FORMATION OF CALCITE BY CHEMOLITHOAUTOTROPHIC BACTERIA – A NEW HYPOTHESIS, BASED ON MICROCRYSTALLINE CAVE PISIOIDS

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Abstract: A new mechanism, stimulating the precipitation of calcite, is postulated. The supersaturation with respect to carbonate minerals is changed, as a result of CO₂ consumption by chemolithoautotrophic, hydrogen-oxidizing bacteria. This mechanism controls the growth of atypical, microcrystalline cave pisoids in Perlová Cave, in Slovakia. The pisoids grow under calm conditions in rimstone pools, where they are bathed continuously in stagnant water. The water is supersaturated, with respect to calcite and aragonite. The bacteria inhabit the outer parts of the pisoids, covered by biofilms. The biofilm influences the supply of the Ca²⁺ ion, slows down the precipitation rate, and favors calcite precipitation over that of aragonite. The calcite initially precipitates as bacterial replicas, which further act as seeds for the growing calcite crystals. This process leads to the obliteration of the primary, bacterial fabrics. Since hydrogen-oxidizing bacteria occur in a wide spectrum of natural habitats, the mechanism of calcification, postulated above, also may operate in other environments.

Key words: microbial carbonates, biomineralization, biofilm, speleothems, Carpathians.

Manuscript received 20 November 2012, accepted 20 December 2012

INTRODUCTION

Bacteria are ubiquitous organisms, existing almost everywhere, from the deep subsurface to the atmosphere. They have the ability to stimulate the precipitation of minerals, both inside and outside their cells (see Ehrlich, 1996, 1999, for review). The role of bacteria in the precipitation of carbonate minerals has been discussed over the last hundred years and it has been confirmed, both in nature and the laboratory (Riding, 2000). Several mechanisms, driven by non-photosynthetic bacteria, lead to the precipitation of carbonate minerals. Most of them involve heterotrophic bacteria (Castanier et al., 2000; Wright and Oren, 2005).

The precipitation of carbonate minerals, under the influence of bacteria, has been recognized in marine and terrestrial environments. In terrestrial settings, this process is operative in soils (Boquet et al., 1973; Monger et al., 1991; Braissant et al., 2003), tufas (Pedley, 2000), travertines (Renaut and Jones, 2000), and caves (e.g., Jones, 2001, 2010, 2011b; Melim et al., 2001; Northup and Lavoie, 2001; Barton and Northup, 2007; Blyth and Frisia, 2008; Baskar et al., 2011). Jones and MacDonald (1989) and Jones (2009) have documented microcrystalline layers in cave pisoids (called cave pearls) from Grand Cayman Island that originated under the influence of microbes. The origin of the majority of the cave pisoids, which are composed of sparry crystals, has been attributed mainly to physicochemical processes that are controlled largely by the supersaturation levels of the parent water, with respect to calcite or aragonite (e.g., Gradziński and Radomska, 1967; Hill and Forti, 1997, p. 84–86; Nader, 2007; Melim and Spilde, 2011).

The present account describes a mechanism, by which chemolithoautotrophic, hydrogen-oxidizing bacteria can influence the precipitation of calcite and in this way play a critical role in the formation of microcrystalline cave pisoids. As well, similar, but as yet unrecognized, mechanisms can operate in other environments.

ENVIRONMENTAL SETTING

Perlová Cave (in Slovak Perlová jaskyňa) is located in Slovakia, in the northern part of the Great Fatra Mountains (in Slovak Velká Fatra), which form part of the Western
Carpathians (Fig. 1). Its entrance is in Belanská Valley, at an altitude of 910 m (19°06’06”E, 48°57’46”N). The cave is developed in bedded, Middle Triassic limestone, belonging to the Krížná unit, which was thrust over a Mesozoic, autochthonous cover of the crystalline core of the Great Fatra Mountains (Mahel, 1968). The area above the cave is covered with a deciduous forest and the thickness of the rocks above the cave is about 10 m.

The cave is 408 m long (Fig. 2; Holúbek and Kleske, 1993). Its internal temperature, according to measurements by the authors, varies between 5.1 ºC and 6.8 ºC. The water is ponded in small, stepped rimstone pools (Fig. 3). The depth of the pools ranges from 2 cm to 6 cm, the largest being 1 × 1.2 m. Each pool contains from about a dozen to several hundred pisoids. The water is supplied only during the rainy season, by dripping and mainly by spilling over the rim from the higher pools to the lower ones. The water in the pools is nearly stagnant. Intact, fragile moonmilk rims testify that the water is never strongly agitated. No pisoids are cemented to the pool bottom.

**MATERIALS AND METHODS**

Water temperature, pH, and specific electrical conductance (SEC) were measured in the field. The total alkalinity (as bicarbonate HCO₃) was determined, using 0.05 molar HCl acid by Gran titration. Chloride (Cl) contents were determined by the method of Mohr, using 0.01 molar AgNO₃, while nitrate (NO₃) contents were determined by the capillary electrophoresis method, using 270 AH-T equipment, a Perkin-Elmer product. The concentrations of other components were determined by inductively coupled plasma-atomic emission spectroscopy (ICP AES), using a Perkin-Elmer product OPTIMA 7300DV. The DIC and equilibria were calculated for water samples, using the program PHREEQC (Parkhurst and Appelo, 1999). The saturation index (SI) has been applied, as a measure of equilibrium, according to the formula: SI = log (IAP/KT), where IAP is an ionic activity product for a given mineral, and KT is a solubility product for that mineral.

Some pisoids were collected aseptically, placed in autoclaved glass flasks, stored in a refrigerator and delivered to the laboratory within 24 hours. For microbiological analysis, 10 g of each sample were centrifuged in physiological
salt, shaken and later incubated at 20 °C and 35 °C from 1 to 21 days. The growth of microorganisms was systematically monitored. The following, microbiological media were used for isolation: Beef Extract – Nutrient Broth – Merck, Trypticase Soy Broth (Soybean-Casein Digest Medium) – BioMerieux, Nutrient Agar – Merck, TSA (Trypticase Soy Agar) – BioMerieux, Soil Extract Agar (Atlas and Parks 1997), Iron Bacteria Isolation Medium (Atlas and Parks, 1997) and Actinomycetes Isolation Agar (Atlas and Parks, 1997). Morphology, Gram stain and biochemical properties of the bacteria were analyzed to identify the microorganisms. Species identification was based on Bergey’s Manual of Determinative Bacteriology and Bergey’s Manual of Systematic Bacteriology (Holt, 1989, 1994). Since there are no standard, biochemical tests for the majority of isolated genera, the biochemical tests were individually selected, according to diagnostic manuals.

The pisoid internal structures were studied under a scanning electron microscope (SEM) JEOL 5410, coupled with a microprobe (EDS) Voyager 3100 (Noran product). To prevent the collapse of the organic structure, some samples were treated, using procedures for biological samples, that is, immediately plunge-frozen in isopentane, cooled by liquid nitrogen and then lyophilized. Organic matter from other samples was removed, using H₂O₂ prior to SEM examination. Standard thin sections were also made from the pisoids. Their mineralogy was determined, using the XRD method and IR spectroscopy.

**RESULTS AND INTERPRETATION**

The water is mainly of the Ca-HCO₃ type (Table 1). All water samples were supersaturated, with respect to calcite, and many were also supersaturated, with respect to aragonite. The pisoids are mostly flattened spheres, up to 2 cm across. They are relatively soft and lack nuclei. Low-Mg, microcrystalline calcite is their only authocthonous carbonate phase. They have rough surfaces and mammilated laminations, with microstromatolitic structures (Fig. 4A). The laminations are visible, owing to concentrations of non-carbonate particles, incorporated into the pisoid cortices, which was confirmed by EDS (Fig. 5; see also Jones, 2009; Gradziński et al., 2010).

The microbiological analyses revealed various strains of bacteria that inhabit the pisoids. Bacteria, belonging to a physiologically defined hydrogen-oxidizing (knallgas) group (Arango and Schlegel, 1991), were identified in each sample studied (Table 2). Species of *Xanthobacter* were the most common. Dinitrogen-fixing bacteria, belonging to the genus *Arthrobacter*, occurred in each sample. No fungi were detected.
Table 1

Chemistry of pool water from Perlová Cave

<table>
<thead>
<tr>
<th>Pool number</th>
<th>t (°C)</th>
<th>pH</th>
<th>Eh (mV)</th>
<th>TDS (mg/L)</th>
<th>HCO₃ (mg/L)</th>
<th>SO₄ (mg/L)</th>
<th>Cl (mg/L)</th>
<th>NO₂ (mg/L)</th>
<th>Ca (mg/L)</th>
<th>Mg (mg/L)</th>
<th>Na (mg/L)</th>
<th>K (mg/L)</th>
<th>DIC (mmol/L)</th>
<th>Si calcite</th>
<th>Si aragonite</th>
</tr>
</thead>
<tbody>
<tr>
<td>P 1</td>
<td>5.9</td>
<td>8.56</td>
<td>423</td>
<td>332.5</td>
<td>215.1</td>
<td>14.06</td>
<td>2.59</td>
<td>16.14</td>
<td>77.88</td>
<td>5.76</td>
<td>&lt;0.2</td>
<td>0.85</td>
<td>3.314</td>
<td>1.02</td>
<td>0.86</td>
</tr>
<tr>
<td>P 4</td>
<td>5.9</td>
<td>8.56</td>
<td>421</td>
<td>342.9</td>
<td>236.9</td>
<td>10.33</td>
<td>3.04</td>
<td>8.46</td>
<td>78.17</td>
<td>5.31</td>
<td>&lt;0.2</td>
<td>0.65</td>
<td>3.608</td>
<td>1.05</td>
<td>0.89</td>
</tr>
<tr>
<td>P 5</td>
<td>5.9</td>
<td>8.50</td>
<td>423</td>
<td>342.4</td>
<td>230.2</td>
<td>12.71</td>
<td>2.88</td>
<td>12.40</td>
<td>77.85</td>
<td>5.54</td>
<td>&lt;0.2</td>
<td>0.74</td>
<td>3.592</td>
<td>0.98</td>
<td>0.82</td>
</tr>
<tr>
<td>P 6</td>
<td>5.7</td>
<td>8.47</td>
<td>427</td>
<td>333.9</td>
<td>208.6</td>
<td>17.49</td>
<td>2.44</td>
<td>23.88</td>
<td>73.97</td>
<td>6.29</td>
<td>&lt;0.2</td>
<td>0.90</td>
<td>3.274</td>
<td>0.89</td>
<td>0.73</td>
</tr>
<tr>
<td>P 8</td>
<td>5.6</td>
<td>8.43</td>
<td>422</td>
<td>354.5</td>
<td>238.0</td>
<td>15.49</td>
<td>2.94</td>
<td>12.25</td>
<td>75.66</td>
<td>9.48</td>
<td>&lt;0.2</td>
<td>1.71</td>
<td>3.733</td>
<td>0.92</td>
<td>0.64</td>
</tr>
<tr>
<td>P 9§</td>
<td>5.4</td>
<td>8.32</td>
<td>418</td>
<td>394.0</td>
<td>245.0</td>
<td>29.20</td>
<td>4.79</td>
<td>24.70</td>
<td>75.16</td>
<td>16.20</td>
<td>&lt;0.2</td>
<td>0.74</td>
<td>3.835</td>
<td>0.95</td>
<td>0.79</td>
</tr>
<tr>
<td>P 10§</td>
<td>5.5</td>
<td>8.39</td>
<td>423</td>
<td>389.6</td>
<td>260.7</td>
<td>16.64</td>
<td>2.55</td>
<td>18.56</td>
<td>74.41</td>
<td>15.95</td>
<td>&lt;0.2</td>
<td>0.75</td>
<td>4.116</td>
<td>0.90</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Note: Unless otherwise stated, mean data from three sampling trips; § – Mean data from two sampling trips

Table 2

Bacterial assemblage in pisoids from Perlová Cave

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>pool number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Agrimonkeyes sp.</td>
<td>+</td>
</tr>
<tr>
<td>Alcaligenes sp.</td>
<td>-</td>
</tr>
<tr>
<td>Arthrobacter crystallopoietes</td>
<td>-</td>
</tr>
<tr>
<td>Arthrobacter sp.</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus alkalophilus</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus azotoformans</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus badius</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus brevis</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas carboxyhydrogena *</td>
<td>+</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>-</td>
</tr>
<tr>
<td>Xanthobacter autotrophicus</td>
<td>-</td>
</tr>
<tr>
<td>Xanthobacter flavus *</td>
<td>+</td>
</tr>
<tr>
<td>Xanthobacter sp. *</td>
<td>+</td>
</tr>
</tbody>
</table>

* presence of given taxon; - absence of given taxon; * hydrogen-oxidizing (knallgas) bacterium

Under the SEM, the pisoid surface revealed a three-dimensional, alveolar-septal biofilm (Fig. 4B), resembling that described by Défarge et al. (1996) from modern stromatolites of the Pacific region. The structure is built predominantly of extracellular, polymeric substances (EPS). It closely resembles the surface of a Xanthobacter colony, which grew in laboratory conditions (Fig. 4C). Since bacteria, belonging to this genus, excrete copious amounts of slime (Wiegel, 1991; Braissant et al., 2003), they probably play a major role in producing the biofilm, covering the pisoids studied.

The pisoids are built of calcite crystals of various shapes and of bodies, formed by organic matter, as suggested by EDS analyses. The rod-like bodies are ~0.5 µm wide and 0.8 to 3 µm long, whereas the globular forms are <1 µm in diameter. They agglomerate in clumps, covered with EPS (Fig. 4D, E). The dimensions and shape of the organic bodies described, along with the presence of living bacteria in the samples studied, suggest that, despite their minute dimensions, the bodies in question represent living, bacterial cells. They occur only in the outer part of the pisoid cortex, up to a few millimeters below the surface (Fig. 6). A similar phenomenon is also typical of the terrestrial oncidoids, described by Jones (2011a).

The largest crystals, up to a few micrometres across, predominantly occur in the central parts of pisoids, whereas small crystals are dominant in the outer parts, close to the surface of the pisoids. Although the former are for the most part irregularly shaped, some exhibit faces and edges. The latter, up to 3 µm long, commonly show rounded edges and display circular cross-sections. Careful examination under the SEM did not reveal such crystals, attached to the surface of the biofilms that cover the pisoids (Fig. 6A). This indicates that these crystals were not trapped and bound by the sticky biofilm, which covers the surface of the pisoids. Thus, they are an autochthonous component, which originated within a pisoid. They are remarkably similar in shape and size, to the bacterial cells, described above, and never exceed significantly their dimensions (Fig. 4F). This similarity suggests that such crystals are three-dimensional calcite replicas of bacterial cells. They formed by the crystallization of calcite around the living cell or just after the death of the organism (see Jones and Kahle, 1986).

The observations under the SEM revealed several generations of calcite crystal formation. Some bacterial cells, although still built of organic matter, are covered with minute, irregular mineral particles, 0.1 µm across, mostly calcite, and reflect an early step of calcite replica formation (Fig. 4H). Later on, the crystallites coalesced (Fig. 4I) and, in consequence, form a continuous crust on the bacterial surface. The replicas and bundles of fibrous calcite subsequently served as the substrate for the further growth of calcite crystals. The biofilm macromolecules limited the growth of crystals to fine, microcrystalline sizes (see Arp et al., 1999). During the decomposition of the biofilm, further growth of crystals is possible.

Apart from the small, anhedral crystals, single, needle-like crystals and filamentous crystals also occur in the outer parts of the pisoids (Fig. 4G). The latter are ~0.2 µm wide. They are curved and closely intertwined, hence their length was difficult to estimate; it probably exceeds 10 µm. Similar, filamentous crystals are known from various, continental carbonates (see Jones and Kahle, 1993; Verrecchia and Verrecchia, 1994 for review) and are regarded as bio-
genic (Gradziński et al., 1997; Loisy et al., 1999; Cañaveras et al., 2006; Bindschedler et al., 2010) or purely abiogenic precipitates (Borsato et al., 2000). Their origin is also ascribed to the precipitation of calcite, due to a solution–precursor–solid mechanism, in the presence of dissolved, organic matter in a parent solution (Olszta et al., 2004; Cañaveras et al., 2006).

Small, anhedral and filamentous crystals were successively overgrown with calcite (Fig. 4J). The process led to complete obliteration of the primary, bacterial fabrics of the pisoids (Fig. 6), as previously described from tufa stromatolites by Szulc and Smyk (1994) and from travertines by Guo and Riding (1994).

**DISCUSSION**

The internal structures of the pisoids studied show that they differed markedly from most speleothems, displaying distinct, crystalline fabrics, even those growing beneath the water level (González et al., 1992; Frisia et al., 2000), including typical cave pisoids (Nader, 2007; Melim and Spilde, 2011). The difference arises, in spite of the fact that the pisoids grew in very similar conditions to other speleothems and are supplied with water of similar chemistry. It implies that the pisoid growth is governed by different factors than that of crystalline speleothems. The pisoids studied bear a strong, structural resemblance to microbial carbonates, which along with the occurrence of living bacteria within the pisoids, indicates that their growth can be promoted microbially. It seems relevant to discuss how bacteria can influence the process of calcification and thus the formation of the pisoids.

The calcification takes place around the bacterial cells, so that the process is of external type (Riding, 1991), which may be driven solely by environmental conditions or by microbial physiology. Rapid degassing of CO$_2$ can be excluded as the main factor, driving calcite precipitation, since the pisoids grow in stable conditions, in a calm-water setting, completely bathed in stagnant pool water. It suggests that another factor, such as bacterial physiology, may stimulate calcite precipitation.

The sequence of crystal growth, described above, from a single, mineralized bacterial cell to a more regular, developed crystal, shows that calcification developed progressively from the mineralized, bacterial cells. Hence, it is similar in style to cyanobacterial calcification in a low DIC–high Ca$^{2+}$ hard-water setting (see Table 1), where the photosynthetic activity causes carbon removal and creates a local shift in supersaturation (Arp et al., 2001, 2010; Shiraishi et al., 2008). Bearing in mind a specific cave environment, such an activity should be ruled out. Thus, the hypothesis can be formulated that a crucial role is played by chemolithotrophic, hydrogen-oxidizing bacteria in the formation of calcite. They actively take up CO$_2$ from their surroundings, because it is their major source of carbon (Ara-
They can live in a speleal environment, where the supply of organic carbon is strongly limited, owing to a chemolithoautotrophic mode of life, depending on inorganic sources of energy. They grow on CO₂, gaseous oxygen, and gaseous hydrogen. Considering the accessibility of the two first components, the supply of gaseous hydrogen seems to be of crucial importance, as it occurs in minute amounts in most natural environments, including caves. In the case studied, it is most probably a by-product of co-occurring, dinitrogen-fixing bacteria, belonging to the genus Arthrobacter (see Smyk and Ettlinger, 1963; Jones and Keddie, 1991).

Biofilms influence the supply of reactants, since they have diffusion-slowing properties (Decho, 2000). In the pisoids studied, the sticky biofilm slows down the transportation of Ca²⁺, which in turn slows the precipitation reaction. This leads to the precipitation of calcite, and inhibits the formation of aragonite, even though the macroenvironment is supersaturated with respect to both minerals. A similar phenomenon was experimentally demonstrated by Buczynski and Chaftz (1991), where the higher viscosity of the medium favored bacterially induced calcite precipitation over that of aragonite.

The process of calcification, induced by chemolithoautotrophic bacteria, postulated above and so far unrecognized, corresponds to the ‘dark CO₂ fixation’, proposed by Krumbein (1979) and Simkiss (1986). The hydrogen-oxidizing bacteria are frequent in a great variety of natural habitats: soils, modern lake sediments, hot-springs and even sea water (Aragno and Schlegel, 1991; Bae et al., 2001; Aguilar et al., 2004). Authigenic carbonate minerals of microcrystalline type are formed in all of these environments. Hence, the influence of hydrogen-oxidizing bacteria may also explain the origin of other, not only speleal, microcrystalline carbonates. Gradziński (2003) also suggested that this type of calcification influences the oxygen stable isotopic signature of calcite.

Nonetheless, the above hypothesis is to some extent speculative. Firstly, it is based only on the classic determination of microbes. Actually, it is known that only a small percentage of microbes in samples from the cavern environment can be cultivated and determined (Northup and Lavioie, 2001). Therefore, in the samples studied, other microbes also may have been present and they could have influenced calcium carbonate precipitation, as well.

Secondly, the hydrogen-oxidizing bacteria, determined in the pisoids, are only facultative autotrophs that also can grow on organic media (Aragno and Schlegel, 1991). The possibility cannot be excluded that in a way of life, other than chemolithoautotrophic, they might induce the precipitation of calcium carbonates. For instance, bacteria, belonging to the genus Xanthobacter, which are common in the pisoids studied, can utilize calcium oxalate and produce calcium carbonate. Such a phenomenon was recognized in a soil extract from Ivory Coast (Braissant et al., 2004). Such bacteria are also known for their capability to stimulate the precipitation of vaterite (Braissant et al., 2003).

Thirdly, there exists a great body of literature on the precipitation of minerals within biofilms and microbial mats (see Dupraz et al., 2009 for review). Several mechanisms of carbonate mineral precipitation are known to occur without the interaction of living organisms (organomineralization sensu Trichet and Défarge, 1995; biologically-influenced mineralization sensu Dupraz et al., 2009). It cannot be ruled out that a part of the precipitation in the pisoids is produced by these autochthonous microbial communities.
CONCLUSIONS

Chemolithoautotrophic, hydrogen-oxidizing bacteria can cause biologically induced calcification. The essence of the process is a shift in calcite supersaturation, due to biogenic CO₂ consumption. Bacterial biofilms, because of their diffusion-slowing properties, inhibit the precipitation of aragonite and thus promote the precipitation of calcite. The presence of a biofilm limits the size of the growing calcite crystals. Thus, the bacteria stimulate the formation of microcrystalline cave pisoids and influence their internal fabrics. However, it must be emphasized that this view is based exclusively on the classic determination of microorganisms. It should be supported additionally by modern, molecular methods of investigation.

Acknowledgments

The paper is an outgrowth of M. Gradziński’s PhD thesis, supervised by the late Professor Andrzej Radomski. Peter Holùbek, Jaga Faber, Mariusz Czop, Renata Jach and Grzegorz Haczewski are thanked for their help. The study was financed by KBN grant 6P04D01914. M. G. was supported by the Foundation for Polish Science (J. Kazmierczak Grant for Researchers). An early version of the manuscript benefited from the constructive comments of Brian Jones. The authors are indebted to reviewers Leslie A. Melim and Tadeusz Peryt, as well as to editors Frank Simpson and Alfred Uchman for their help in improving the manuscript.

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