

# Aerobic microbial dolomite at the nanometer scale: Implications for the geologic record

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## ABSTRACT

**Microbial experiments are the only proven approach to produce experimental dolomite under Earth's surface conditions. Although microbial metabolisms are known to induce dolomite precipitation by favoring dolomite growth kinetics, the involvement of microbes in the dolomite nucleation process is poorly understood. In particular, the nucleation of microbially mediated dolomite remains a matter for investigation because the metabolic diversity involved in this process has not been fully explored. Herein we demonstrate that *Halomonas meridiana* and *Virgibacillus marismortui*, two moderately halophilic aerobic bacteria, mediate primary precipitation of dolomite at low temperatures (25, 35 °C). This report emphasizes the biomineralogical implications for dolomite formation at the nanometer scale. We describe nucleation of dolomite on nanoglobules in intimate association with the bacterial cell surface. A combination of both laboratory culture experiments and natural samples reveals that these nanoglobule structures may be: (1) the initial step for dolomite nucleation, (2) preserved in the geologic record, and (3) used as microbial tracers through time and/or as a proxy for ancient microbial dolomite, as well as other carbonate minerals.**

## INTRODUCTION

The ability of living microbes to control and direct precipitation of minerals is undoubtedly an important geobiological process. Microbially induced carbonate precipitation has been inferred in continental and marine settings under oxic conditions (Casanova et al., 1999). Nucleation of the carbonate mineral dolomite [CaMg(CO<sub>3</sub>)<sub>2</sub>] is an excellent example of how microbes are able to overcome kinetic barriers to facilitate precipitation (Vasconcelos et al., 1995; Roberts et al., 2004; Wright and Wacey, 2005; Sánchez-Román, 2006).

Despite comprehensive studies on dolomite formation at low temperatures, little is known about the role of microorganisms in its formation and nucleation. The observed relationships between microbial cells and carbonate minerals in natural environments indicate that microbes directly participate in nucleation processes (Vasconcelos et al., 1995; van Lith et al., 2003; Sánchez-Román, 2006). Laboratory experiments provide evidence supporting this hypothesis (Warthmann et al., 2000; Bosak and Newman, 2003; Sánchez-Román et al., 2007; Bontognali et al., 2008). Microbial cell surfaces and excreted extracellular polymeric substances (EPS), which carry a net negative electric charge and have the capacity to bind Ca<sup>2+</sup> ions, are frequently cited as being the sites of carbonate nucleation (Rivadeneyra et al., 1996; Dupraz et al., 2004; Sánchez-Román

et al., 2007). Aloisi et al. (2006) reported that nucleation of Ca carbonate occurs on nanoglobules produced by sulfate-reducing bacteria and hypothesized that nucleation of carbonates on microbial cell material may have been the dominant mode of microbial carbonate formation throughout the geologic record.

Here we report the results of an investigation of how microbes mediate the nucleation and formation of dolomite using aerobic culture experiments at 25 and 35 °C. We present a high-resolution transmission electron microscopy (TEM), atomic force microscopy (AFM), scanning electron microscopy (SEM), and mineralogical study of the microbial dolomite precipitates. A nanoscale comparative study of modern and ancient dolomite samples was carried out to test the validity of the nanoglobule formation process and determine if the process can be observed in geological samples. Because dolomite is: (1) the most stable carbonate mineral in the oceans, (2) a common mineral in ancient sedimentary rocks, and (3) rarely found to precipitate in modern environments, these observations can provide insight into an important microbial process in the geologic record.

## MATERIAL AND METHODS

The experiments were designed with two moderately halophilic aerobic bacteria, *Halomonas meridiana* ACAM 246 and *Virgibacillus marismortui* AJ009793 (see the GSA Data Repository<sup>1</sup>). These bacterial strains were surface inoculated on plates of solid medium D-1 (see the

Data Repository) and incubated at 25 and 35 °C. In order to detect the presence of precipitates, the plates were examined periodically with light microscopy. We performed pH measurements at the end of mineral formation experiments by direct application of pH-indicator paper (Merck Spezial-Indikatorpapier) on the semisolid surface. Parallel control experiments without bacteria and with dead bacterial cells were run for all conditions. After 30 days of incubation, crystal precipitates were recovered from both cultures.

To identify the mineral composition, X-ray diffraction (XRD) patterns of the precipitates were produced using a Bruker AXS D8 Advance Bragg-Brentano diffractometer with Cu K $\alpha$  radiation. A LEO 1530 scanning electron microscope equipped with an energy dispersive spectrometer (EDS) was used for imaging and elemental analysis of single crystals of dolomite samples from culture experiments and modern and ancient environments (see the Data Repository).

To investigate the involvement of *H. meridiana* in the nucleation of dolomite, we carried out TEM, AFM, and Raman microscopy studies (see the Data Repository).

## Experimental Results

Both *V. marismortui* and *H. meridiana* induce the precipitation of dolomite [CaMg(CO<sub>3</sub>)<sub>2</sub>] and hydromagnesite [Mg<sub>5</sub>(CO<sub>3</sub>)<sub>4</sub>(OH)<sub>2</sub>·4H<sub>2</sub>O] at 25 and 35 °C (Fig. DR1 in the Data Repository). Dolomite was found to be a major constituent in all culture experiments. No precipitate formation was observed in the control experiments. In all of the culture experiments, the time required for the initiation and extensive precipitation decreased with increased temperature (Table DR1). In all cultures, the quantity of crystals increased with increasing incubation time. The rate of crys-

<sup>1</sup>GSA Data Repository item 2008225, supplementary methods, Table DR1 (biochemical conditions of the culture media), Figure DR1 (X-ray diffractograms of the crystal formed in culture experiments), Figure DR2 (Raman spectrum of the extracellular organic film), and Figure DR3 (scanning electron microscope images of dolomite nanocrystal aggregates showing granulated texture), is available online at [www.geosociety.org/pubs/ft2008.htm](http://www.geosociety.org/pubs/ft2008.htm), or on request from [editing@geosociety.org](mailto:editing@geosociety.org) or Documents Secretary, GSA, P.O. Box 9140, Boulder, CO 80301, USA.

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tal growth was higher at 35 °C than 25 °C, as observed by optical microscopy. A significant increase in pH occurred in the cultures with living bacteria, from the original pH 7.2 of the D-1 medium up to 9 (Table DR1). No change in pH was detected in the control experiments.

AFM observations show mineral precipitates <200 nm in diameter, termed nanoglobules (Fig. 1A). The size and distribution are irregular. Most of the nanoglobules are 50–100 nm and the remaining globules are 100–200 nm (large globules). The nanoglobules are composed of dolomite (Fig. 1C) and occur attached to the surface of *H. meridiana* cells, where they are in some cases embedded in a thin organic film that envelops the cells. This organic film is produced by *H. meridiana* during its growth and it is composed of EPS, as demonstrated by Raman spectroscopy analyses (see the Data Repository and Fig. DR2). In a detailed view (Fig. 1B), we can observe that 100% of the surface of the *H. meridiana* cell is covered by nanoglobules.

Figure 1C shows the average of three Raman spectra collected from nanoglobules formed in culture experiments and from biogenic dolomite (used as a reference material), respectively. The spectra were collected with an acquisition time of 60 s each. In order to remove the broad fluorescence background, a baseline correction was performed. For precise positioning, an AFM image was recorded, the AFM tip was parked on a nanoglobule, and the laser beam of a combined AFM-confocal Raman setup (see the Data

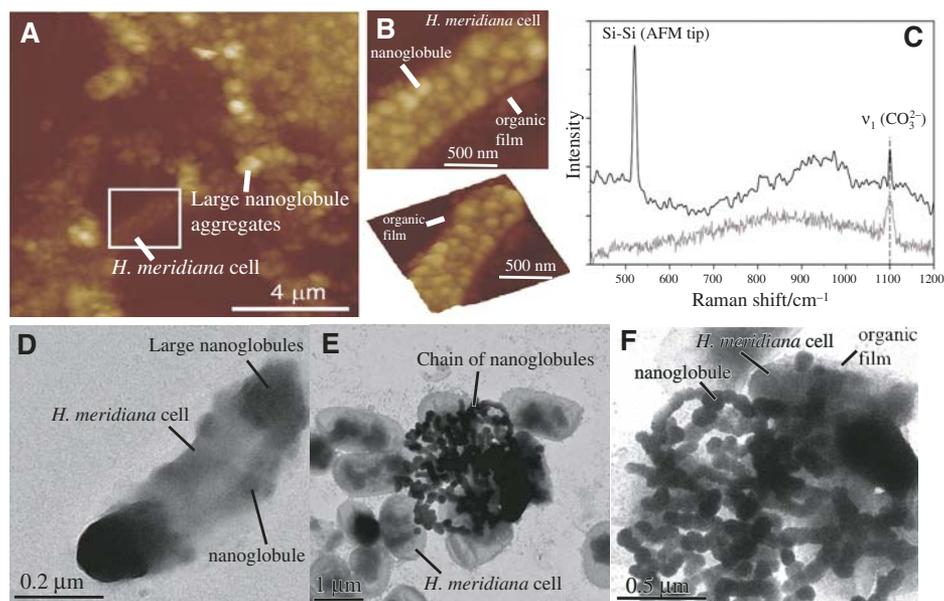
Repository) was focused onto the tip apex. The spectrum consists of two Raman bands. First, the band at 520 cm<sup>-1</sup> can be assigned to the Si-Si stretching vibration of the AFM tip material. Second, the band at 1100 cm<sup>-1</sup> is the most prominent band in the Raman spectrum of dolomite. This was confirmed by a reference measurement of biogenic dolomite powder (see spectrum in Fig. 1C). The band at 1100 cm<sup>-1</sup> in both spectra (nanoglobules and reference material) is in good agreement with the symmetric stretching vibration of CO<sub>3</sub><sup>2-</sup> (ν<sub>1</sub>) in dolomite as described in the literature (Edwards et al., 2005).

The TEM investigation of the microbial precipitates shows large (100 nm) and small (50 nm) nanoglobules attached to the surface of *H. meridiana* cells (Fig. 1D), as observed with AFM. Also, chains of nanoglobules are seen attached to *H. meridiana* surface cells (Figs. 1E and 1F). Figure 1F shows that *H. meridiana* cells and nanoglobules are embedded in a thin organic (EPS) film. Most of these observations are very similar to results recently reported for cultures using sulfate-reducing bacteria (Aloisi et al., 2006; Bontognali et al., 2008).

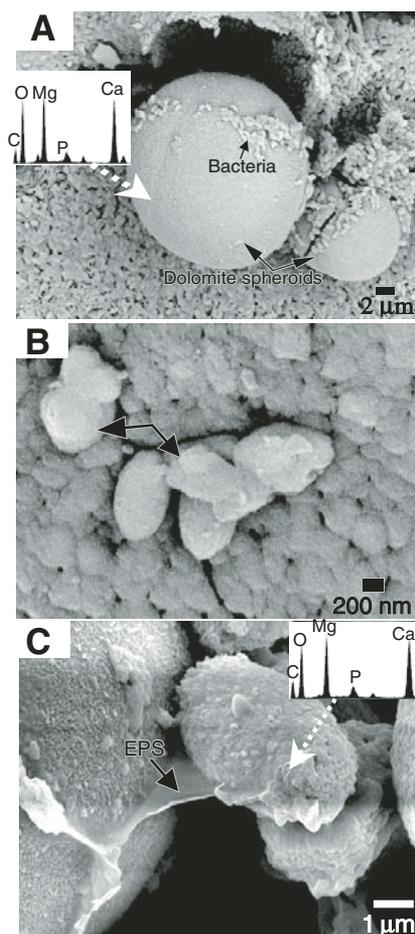
An SEM investigation of the studied precipitates shows that in all culture experiments, we obtained dolomite with spheroidal and ovoidal morphology (Fig. 2). These objects are as much as 15 μm in diameter, and appear to be formed by aggregates of nanoglobules (Figs. 2B and 2C). These nanoglobules are morphologically similar to the nanoglobules

observed with AFM and TEM (Fig. 1), suggesting that these microspheres and ovoids are composed of aggregates of single dolomite nanoglobules. In some cases, mineralized bacteria can be clearly recognized (Figs. 2B). EDS microanalyses of the crystals and nanoglobules confirm the XRD and Raman analyses results, showing the presence of dolomite in all samples. The stoichiometry of dolomite crystals is well defined by a constant intensity ratio of Mg and Ca peaks in EDS spectra (Fig. 2; see formula for dolomite in Table DR1).

SEM observations of modern dolomite and ancient dolomite reveal the presence of spheroidal nanostructures showing a granulated



**Figure 1.** Atomic force microscopy (A, B), Raman spectra (C), and transmission electron microscopy (D, E, F) images of dolomite nanoglobules formed in *Halomonas meridiana* culture. A: General view showing aggregates of nanoglobules. Most of field of view is occupied by aggregates of large globules. B: Detail of *H. meridiana* cell covered by nanoglobules (<200 nm), which are embedded in thin organic film. C: Raman spectra of both nanoglobules formed in culture experiments and biogenic dolomite (reference material). D: Detail of *H. meridiana* cell in intimate association with large and small nanoglobules. E: *H. meridiana* cells covered by nanoglobule chain. F: Detail of the nanoglobule chain shown in E.



**Figure 2.** Scanning electron microscope photomicrographs of microbial dolomite precipitates. A: Dolomite spheroids precipitated in *Halomonas meridiana* culture at 35 °C. Note that bacteria are closely related to the dolomite spheroids (arrows). B: Detail of surface (A) with dolomitized bacteria (arrows) appearing to rest on nanoglobular crystals. C: Ovoidal dolomite, precipitated in *Virgibacillus marismortui* culture at 25 °C, embedded in organic film and covered with nanocrystals. Energy dispersive spectrometry scans indicate the relatively equal proportions of Mg and Ca in the selected samples (dotted arrow). EPS—extracellular polymeric substances.

texture. The majority of these structures consist of nanoglobule aggregates (Figs. 3A and 3B). Possible nanoglobules can be seen on the surfaces of ancient dolomites (Figs. 3C and 3D). These nanoglobules are similar in size (~80 nm in diameter) to those observed in dolomite precipitates from our culture experiments (see Figs. 1, 2B, and 2C).

## DOLOMITE PRECIPITATION AND NUCLEATION

Because no precipitation occurred in the sterile cultures (without bacteria and with dead bacteria cells), globule formation apparently takes place only in the presence of bacteria and in a chemical environment favoring carbonate precipitation. *H. meridiana* and *V. marismortui* metabolic activity involves production of  $\text{NH}_3$  by means of oxidative deamination of amino acids.  $\text{NH}_3$  creates an alkaline microenvironment around a cell. This is consistent with the pH rise we observed from an initial value of 7 to final values of 8.5–9 (Table DR1).  $\text{CO}_2$  is also produced by the bacteria;  $\text{CO}_2$  dissolves and transforms into either  $\text{HCO}_3^-$  or  $\text{CO}_3^{2-}$  at higher pH. In the presence of  $\text{Ca}_{(\text{aq})}^{2+}$  and  $\text{Mg}_{(\text{aq})}^{2+}$ , at some point the culture medium becomes supersaturated with respect to dolomite. All these physicochemical changes show the influence of the bacteria in the geochemical alteration of their close environment and, undoubtedly, in the mediation of dolomite precipitation. The same mechanism could explain the formation of dolomite under aerobic conditions in natural

environments rich in nitrogenated organic matter, provided that there is enough  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  available. This explanation is supported by the fact that *V. marismortui* was isolated from a dolomitic sediment rich in organic matter, with a pH approaching 9 (Sánchez-Román, 2006).

Our AFM and SEM studies demonstrate that dolomite crystals nucleate on bacterial nanoglobules, as previously suggested for calcium carbonate precipitation (Aloisi et al., 2006). The initial steps of nanoglobule formation occur in the external bacterial envelopes and in some cases within an EPS film in intimate association with the bacteria cell surface (Fig. 1). This EPS film originates during the bacterial growth and has the capacity to bind metal ions. It is, thus, another possible candidate to form globule nuclei together with the external bacterial envelopes. We have shown that metabolically active moderately halophilic aerobic bacteria are capable of nucleating dolomite extensively and we have identified the site of dolomite formation.

The nanoglobules attached to the bacteria cell surfaces (Fig. 1), and some dolomitized bacteria within some dolomite crystals (Fig. 2B) provide evidence that bacterial precipitation of dolomite may begin with the accumulation of equal amounts of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the external bacterial cellular envelopes and/or in the organic film (EPS) and be followed by precipitation on nanoglobules on the cell surface. Bontognali et al. (2008) demonstrated the essential role of EPS in dolomite precipitation in anaerobic culture experiments. We assume

that EPS likewise plays an important nucleation role in our aerobic cultures in conjunction with the cellular surface.

Based on the results of our culture experiments, we propose that the presence of aerobic bacteria can mediate dolomite precipitation and overcome the recognized kinetic barriers to dolomite formation (Land, 1998). Furthermore, these aerobes may play an active role in the formation of this mineral in natural environments.

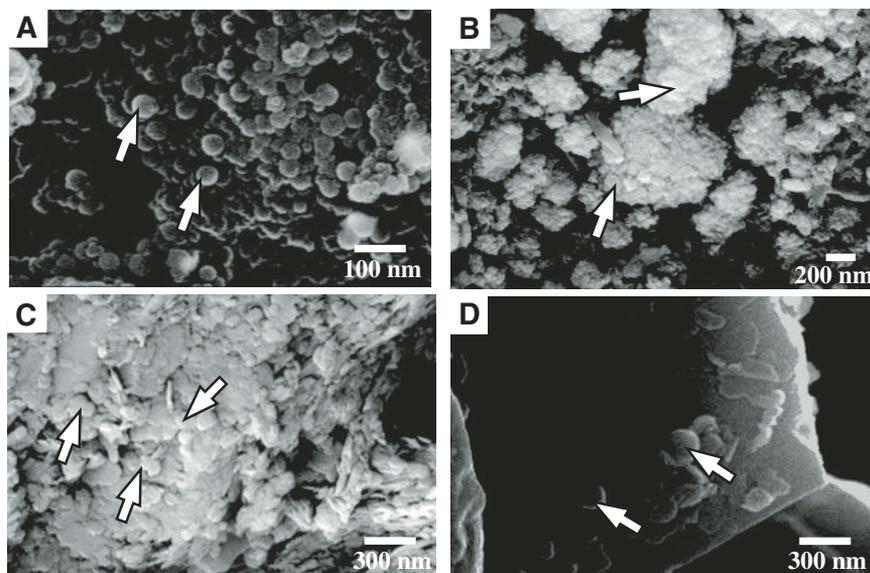
## Crystal Morphology Versus Nanoglobules in Geologic Samples

Folk (1993) first reported 20–200 nm nanobacteria in calcite and aragonite crystals observed in SEM. The term nanobacteria was used because they occur in chains or clusters and have been initially associated with dwarf forms of bacteria having a diameter only 1/100th of ordinary bacteria (Folk, 1993; McKay et al., 1996). Folk and Chafetz (2000) proposed that nanobacteria are not stress forms of normal bacteria, but may be a class of organisms in their own right. They examined the quantitative occurrence of the nanobacteria in various limestone components and extended their occurrence through geologic time.

We have found nanometer spheres occurring with granulated texture in natural dolomite from different locations of various geologic ages. The geological samples are (1) dolomite from modern environments (Figs. 3A and 3B; Figs. DR3A, DR3B, and DR3C), (2) Triassic dolomite (Fig. 3C), (3) Proterozoic dolomite (Fig. 3D), and (4) Archean dolomite (Fig. DR3D). The occurrence of nanostructures in all of these samples could be related to microbial mediation as observed in dolomite formed in aerobic cultures (present work) and in carbonates formed in anaerobic cultures (Aloisi et al., 2006; Bontognali et al., 2008).

The development of nanoglobules is an important process in the nucleation of carbonate minerals. Furthermore, the preservation of such structures in the rock record could provide a record to trace microbial processes through geologic time. The controversy regarding the existence of nanobacteria fossils (Folk, 1993, 1999) remains because a definitive argument against this hypothesis has never been given. However, recent studies (Aloisi et al., 2006; Benzerara et al., 2006) provide an alternative explanation to interpret nanostructures and nanoscale organic signatures in carbonates. Furthermore, our study, using SEM, AFM, and TEM techniques at the nanometer scale, may better explain the remarkable observations of Folk (1993, 1999) concerning the involvement of microorganisms in mineral nucleation.

The spheroidal growth morphology we observe here is similar to that of natural dolomites found in many modern settings (e.g.,



**Figure 3. Scanning electron microscope photomicrographs of dolomite nanocrystal aggregates showing granulated texture. Samples are from modern environments. A: Modern dolomitic stromatolites from Lagoa Vermelha. B: Dolomite from Coorong lagoon, South Australia. C: Triassic dolomite from the Hauptdolomit Formation of Engadin region of Switzerland. D: Proterozoic dolomite from Omkyk Member, Nama Group, Namibia. Note that dolomite crystals are formed by aggregates of nanoglobules (indicated by arrows). These nanoglobules range from 30 to 100 nm in diameter.**

von der Borch and Jones, 1976) and in the rock record (Mastandrea et al., 2006). The similarity between the morphology of the dolomite formed in culture experiments and some natural dolomites can be considered as further evidence favoring the microbial origin of such dolomites. The similarity of the nanoglobule aggregates (Fig. 3) to the nanoglobules (Figs. 1 and 2) and chains of nanoglobules attached to the cell surface (Figs. 1E and 1F) suggests that dolomite microspheres are composed of aggregates of nanoglobules and/or of chains of nanoglobules. Previously, similar nanometer spheres have been found in hydroxyapatite and Ca carbonate nucleated in laboratory culture experiments (Vali et al., 2001; Benzerara et al., 2004; Aloisi et al., 2006), in dolomite and aragonite from modern environments (von der Borch and Jones, 1976; Benzerara et al., 2006), and in Triassic dolomitic stromatolites (Perri and Tucker, 2007). In summary, we propose that the similar nanometer-sized calcified spheres from sedimentary environments, previously described as nanobacteria (Vasconcelos et al., 1995; Dupraz et al., 2004; Folk, 1993, 1999), most probably are nanocrystals of carbonate minerals with globular morphology (i.e., nanoglobules) at the early stage of nucleation. Furthermore, the granulated texture observed in modern and ancient dolomites probably represents an agglomeration of dolomite nanoglobules.

## CONCLUSIONS

Studies at the nanoscale show that dolomite nucleation occurs on nanoglobules attached to microbial cell surfaces. Our results have major implications for understanding microbially induced dolomite and other carbonate formation at Earth's sedimentary conditions. These results provide significant evidence for the origin of some sedimentary carbonate deposits and suggest that microbial signatures could be preserved in the rock record. Furthermore, our observations provide an alternative interpretation for nanometer objects frequently found in dolomite crystals and in other types of carbonate crystals. To interpret nanoglobules as biosignatures, however, requires better insight into the mechanisms occurring at the organic-inorganic boundary.

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