Nucleation of calcium carbonate on bacterial nanoglobules

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ABSTRACT

Nucleation of calcium carbonate on microbial cell material may have been the dominant mode of microbial carbonate formation during most of Earth's history. Current knowledge predicts that nucleation takes place on the cell surface or on extracellular polymeric substances. However, the initial nucleation steps have not been described in detail and the process remains elusive. Here we describe the bacterial nucleation of calcium carbonate at the nanometer scale. In our precipitation experiment with sulfate reducing bacteria (SRB), the bulk of calcium carbonate precipitates on hundreds of individual globules 60–200 nm in diameter. Globules originate from the SRB cell surface but calcify significantly only when released to the culture medium. Similar globules have been observed, albeit at a much larger scale, in other bacterial precipitation experiments and in many natural microbial carbonates, suggesting that the process we describe could be an important step in microbial calcification.

Keywords: bacteria, nucleation, carbonates, precipitation experiment, electron energy loss spectroscopy, transmission electron microscopy.

INTRODUCTION

Microbes are involved in the precipitation of carbonate minerals in a wide range of modern and ancient geological environments (Riding, 2000; Krumbein et al., 2003). Their involvement is twofold. The effect of metabolic reactions that increase the alkalinity of the aquatic environment has been investigated extensively (Castanier et al., 1999). Less is known about how microbes affect the nucleation of carbonates (Schultze-Lam et al., 1996). Geochemical modeling and laboratory experiments show that nucleation processes, rather than microbial metabolisms, controlled microbial carbonate formation for most of Earth's history (Bosak and Newman, 2003).

In natural environments, the close spatial association of microbial cells and carbonate minerals is used as evidence that microbes participate in nucleation processes (van Lith et al., 2003a). Laboratory experiments provide indirect evidence that supports this idea (Warthmann et al., 2000; Bosak and Newman, 2003). Microbial cell surfaces and excreted extracellular polymeric substances (EPS), which carry a net negative electric charge and have the capacity to bind Ca^{2+} ions, are frequently cited as being the sites of carbonate nucleation (van Lith et al., 2003a; Dupraz et al., 2004). However, direct observations of the nucleation process are lacking and the nucleation sites remain elusive.

Should calcium carbonate nucleation take place on cell surfaces, it would likely lead to cell entombment and death of the organism (Morita, 1980; Southam and Donald, 1999). Thus, the locus of crystal nucleation is of vital importance for microbial cells. Furthermore, nucleation processes are likely to control the shape of carbonate precipitates. The search for life in ancient rocks, as well as on extraterrestrial planets, requires the development of morphological proxies indicative of microbial involvement in mineral formation (Allen et al., 2000). Understanding how microbes affect nucleation processes is a fundamental step in defining such proxies.

In this paper we investigate the process of calcium carbonate nucleation by sulfate reducing bacteria (SRB) through a laboratory experiment followed by a high-resolution transmission electron microscope (TEM) and chemical study of the microbial carbonate precipitate.

METHODS

Organism

We used the sulfate-reducing bacterium *Desulfonatronum lacustre* (Pikuta et al., 1998) in our precipitation experiment. *D. lacustre* is a gram-negative, alkaliphilic sulfate reducer that is phylogenetically related to the carbonate-precipitating sulfate reducer *Desulfonatronovibrio hydrogenovorans* (Zhilina et al., 1997). A pure culture of *D. lacustre* was prepared in liquid medium 813 from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) at 30 °C and pH 8 using formate as a substrate (Table 1). Although the alkalinity is elevated in this medium, and is bound to increase due to sulfate reduction during culturing, the medium contains no dissolved calcium. Therefore, calcium carbonate precipitation cannot take place in the *D. lacustre* culture.

Precipitation Experiment

We added 1 mL of *D. lacustre* culture to 15 mL of LV medium at 30 °C in a test tube; the LV medium (Table 1) reproduces the chemical conditions of Lagoa Vermelha, a hypersaline lagoon where SRB currently promote the formation of carbonate minerals (Warthmann et al., 2000). The LV medium is strongly supersaturated with calcite and aragonite ($\Omega \sim 10$–15). In nature, the saturation state with respect to carbonates is often controlled by a combination of metabolic processes. Rather than trying to reproduce a particular combination of

TABLE 1. CHEMICAL COMPOSITION OF LAGOA VERMELHA (LV) AND *DESULFONATRONUM LACUSTRE* CULTURE MEDIA

	LV (mM)	*D. lacustre culture* (mM)
Cl^-	707	192
Na^+	737	312
Mg^{2+}	80	0.49
SO_4^{2-}	10	35.2
K^+	7.2	4.3
Ca^{2+}	13	0
PO_4^{3-}	0.9	1.6
$NH4^+$	4.7	18.7
HCO_3^-	~30	~30
HS^-	26	5
pH	8.0	8.8
Formate	80	80

microbial metabolisms occurring in nature, we preferred to simplify the system, inducing supersaturation chemically. Thus, rather than the effect of microbial metabolism on carbonate mineral stability, we test the capacity of the *D. lacustre* inoculum to provide nucleation centers in the LV medium. Nevertheless, our observations of the nucleation process should be applicable to natural systems where supersaturation with respect to carbonate minerals results from metabolic activity.

Sterile control experiments were run to check if the mixtures of DSMZ 813 and LV media we use in our precipitation experiment promote the precipitation of carbonates in the absence of microbial cells. Three sterile controls were (1) LV medium only, (2) DSMZ 813 medium (1 mL) mixed with LV medium (15 mL), and (3) DSMZ 813 medium (1 mL), prepared by filtration of a *D. lacustre* culture through a 0.2 μm filter, mixed with LV medium (15 mL). All sterile control experiments were run at 30 °C and the media used contained formate in concentrations comparable to those of the precipitation experiment.

RESULTS

Precipitation of Carbonates by *D. lacustre*

During the first hour after inoculation of the LV medium with the *D. lacustre* culture, white floccules appeared in the inoculated LV medium. When the floccules settled in the lower part of the test tube, they formed a white layer ~0.5 mL in volume. Filtered and dried floccules react readily with dilute (0.5 M) HCl, indicating that they contain carbonate material. Although the LV medium is supersaturated with calcium carbonate, the sterile control experiments failed to produce a precipitate in four months, indicating that a purely abiotic precipitation process can be excluded in our *D. lacustre* precipitation experiment. Instead, our observations indicate that carbonate precipitation occurs in the LV medium because the *D. lacustre* inoculum is promoting the nucleation of carbonates.

Discovery of Globules Associated with *D. lacustre* Cells

To investigate the involvement of *D. lacustre* in the nucleation of carbonates, we carried out a high-magnification TEM study of the carbonate floccules (see GSA Data Repository[1]). Figure 1A is a TEM view of the material forming the floccules and is representative of a high number of floccule observations. The floccules are composed of a mixture of *D. lacustre* cells and spherical objects (hereafter globules) 60–200 nm in diameter. The size distribution of globules is irregular; a large number of globules are in the 80–100 nm size class (small globules) and most of the remaining globules are 120–200 nm (large globules) (Fig. 1A, inset). The spatial distribution of globules seems to be related to their size (Fig. 1B). Small globules occur only attached to the surface of *D. lacustre* cells where they are embedded in a thin (<20 nm) film of material of low electron density that envelops *D. lacustre* cells (Fig. 1B, inset). This material rapidly degrades under the electron beam and probably consists of EPS secreted by *D. lacustre*. At least 50% of the surface of most *D. lacustre* cells is covered by small globules and in some cases small globules can be so abundant that the bacterial cell surface is no longer visible (Fig. 1B). Large globules occur mostly separated from *D. lacustre* cells. They form aggregates of as much as several hundred units that form independent entities on the TEM mesh or can be partially attached to polar ends of *D. lacustre* cells (Figs. 1B, 1C). Individual large globules are roughly five times larger in volume than small globules. Furthermore, large globules are the most electron dense objects we observed, indicating that they concentrate most of the material that makes up the floccules.

We did not observe globules with the TEM in the DSMZ 813 medium or in a mixture of

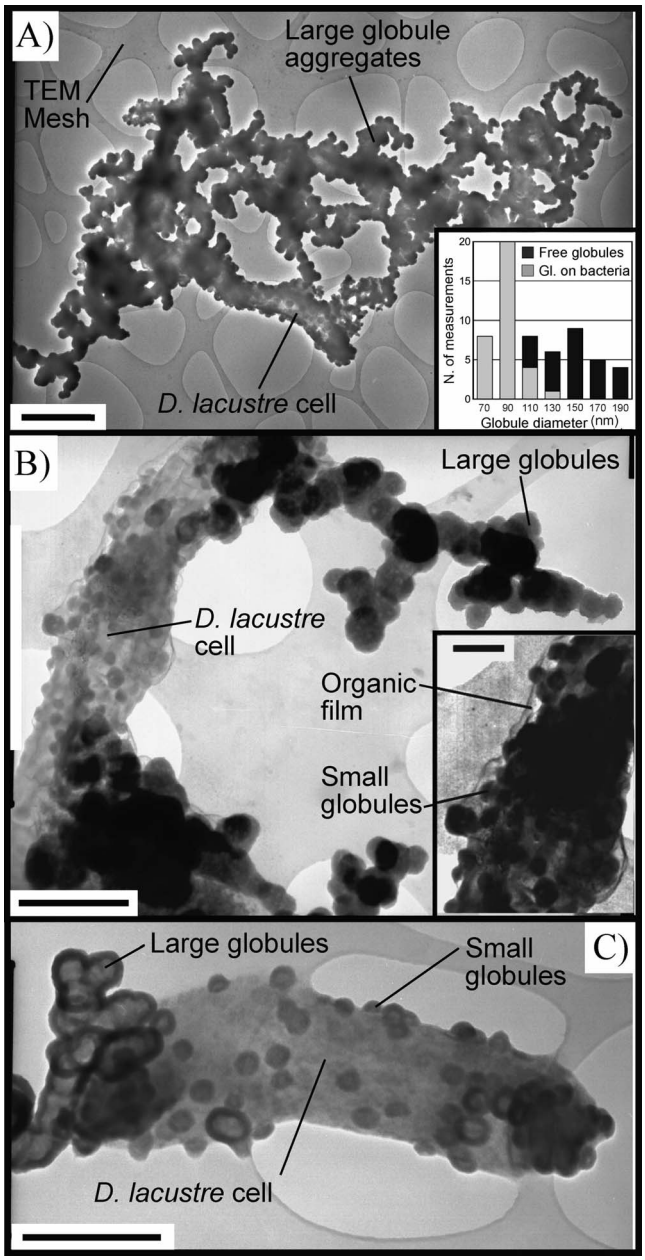

Figure 1. Transmission electron microscope (TEM) images of carbonate floccules formed in precipitation experiment with sulfate-reducing bacterium *Desulfonatronum lacustre*. A: General view. Most of field of view is occupied by aggregates of large globules. One *D. lacustre* cell occurs in central-lower part of field of view and is covered by small globules (scale bar 1 μm). Inset: Size distribution of globules observed in *D. lacustre* precipitation experiment (scale bar 300 nm). B: Detail of *D. lacustre* cell completely covered by small globules. Small globules are embedded in thin (<20-nm-thick) layer of low electron-dense material probably composed of extracellular polymeric substances (EPS) excreted by *D. lacustre*. Large globules form aggregates that are partially attached to polar edges of *D. lacustre* cell (scale bar 500 nm). Inset: Small globules attached to *D. lacustre* cell and covered by EPS (scale bar 200 nm). C: Detail of *D. lacustre* cell showing aggregates of large globules attached to polar end of bacterial cells (scale bar 500 nm).

[1]GSA Data Repository item 2006223, methods of the transmission electron microscopy and electron energy loss spectroscopy investigations, and Figure DR1, TEM image of *D. lacustre* cell in DSMZ nr. 813 culture medium, is available online at www.geosociety.org/pubs/ft2006.htm, or on request from editing@geosociety.org or Documents Secretary, GSA, P.O. Box 9140, Boulder, CO 80301-9140, USA.

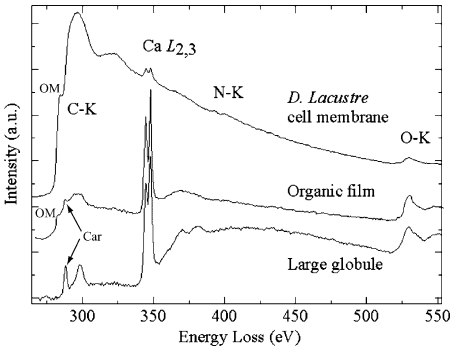

Figure 2. Electron energy loss spectroscopy (EELS) spectra of *Desulfonatronum lacustre* cell membrane, organic film surrounding *D. lacustre* cells, and large globules produced in calcification experiment. In C-K edge, peaks at 288.5 eV are diagnostic of organic matter (OM) and peaks at 290–290.5 eV are diagnostic of carbonate ion (Car) (Benzerara et al., 2005).

DSMZ 813 medium and formate (4 g/L), excluding the possibility that globules form these materials during sample dessication in the TEM. Furthermore, we did not observe globules in the *D. lacustre* culture prepared in the DSMZ 813 medium (Fig. DR1; see footnote 1), indicating that they do not originate from the dessication in the TEM of the EPS that surrounds the bacterial cells. Together with the failure to produce a calcium carbonate precipitate in the sterile culture experiments, these observations indicate that the globules are not an artifact of sample preparation and imply that globule formation takes place only in the presence of bacteria and of a chemical environment favoring calcium carbonate precipitation.

Calcium Carbonate Precipitation on Globules

To identify the sites of carbonate precipitation, we investigated the chemistry of *D. lacustre* cell surfaces, the thin layer enveloping bacterial cells, and the globules using electron energy loss spectroscopy (EELS). Figure 2 shows EELS spectra that are representative of the three investigated materials. In the EELS spectrum from the *D. lacustre* cell surface, the C-K edge resembles that of a poorly ordered carbon-based structure and is typical of organic carbon analyzed with limited energy resolution or after being altered by the electron beam (Chan et al., 2004; Braun et al., 2005). Furthermore, the N-K edge indicates the presence of nitrogen with a C/N ratio close to 1/20. Both observations are consistent with the presence of biomass in the cellular membrane (Watteau et al., 1996). A small Ca *L*2,3 edge located at ~346 eV is also present, indicating that some calcium is bound to *D. lacustre* cell surfaces. No carbonate ions were detected, however, implying that the surface of *D. lacustre* cells is not the site of carbonate precipitation. The EELS spectrum from the thin film enveloping *D. lacustre* cells shows a well-developed Ca *L*2,3 edge, indicating the presence of calcium, and a C-K peak with a maximum at 288.5 eV and a small sharp peak at 290–290.5 eV. The first peak corresponds to organic carbon and the sharper transition is due to a carbonate bonding (Benzerara et al., 2005). These observations confirm that the thin film around *D. lacustre* cells is made of EPS and indicate that calcium, but also carbonate ions, are bound to it. The EELS spectrum from both small and large globules shows a well-defined C-K edge with a maximum of the sharp π^* transition at 290.5 eV, which is typical of the carbonate ion (Garvie et al., 1994; Benzerara et al., 2005). In addition, a large Ca *L*2,3 edge (Fig. 2) and a P *L*2,3 edge (at 138 eV, data not shown) always appear. Several hundred EELS analyses of globules indicate that the carbonate material present in the globules has approximate elemental ratios of Ca/C = 1.2, Ca/P = 2.6, and Ca/O = 0.3 that likely correspond to a complex mixture of carbonates, phosphates, and organic carbon. No electron diffraction pattern is produced by the globules, implying that the calcium carbonate they contain is amorphous. Although minor amounts of calcium carbonate are bound to EPS and to small globules, both the abundance of large globules and their elevated carbonate content indicate that the bulk of carbonate precipitation takes place on large globules.

DISCUSSION

We have shown that *D. lacustre* promotes calcium carbonate precipitation by producing spherical nucleation centers that calcify extensively when released to the culture medium. The initial steps of globule formation occur under an EPS film in intimate association with the bacterial cell surface. Several biological materials could compose the nuclei of the globules. Nanometer-scale spheres similar to the *D. lacustre* globules have been formed in laboratory experiments by nucleation of hydroxyapatite on biologic macromolecules such as phospholipids and proteins (Cisar et al., 2000; Vali et al., 2001; Benzerara et al., 2004). Alternatively, globule formation could commence on EPS fragments or on membrane vesicles (Beveridge, 1999) originating from the *D. lacustre* cell wall. All of these materials share the origin from the bacterial surface and the capacity to nucleate metal ions and are thus possible candidates as globule nuclei.

Regardless of the nature of the globule nuclei, the nucleation process we describe clarifies a controversial issue of microbial calcification. The binding of metal ions on metabolically active microbial cells is inhibited because the cell envelope is protonated (Urrutia et al., 1992); in our experiment very limited calcification takes place on the cell surface and on the materials adjacent to it. In contrast to this picture, laboratory experiments have shown that metabolically active SRB are capable of nucleating carbonates extensively (van Lith et al., 2003b), although these experiments failed to identify the site of carbonate precipitation. Calcium carbonate precipitation on globules in the environmental fluid, away from the cell surface, solves this apparent ambiguity.

The fact that precipitation takes place on the globules instead of on the bacterial cell wall could be beneficial to *D. lacustre* because it decreases the chances of cell entombment. In addition, due to carbonate precipitation, the concentration of alkalinity and calcium in the aquatic environment surrounding the SRB cells decreases, leading to favorable chemical conditions for bacterial life (Hammes and Verstraete, 2002). However, it remains to be understood whether globule formation is a physiological mechanism involved in the prevention of cell entombment or if it takes place in response to conditions independent of the saturation state of the medium with respect to carbonate minerals.

Nano-scale carbonate spheres have already been observed in microbial carbonate precipitation experiments (Vasconcelos et al., 1995; Warthmann et al., 2000). However, these observations were conducted at a larger scale than our TEM-EELS investigation, such that the origin and involvement of the nanospheres in carbonate nucleation could not be elucidated. In the experiment carried out by Warthmann et al. (2000, their Fig. 2A), in the first stages of carbonate ''dumbell'' development, nanometer carbonate grains form at the polar end of SRB cells. The similarity with the aggregates of large globules attached to the polar ends of *D. lacustre* is striking (Figs. 1B, 1C), and suggests that the initial stages of carbonate precipitation of Warthmann et al. (2000) are controlled by the nucleation process we describe in our experiment. In addition, nanometer-sized calcified spheres have been described extensively from sedimentary environments (Folk, 1993, 1999; Vasconcelos and McKenzie, 1997; Dupraz et al., 2004) and have controversially been interpreted as nanobacteria (Folk, 1993, 1999). This idea has been under serious criticism because the described forms are smaller than the theoretical lower size limit for microbial life (200–300 nm) (Maniloff, 1997). The globules produced in our precipitation experiment share the morphological and chemical character of putative

nanobacteria described from sediments, including the capacity to form colonies. Globule formation by bona fide bacteria is an alternative process to enzyme-driven tissue decay (Schieber and Arnott, 2003) that could explain the origin of nanobacteria-like objects in sedimentary environments. Taken together, the widespread occurrence of nano-scale calcified spheres in the natural environment and the results of our nucleation experiment suggest that nucleation of calcium carbonate on microbially derived nanoglobules might be an important step in microbial calcification.

CONCLUSION

We described a new mode of microbial carbonate nucleation at the nanometer scale. Under our experimental conditions, the bulk of carbonate precipitation takes place on globules formed near the microbial cell wall that calcify significantly only when released in the aquatic environment surrounding microbial cells. This mode of precipitation may explain the origin of nano-sized calcified spheres that have been observed in microbial precipitation experiments and in many natural environments and could represent and important step in microbial calcification.

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