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RESEARCH ARTICLE

Calcium carbonate precipitation induced by a bacterium strain isolated from an oligotrophic cave in Central China

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Abstract A heterotrophic *Bacillus* sp. strain (5C-1) was isolated from Heshang cave, an oligotrophic karst cave in the middle reaches of Yangtze River, and identified by BIOLOG and 16S rDNA sequencing. Bacterially induced formation of calcium carbonate by 5C-1 was investigated in several comparative experimental sets with or without the cell and extracellular enzymes. The temporal variations of both the amount of the precipitates and the pH values of the solution were measured by a spectrophotometer and a pH meter, respectively. The morphological characteristics of the calcium carbonate precipitates were observed with environmental scanning electronic microscopy (ESEM). The growth of 5C-1 was found to greatly promote the pH value of the liquid medium in the first 2 days, which favors the formation of calcium carbonate. No precipitates were formed with the pH value lower than 8.6, though the pH value was demonstrated to be not the only factor controlling the formation of the calcium carbonate. The accumulation of extracellular polysaccharide substance was observed to favor the precipitate formation. Only when both factors reached a threshold did the precipitates form with the addition of $CaCl_2$. Cells and extracellular enzymes were not the factors that limit the precipitate formation in our microbial systems. The precipitates of a variety of morphological features including dumb bells, peanuts, irregular and spherical and rhombic forms were mainly observed in our microbial systems but not in the chemical control system. Interestingly, imprints of bacterial cells and spores were observed to be present on the surface of the precipitates of a peanut or a dumb bell form, probably indicative of the microbial escaping mechanism during the mineralization of calcium carbonate.

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1 Introduction

In the past decade great achievements were made on the interaction between microbes and minerals, including the microbial dissolution and the microbially induced formation of minerals (Boquet et al., 1973; Ransom et al., 1999; Lower et al., 2001; Edwards et al., 2005; Benzerara et al. 2006; Perdria et al., 2009). Much work was conducted on carbonate minerals due to their important roles in the global carbon cycle in Earth's history. To date, both autotrophic (i.e., cyanobacteria) and heterotrophic (i.e., sulfate reducing bacteria) bacteria (Riding, 2000; Visscher et al., 2000; Ben et al., 2004; Dupraz et al., 2004; Wright and Wacey, 2005; Baumgartner et al., 2006) are demonstrated to induce or mediate the carbonate mineralization in sediments and during rock formation. These geomicrobiological processes could play a key role in biogeochemical cycles of carbon throughout geological history. However, less is known about the microbially-induced calcareous mineralization in karst ecosystems, especially in the dark and oligotrophic caves. Some fundamental issues are still quite open to both geologists and microbiologists. For example, are there microbes living in the dark caves? What are they doing there? Do they influence the formation of the stalagmite in which high-resolution paleoclimatic signals are well recorded by oxygen isotope composition? Can they change the chemistry of dripping water, pool water, and the carbonate deposits? Here we reported our work on bacteria living in the Heshang oligotrophic cave in central China, to see if they could play an important role in the formation of calcium carbonate. Morphological characteristics of the microbially induced calcium carbonate and

the mechanism of how microbes induce the mineralization will be investigated.

2 Materials and methods

2.1 Sampling and isolation of the strain

The sample of calcareous precipitate from which 5C-1 was isolated was collected from Heshang cave in the Qingjiang Valley of the middle reaches of the Yangtze River (30°27′N, 110°25′E; 294 m) in central China . The cave is 205 m above sea level, with a length of 250 m and a width between 10 m and 30 m. The sampling site is in the aphotic zone within the cave, 100 m away from the cave entrance. Water drips to the surface of the calcareous precipitation.

Calcareous precipitates were aseptically collected with a chisel and a hammer, kept in a 50 mL sterilized centrifuge tube, and taken to the geomicrobiology lab on ice. The precipitates were crushed in a sterilized mortar and pestle in an ultra-clean laminar hood. As much as 2 g of the crushed sample was transferred into 200 ml liquid medium (in 1 L distilled water with beef extract 3 g, peptone 10 g, and NaCl 5 g) and incubated at 36°C with 170 rmp in a rotator. Pure isolates were conducted several times on steak plates till the individual pure colonies were obtained. BIOLOG (Biometra) and 16S rDNA sequencing methods were exploited for the identification of the strain isolated, 5C-1.

2.2 Experimental setup

The 5C-1 strain was incubated in liquid medium to different growth stages, and the cultures were then treated in different ways periodically. Cells were got rid of from Group A by centrifugation of 5000 rpm, leaving only extracellular enzymes active in the culture. Group B was centrifuged to remove the bacterial cells, and further boiled to denature the extracellular enzymes. In contrast, group C was not centrifuged and boiled, and both the cells and extracellular enzymes were left active in the culture. A chemical control set without any bacterial inoculum was also conducted to compare with the microbial systems, favoring the elucidation of the contribution of bacterial metabolism to the chemical changes of the solutions. $CaCl_2$ was added to the above cultures with a final concentration of 0.2 M to evaluate the formation process of calcium carbonate.

The amount of the calcium carbonate precipitates was counted by a spectrophotometer (TU1800, Beijing Purkinje general instrument Co. Ltd) periodically. The temporal variation of the pH values of the culture solution was measured by a pH meter. Morphological features of the precipitates were observed under light microscopy and scanning electronic microscopy (SEM) (Quanta200FEG, FEI) in Peking University.

3 Results

Both BIOLOG and 16S rDNA data confirmed that the 5C-1 strain belongs to *Bacillus* sp. The strain grew fast and multiplied with an exponential rate after 5 hrs incubation in the liquid medium. The growth rate leveled off 20 hrs later, indicative of the presence of the stationary stage. With the growth of the 5C-1 strain, the pH value of the culture solution was observed to increase remarkably from 7.2 to 9.3 throughout the bacterial experiments. The pH value in the chemical control set was found to remain quite stable, around 6.9 to 7.0 (Fig. 1).

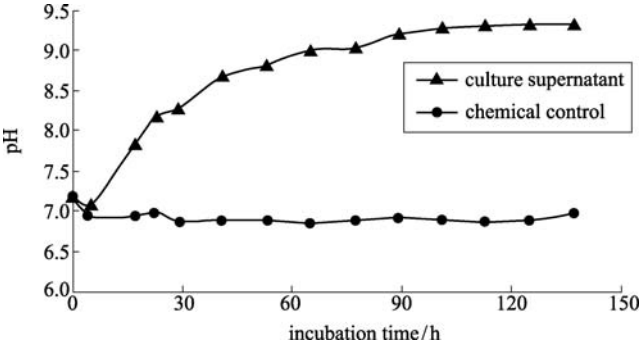

Fig. 1 Temporal variation of the pH value during the growth of 5C-1 strain

No calcium carbonate precipitates were found in the chemical control set throughout the whole experimental course. However, precipitates were observed in the microbial systems, including groups A, B and C, when the pH values of the cultures increased over 8.6. This inferred the presence of the microbial contribution to the calcium carbonate precipitation by promoting the pH values of the culture solution. The occurrence of the precipitates in the boiled supernatant suggests that neither bacterial cells nor the extracellular enzymes could limit calcium carbonate formation in our systems when the pH value is over 8.6.

The amount of the calcite precipitates is further counted in group A on the basis of the difference of the absorbance of the supernatant before and after $CaCl_2$ addition. A positive correlation was observed between the amount of the calcite precipitates and the pH values in the range of 8.7 to 9.3 (Fig. 2).

Morphological features of the calcite precipitates were found to vary with experimental conditions. Peanut or dumbbell calcium carbonate of different sizes was commonly found in all conditions (Fig. 3), indicative of the process of the particle formation and growth (Fig. 3(c)). Bacterial imprints as well as bacterial spores were observed on the surface of calcium carbonate particles (Figs. 3 (a) and (b)) which indicated the nucleation on the surface of bacteria. Irregular calcite, spherical calcite of

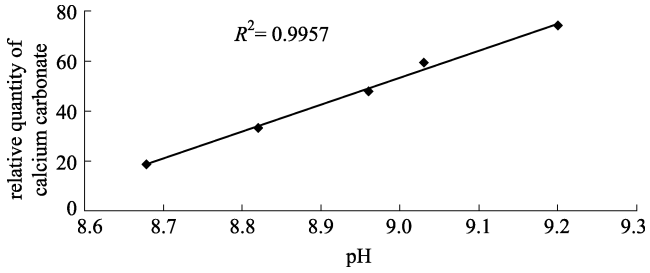

Fig. 2 Positive linear relationship between the amount of calcium carbonate and the pH value

6–8 μm diameter, and rhombic calcium carbonate of 6 μm in the long axis were also observed among the precipitates.

4 Discussion

It is acknowledged that calcite precipitation is impacted by both chemical and biological factors. Chemical factors include dissolved inorganic matter (DIC), pCO_2, Ca^{2+} concentration and the pH value, while the biological factors concern the metabolic activity such as photosynthesis (Riding 2009), sulfate reduction (Wright, 1999), urea degradation and EPS (Braissant et al., 2007). In our current systems, most of the chemical parameters were fixed except for the pH value. The impact of the pH value on the calcite precipitation is obvious. No precipitates were formed under pH values lower than 8.6. However, the variation of the pH values in our work is clearly induced by bacteria via metabolic activity.

Interestingly, the pH value of 8.6 seems to be a threshold for the formation of calcium carbonate in microbial systems. However it is not the only factor for controlling the precipitate formation since no precipitate was observed when the pH value reached 8.6 during the bacteria growth from the beginning of the experiments. Only when the pH value was over 8.6 and re-adjusted back to this critical value was calcite observed to form in the medium with the addition of $CaCl_2$. Since the Ca^{2+} concentration and the pH values were kept the same in the two cases, the difference in the amount of metabolite, especially the extracellular substance in the supernatant of group A, should be another important factor to control the calcite formation.

Since *Bacillus* is a genus of aerobic, gram positive and heterotrophic bacteria, it does not photosynthesize in nature. It is impossible for sulfate reduction and urea degradation to occur in our systems. The extracellular substance such as EPS besides the cell surface would be responsible for the nucleation of calcite precipitation in this investigation. It was demonstrated that the calcite nucleation

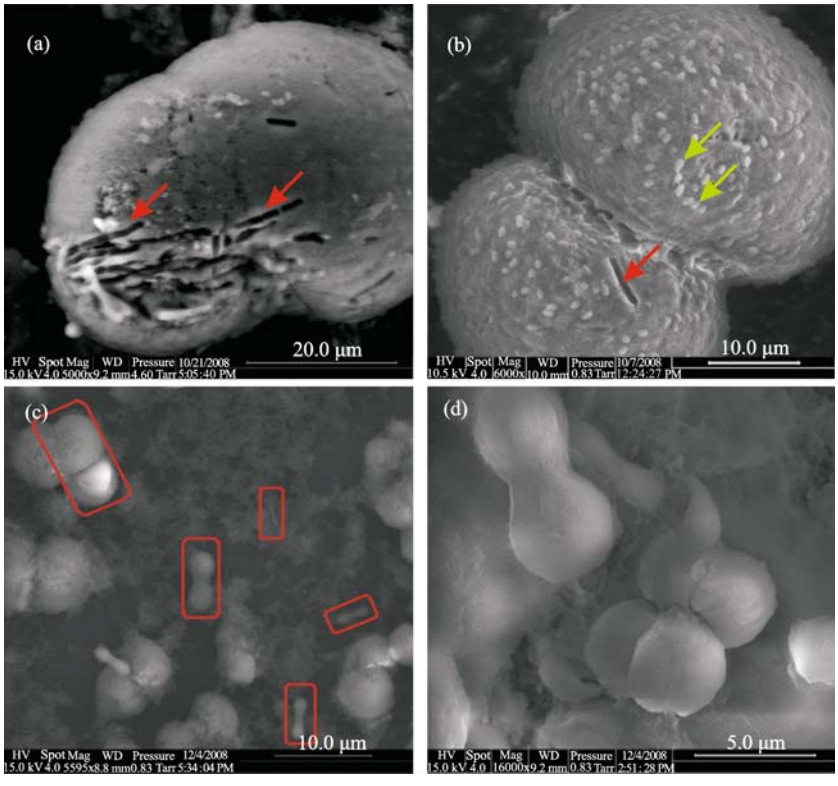

Fig. 3 SEM images of calcium carbonate precipitates by a bacterial strain 5C-1 isolated from an oligotrophic cave.
(a) Bacterial imprints (indicated by red arrow) and (b) bacterial spores (indicated by green arrow) on the surface of calcium carbonate particle; (c) peanut calcium carbonate particles of different sizes (within the red blocks); (d) peanut and spherical calcium carbonate particles with smooth surface

initiated on the cell surface to form 60–200 nm globules, and significant calcification took place when these globules on the cell surface were released to the medium (Aloisi et al., 2006). Further investigation showed that most cells were found to locate outside the EPS aggregation where calcite mineral growth mainly took place; this avoids having the cells be entombed within the minerals (Bontognali et al., 2008). This geomicrobiological process of calcification might explain the rareness of fossilized cells in carbonate rocks despite the extensive microbial involvement during the mineral precipitation in ancient oceans. Nanoglobules and the imprints of bacterial cells present on the surface of calcite particles in our investigation might provide compelling evidence of the escape mechanism of the 5C-1 strain from being entombed during the mineral formation. However, in some cases, bacterial cells were indeed entombed in the minerals during the vaterite mineralization (Rodriguz-Navarro et al., 2007).

5 Conclusions

Heterotrophic bacterium *Bacillus* sp. isolated from an oligotrophic cave can mediate calcium carbonate precipitation by enhancing the pH values of the solution and providing the nucleation sites. Bacterial cells and extracellular enzymes are not requisite for the formation of calcite precipitates under the pH values greater than 8.6. Bacterial cells could escape from being entombed by staying out of the main mineralizing site, the "EPS aggregation", or survive via sporulation. Our work confirmed that bacterially induced or mediated mineralization is a widespread process in natural environments though few cells might be fossilized in the rocks.

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