystals were analysed using Field Emission Scanning Electron Microscopy (FESEM) (FEI, Sirion 200) and X-ray diffractometry (XRD) (PANalytical B.V., X'Pert PRO) to determine morphology and mineralogical composition.

Results

Change in Ca^{2+} concentration

The descending trend in the concentration of Ca^{2+} was similar in the control groups as well as in the test groups for both experimental systems (Figure 1). The concentration of Ca^{2+} all decreased sharply during the first 3 h of

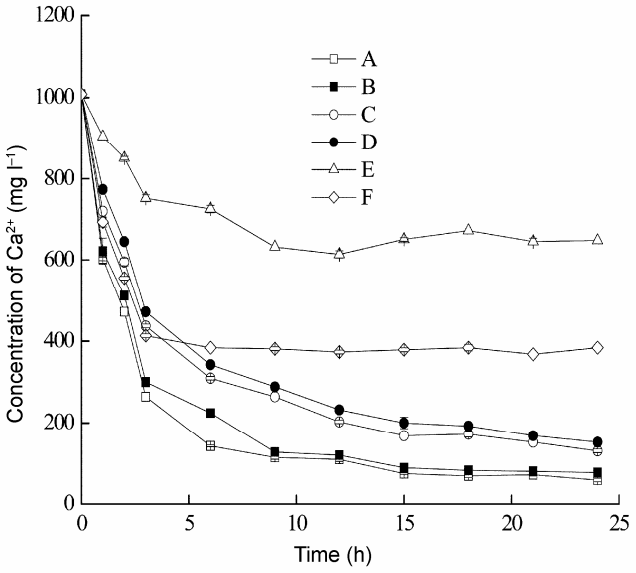

Figure 1. Change of Ca^{2+} with time under different treatments. A, Bacteria culture group; B, Bacteria culture + CA inhibitor group; C, Culture solution group; D, Culture solution + CA inhibitor group; E, Medium control group and F, Water control group. Bars represent mean ± standard deviation (n = 3).

the experiment. After 3 h, the concentration of Ca^{2+} decreased gradually and then approached equilibrium in each group, except for group F. The concentration of Ca^{2+} in group F was almost stable around 380 mg l^{-1} after 3 h. In general, the concentration of Ca^{2+} in the test groups in the presence of bacteria (groups A and B) decreased more rapidly than that in the absence of bacteria (groups C and D). The average concentration of Ca^{2+} between 15 and 24 h in the test groups A and B was 50% over or above lower than that in the test groups C and D, and 78% over or above lower than that in the group F. In the test groups in the presence of bacteria, the concentration of Ca^{2+} decreased more rapidly in group A than in group B. The total amount of deposited Ca^{2+} in group A was 10.0%, 1.7% and 2.1% higher than that in group B at 6 h, 15 h and 24 h respectively. In the test groups in the absence of bacteria, the concentration of Ca^{2+} decreased more rapidly in the group C than in group D. The total amount of deposited Ca^{2+} in group C was 4.8%, 3.8% and 2.4% higher than that in group D at 6 h, 15 h and 24 h respectively. Although the concentration of Ca^{2+} in group F decreased more quickly than that in the test groups in the absence of bacteria in the first 3 h of the experiment, it reached equilibrium soon after 3 h of the experiment and did not continue to decrease. The concentration of Ca^{2+} in the equilibrium phase in group F was far higher than that in the test groups in both the presence and the absence of bacteria. The descending rate of Ca^{2+} concentration in the group E was the lowest, and the concentration of Ca^{2+} in group E was far higher than that in the other groups, including group F.

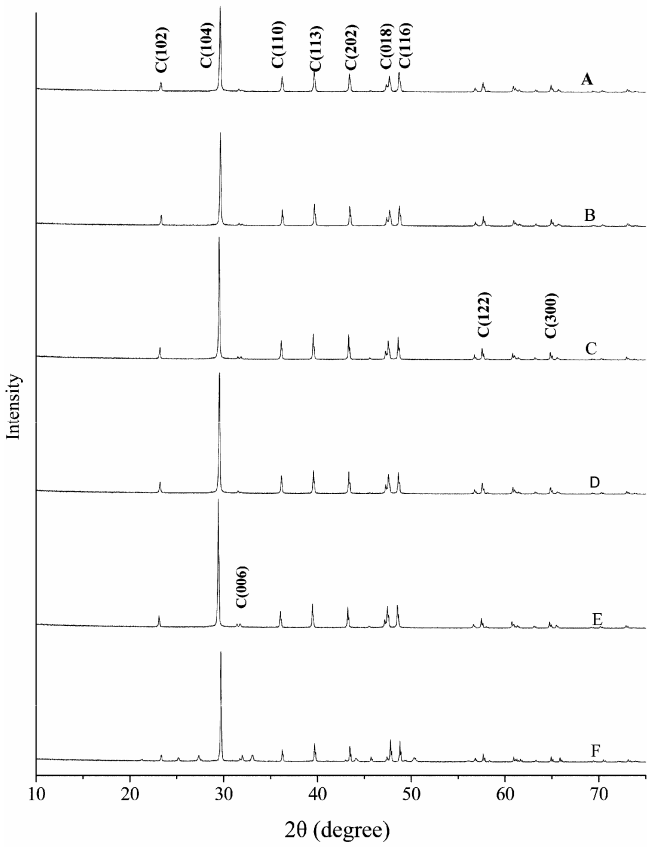

Figure 2. X-ray diffraction patterns of precipitates collected from different groups. Numbers in parentheses indicate the Miller indices, where C denotes calcite. A, Bacteria culture group; B, Bacteria culture + CA inhibitor group; C, Culture solution group; D, Culture solution + CA inhibitor group; E, Medium control group, and F, Water control group.

Comparison of XRD patterns among different treatments

Calcite was precipitated in the test groups in both the presence and the absence of bacteria as well as in the control groups, as confirmed by the XRD analysis (Figure 2). In the XRD patterns, the characteristic diffraction peaks occurred at $2\theta = 23.3°$, 29.6°, 36.2°, 39.6°, 43.4°, 47.7° and 48.7°, and the strongest reflection occurred at $2\theta = 29.6°$. XRD analysis of the precipitates showed that calcite with various morphologies formed in all the test groups and the control groups. The calcite Miller indices [(102), (104), (110), (113), (202), (018) and (116)] were almost the same.

Comparison of crystals among different treatments

During the process of deposition visible $CaCO_3$ precipitates were clearly observed within 1 h in the test groups A and B and in group F, and obvious precipitates occurred at 2 h in the test groups C and D, and at 3 h in group E. There were obvious differences in the size and shape among different treatments according to FESEM. The sizes of the crystals formed in the test groups A and B were 20–40 μm in diameter, and those in the test groups C and D were 10–40 μm in diameter, whereas the sizes of the crystals formed in the two types of control groups were the smallest, only 10–20 μm.

In the test groups A and B, calcite grains were spherical with peanut morphologies (A1 and B1, Figure 3), which had smooth (A1 and B2, Figure 3) and rough surfaces (A2 and B2, Figure 3). There were no obvious differences in the shape and size of calcite crystals between the groups A and B. The number of crystals with coarse surface was relatively more abundant than that with smooth surface. Moreover, some imprints about 1 μm in length on the surface of calcite crystals were observed in both groups A and B. These imprints might have been left behind by bacterial cells which had been washed away during sample preparation. Furthermore, it was also observed that the calcite grains were accumulated by irregular structure of layer flake (A3 and B3, Figure 3). Grains with smooth surface showed much less angularity than those with coarse surface.

The calcite crystals formed in the test groups C and D were irregular, and approximately of ellipsoidal or square

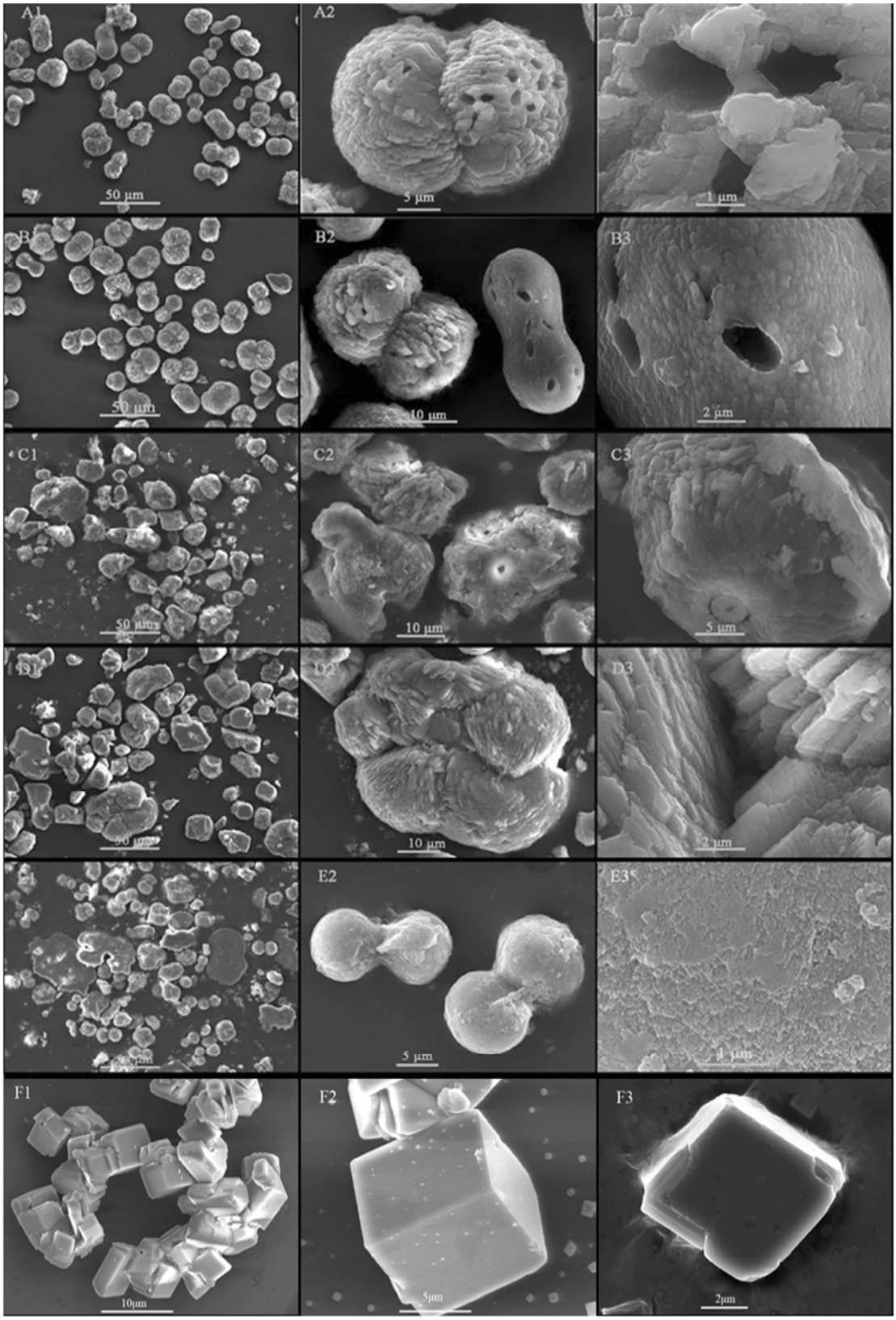

Figure 3. Field emission scanning electron microscopic observations of calcite collected from groups A–F. A1–E1, General overview of calcite grains; A2, B2, Peanut calcite grains and their holes; A3, B3, Detail of a calcite peanut. C2, Several calcite grains seen in the culture solution group; C3, A calcite grain seen in the culture solution group; D2, A calcite grain seen in the culture solution + CA inhibitor group; D3, A calcite grain seen in the culture solution + CA inhibitor group; E2, Peanut calcite grains seen in the medium control group; E3, A calcite grain surface seen in the medium control group; F1, Rhombohedral calcite grains seen in the water control group and F2, F3, Calcite grains seen in the water control group.

morphology (C1, C2, D1 and D2, Figure 3). There were no imprints found on the surface of the crystals, unlike the test groups in the presence of bacteria. Moreover, the form of the crystals was rough (C2 and D2, Figure 3). The calcite crystals were also composed of layer flake structure (C3 and D3, Figure 3), and the layer flakes accumulated compactly.

The calcite crystals formed in group E were mainly spherical and peanut morphology as well as spherical superposition morphology (E1 and E2, Figure 3), and most of the calcite crystals were spherical. The calcite crystals were also composed of layer flake structure (E3, Figure 3), however, their texture was finer compared to that of the test groups. The calcite crystals formed in the water control group were rhombohedral single crystals, twin crystals and their aggregates (F1, F2 and F3, Figure 3). Since the crystals were formed in the pure water chemical environment, the crystals developed well in rhombohedral structure and crystal morphology were more regular. Rhombohedral calcite was also composed of layer flake structure (F3, Figure 3). However, its surface was smooth and had no angularity.

Discussion

Bacteria serving as nucleation sites of $CaCO_3$

Compared to the experimental system without bacteria, the precipitation rate of Ca^{2+} was significantly faster in the experimental system with bacteria. Moreover, FESEM analysis indicated that there were some bacterial imprints on the surface of calcite crystals in the experimental system with the bacteria. These results suggested that bacteria might serve as nucleation sites for calcite precipitation, which is in agreement with the earlier results of other researchers. The bacterial cell surface with various ions could nonspecifically induce mineral deposition by providing nucleation sites[22]. Lian *et al.*[23] demonstrated that the process of carbonate crystal formation by *Bacillus megaterium* involved the nucleation of calcite on the bacterial cell walls. It was hypothesized that every cell could serve as a nucleation site for $CaCO_3$ precipitation when the cell concentration was low[24]. Possible biochemical reactions in the H_2O–CO_2–$CaCl_2$ system inoculated with the bacteria to precipitate $CaCO_3$ at the cell surface are presumed as follows:

$$Ca^{2+}_{(l)} + \text{Cell} \rightarrow \text{Cell-}Ca^{2+}_{(l)},$$

$$HCO^{-}_{3(l)} \Leftrightarrow H^{+}_{(l)} + CO^{2-}_{3(l)},$$

$$\text{Cell-}Ca^{2+}_{(l)} + CO^{2-}_{3(l)} \rightarrow \text{Cell-}CaCO_{3(s)}.$$

In the bacterial culture medium, Ca^{2+} is not likely utilized by bacterial metabolic processes, it just accumulates outside the cell[25]. As a result of enzymatic reversible hydration of CO_2, CO_2 or HCO_3^- is produced and the dissolved CO_2 transforms to CO_3^{2-} or HCO_3^- around the cell, commencing the growth of $CaCO_3$ crystals around the cell.

Determination of bacterial CA involvement in calcite precipitation

In the H_2O–CO_2–$CaCO_3$ system, the slow reaction $HCO_3^- + H^+ \rightarrow H_2O + CO_2$ was considered as one of the rate-limiting steps for the precipitation rate of calcite from supersaturated solutions[26]. CA can catalyse the interconversion of CO_2 and HCO_3^- (ref. 27), so that the rate of conversion to CO_2 may increase. Therefore, CA may aid in the precipitation of $CaCO_3$, following the equations:

$$HCO_3^- + H^+ \rightarrow H_2O + CO_2,$$

$$Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3 + H^+ + HCO_3^- \rightarrow CaCO_3 + H_2O + CO_2.$$

The loss of CO_2 from the system results in an increase in pH and promotes precipitation of $CaCO_3$. Dreybrodt *et al.*[26] added bovine CA to the H_2O–CO_2–$CaCO_3$ system, and found that the conversion of HCO_3^- into CO_2 was enhanced, and precipitation rate of $CaCO_3$ increased. Liu *et al.*[28] added bovine CA to brines, and the results showed that the enzyme increased the rate of precipitation of $CaCO_3$. Mirjafari *et al.*[29] also demonstrated that bovine CA promoted the formation of $CaCO_3$. There are additional studies dealing with the presence and the role of CA in the $CaCO_3$ deposition process of invertebrates, where $CaCO_3$ is the major component in their calcified skeletal structures. For example, Miyamoto *et al.*[30] first discovered a CA domain within nacrein, a soluble organic matrix protein of the nacreous layer in the mollusc *Pinctada fucata*. Watanabe *et al.*[31] found an internal sequence in *Tubastrea aurea* that exhibited similarity to a part of the CA sequences. At this point, however, the role of CA from microorganisms in the precipitation of carbonate has been less reported. We had found that CA activity could be detected in most of the studied soil microorganisms from the karst soils[32,33] and explored the effects of microbial extracellular CA on limestone dissolution[19,20,34]. In this article, we studied the effects of CA from *Bacillus* sp. on calcite precipitation through the inhibition of CA activity in the experimental system with and without bacteria. Both experimental systems showed the inhibition of calcium deposition in the presence of CA inhibitor. However, the differences between the treatments with and without the CA inhibitor were not significant. One of the possible reasons may be that the bacterial culture or culture solution (after removal of the bacteria) had other components except CA, which may also have some

effects on calcite precipitation. Nevertheless, CA from bacteria may be involved in calcite precipitation. Tambutté *et al.*[35] also showed that CA was involved in the calcification process of the azooxanthellate coral, *T. aurea* through the inhibition of calcium deposition into the skeleton in the presence of CA inhibitor. Further studies are needed to demonstrate the promoting role of CA from bacteria in calcite precipitation through separating and purifying bacterial extracellular CA from the bacterial culture solution.

Although the present finding that bacterial CA may promote calcite precipitation as an activator is from the laboratory experiment, there are wide implications for natural carbonate precipitation, since bacteria are ubiquitous in nature, and CA is widespread in prokaryotes and certain eukaryotes[27,33]. Thus, the contribution of bacterial CA to the carbon cycle needs to be examined while studying the role of microorganisms in the carbon cycle.

Effects of other components on calcite precipitation

In the present experiments, we adopted both bacterial culture and culture solution as precipitation medium. CA activity could be detected in both the media, which indicated that both media had bacterial extracellular CA. In the media, there were other metabolites produced and secreted by the bacteria during the growth and metabolic process, as well as residual medium ingredients. The organic matter containing proteins (excluding CA), polysaccharides, lipids, etc., might also have some effects on carbonate precipitation. These effects may be numerous: promotion or inhibition of carbonate crystal growth, crystal morphology and calcium binding. A few studies have reported that the organic matrix extracted from the calcified skeletal structures of invertebrates or biomineralized materials could regulate carbonate crystallization and crystal growth. Watanabe *et al.*[31] summarized the possible role of matrix proteins in the formation of calcified hard tissues in invertebrates. They pointed out that matrix proteins may promote nucleation of calcium crystals, inhibit the crystal growth and determine the polymorph of $CaCO_3$. Ozaki *et al.*[36] isolated coccolith matrix acidic polysaccharide (CMAP) from the coccolith of a coccolithophorid alga, *Pleurochrysis haptonemofera*, and found that CMAP showed a strong inhibitory activity on $CaCO_3$ precipitation. Furthermore, Braissant *et al.*[37] found that amino acids induced vaterite precipitation with traces of calcite at high xanthan concentrations. At present, however, there is no report about the influence of organic matter such as extracellular proteins (excluding CA), exopolysaccharides, etc. obtained directly from microbial culture solution on calcite precipitation. In our experiments, the total precipitation amount of Ca^{2+} in the CA-inhibited groups was still higher than that in the water control group and the medium control group, suggesting that the organic matter except CA, in the bacterial culture or culture solution group also had a positive effect on calcite precipitation. However, further questions remain, such as which kind of organic matter plays a leading promoting role in calcite precipitation, or whether the promoting effect results from the synthetical role of these organic matters.

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