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

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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 CO_2 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 Ca^{2+} 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.

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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 Radomski, 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

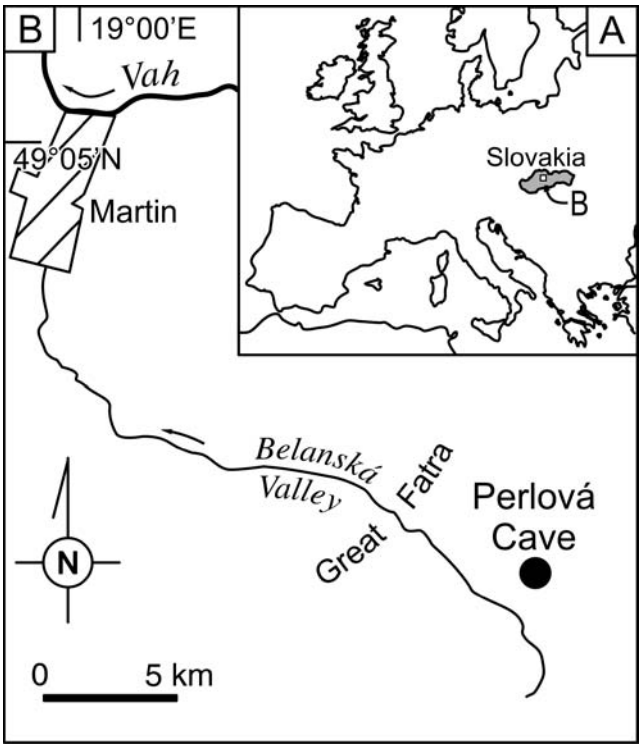

Fig. 1. Location of Perlová Cave

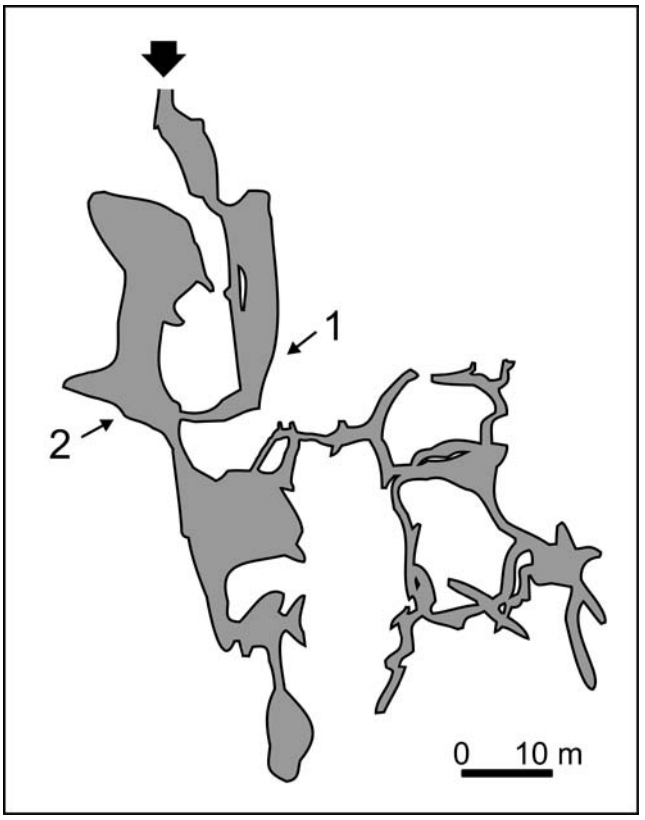

Fig. 2. Map of Perlová Cave, after Holúbek and Kleskeň (1993), simplified; big arrow indicates cave entrance, arrows indicate sampling sites: 1. Pearl Passage (Perlová chodba) – pools 1, 4–6; 2. Parliament Chamber (Parlament) – pools 8–10

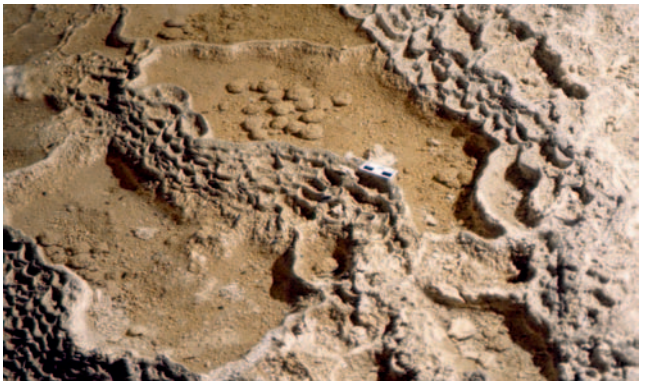

Fig. 3. Stepped pools with pisoids, Pearl Passage, scale bar is 3 cm long. Photograph from Gradziński (2001)

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 thrusted over a Mesozoic, autochthonous cover of the crystalline core of the Great Fatra Mountains (Maheľ, 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 Kleskeň, 1993). Its internal temperature, according to measurements by the authors, varies between 5.1 °C and 6.8 °C. 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 HCO_3) 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 $AgNO_3$, while nitrate (NO_3) 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

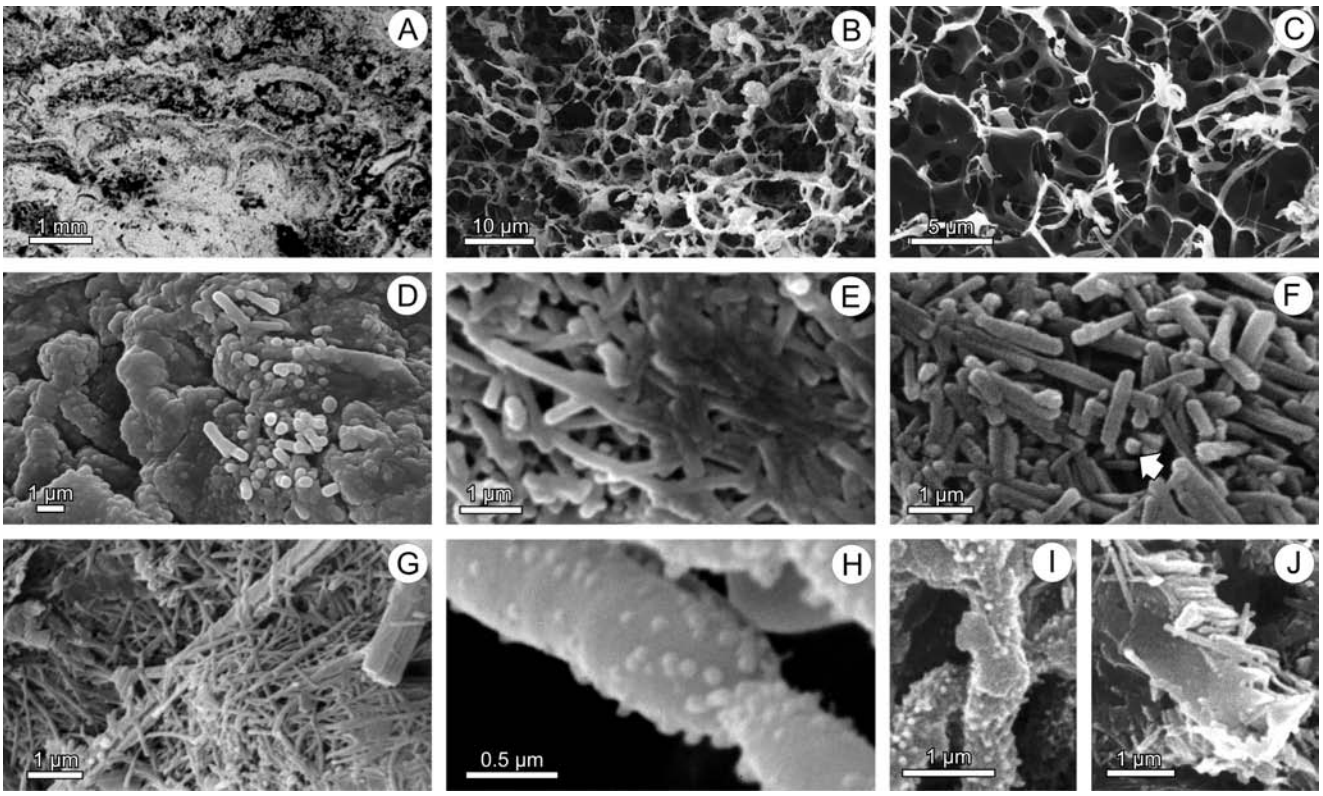

Fig. 4. Internal structure of cave pisoids. **A** – Irregular lamination of pisoid. **B** – EPS building alveolar-septal framework on pisod surface. **C** – Surface of *Xanthobacter* colony, growing in laboratory. **D** – Bacterial fabrics of pisoid. **E** – Rod-shaped, bacterial cells, partly covered by EPS. **F** – Calcite replicas of bacterial cells; note circular cross-sections of replicas (arrow). **G** – Needle and filamentous calcite crystals. **H** – Bacterial cells with small calcite particles, the first step of replica formation. **I** – Calcite crust, growing on bacterial surface. **J –** Overgrowth of small crystals with calcite, leading to formation of largest crystal. A – thin section, B–J under SEM. Samples in B–E and G–J were plunge-frozen in isopentane, cooled by liquid nitrogen and then lyophilized; sample in F was treated with H_2O_2 to remove organic matter. Photographs A, D, F, J from Gradziński (2001)

salt, shaken and later incubated at 20 °C and 35 °C from 1 to 21 days. The growth of micro-organisms was systematically monitored. The following, microbiological media were used for isolation: Beaf Extract – Nutrient Broth – Merck, Trypticase Soy Broth (Soybean-Casein Digest Medium) – BioMerieux, Nutrient Agar – Merck, TSA (Trypcase 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 proprieties of the bacteria were analyzed to identify the micro-organisms. 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 H_2O_2 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-HCO_3 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 autochthonous carbonate phase. They have rough surfaces and mammilated lamination, with microstromatolitic structures (Fig. 4A). The lamination is 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 (Aragno and Schlegel, 1991), were identified in each sample studied (Table 2). Species of *Xanthobacter* were the most common. Dinitrogen-fixing bacteria, belonging to the genera *Arthrobacter*, occurred in each sample. No fungi were detected.

Table 1

Chemistry of pool water from Perlová Cave

Pool number	t (°C)	pH	Eh (mV)	TDS (mg/L)	HCO_3 mg/L)	SO_4 (mg/L)	Cl (mg/L)	NO_3 (mg/L)	Ca (mg/L)	Mg (mg/L)	Na (mg/L)	K (mg/L)	DIC (mmol/L)	SI calcite	SI aragonite
P 1	5.9	8.56	423	332.5	215.1	14.06	2.59	16.14	77.88	5.76	<0.2	0.85	3.314	1.02	0.86
P 4	5.9	8.56	421	342.9	236.9	10.33	3.04	8.46	78.17	5.31	<0.2	0.65	3.608	1.05	0.89
P 5	5.9	8.50	423	342.4	230.2	12.71	2.88	12.40	77.85	5.54	<0.2	0.74	3.592	0.98	0.82
P 6	5.7	8.47	427	333.9	208.6	17.49	2.44	23.88	73.97	6.29	<0.2	0.90	3.274	0.89	0.73
P 8	5.6	8.43	422	354.5	238.0	15.49	2.94	12.25	75.66	9.48	<0.2	1.71	3.733	0.92	0.64
P 9§	5.4	8.32	418	394.0	245.0	29.20	4.79	24.70	75.16	16.20	<0.2	0.74	3.835	0.95	0.79
P 10§	5.5	8.39	423	389.6	260.7	16.64	2.55	18.56	74.41	15.95	<0.2	0.75	4.116	0.90	0.75

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

Bacteria	pool number					
	1	4	5	6	9	10
Agromyces sp.	+	+	+	–	–	–
Alcaligenes sp.	–	–	–	–	+	–
Arthrobacter crystallopoietes	–	+	+	+	–	+
Arthrobacter sp.	+	+	+	+	+	+
Bacillus alcalophilus	+	–	–	–	+	+
Bacillus azotoformans	–	–	–	+	–	+
Bacillus badius	–	–	–	+	–	–
Bacillus brevis	–	+	–	+	–	–
Bacillus megaterium	+	+	+	+	–	–
Pseudomonas carboxyhydrogena *	+	–	+	+	–	–
Pseudomnas sp.	–	–	+	–	+	–
Xanthobacter autotrophicus *	–	–	–	+	+	+
Xanthobacter flavus *	+	+	+	–	+	–
Xanthobacter sp. *	+	–	–	–	+	–

+ 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 oncoids, 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, both 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, most probably 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-

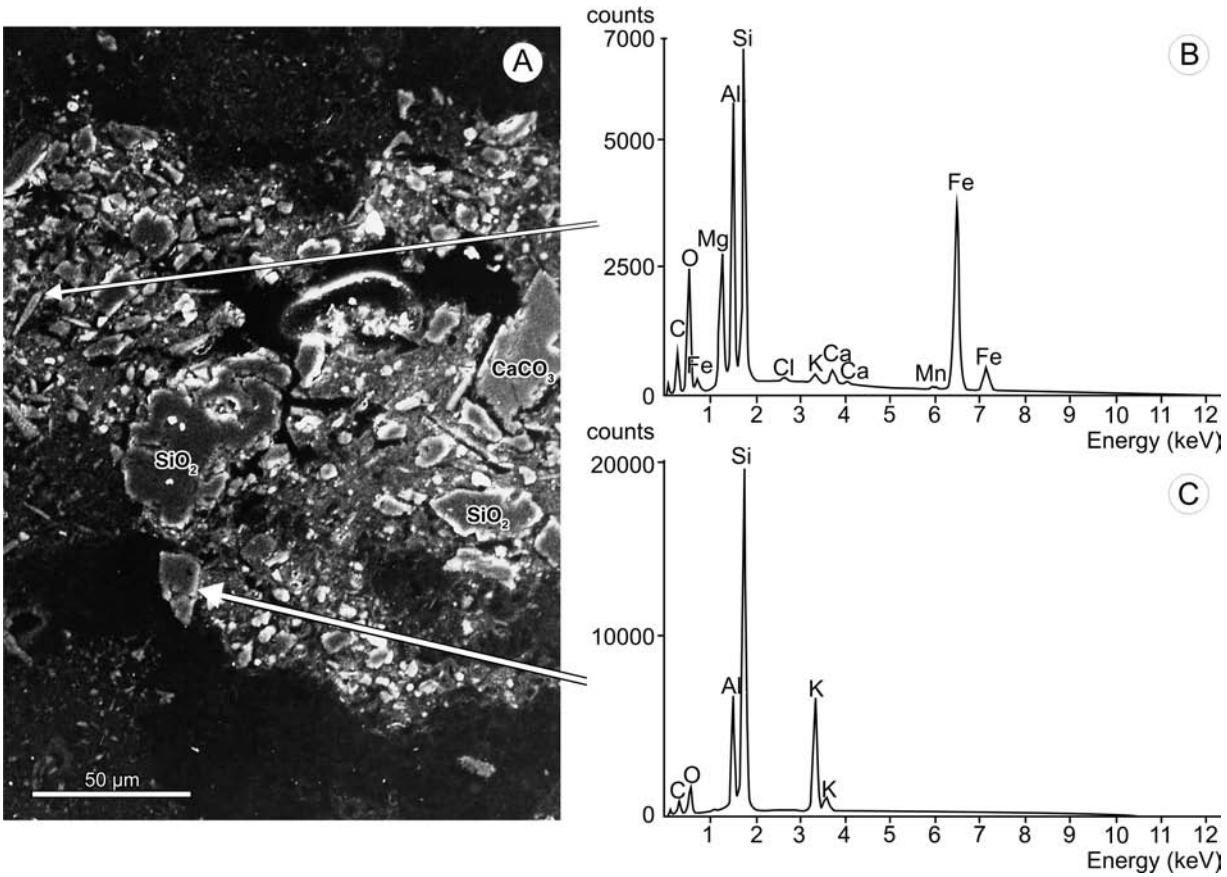

Fig. 5. Laminae, composed of detrital grains within pisoid. **A** – polished thin section under SEM, chemical composition of some grains is indicated. **B, C** – EDS spectra of alumnosilicate, detrital grains

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 chemoautolithotrophic, 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-

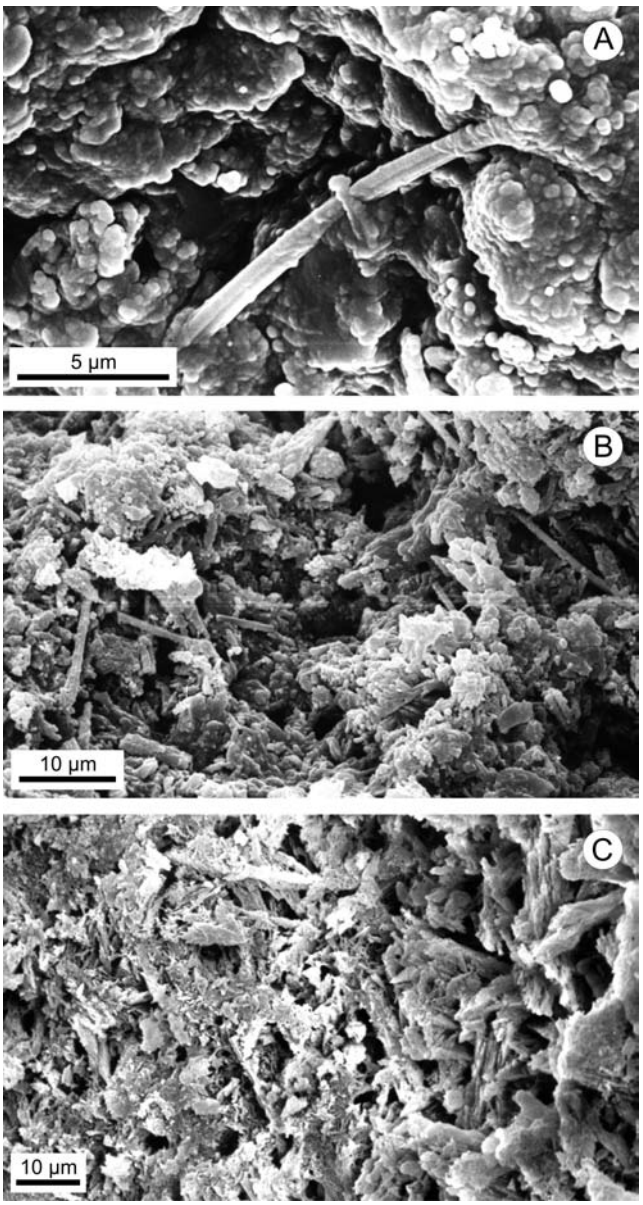

Fig. 6. Contrasting fabrics of different parts of pisoid. **A** – outermost part of pisoid, composed of irregular clumps of globular and rod-like bodies, covered with EPS and needle-fibre calcite. **B** – well developed needle-fibre calcite and microcrystalline calcite aggregates in outer part of pisoid (around 3 mm beneath the surface). **C** – aggregates of spiky calcite crystals aligned along their long axes and microcrystalline calcite aggregates, central part of pisoid. Samples were plunge-frozen in isopentane, cooled by liquid nitrogen and then lyophilized

gno and Schlegel, 1991). Thus, they cause depletion of dissolved CO_2 in their pericellular region, which leads to rapid conversion of HCO_3 to CO_2. This process results in alkalization of the microenvironment, and thus results in the creation of CO_3^{2-} ions, which finally causes calcite crystallization (Buhmann and Dreybrodt, 1985).

According to the above hypothesis, the rate of the process is controlled by the activity of hydrogen-oxidizing bacteria. They can live in a spelean 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 CO_2, 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 Ca^{2+}, 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 Chafetz (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 CO_2 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; Aguiar *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 spelean, 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 Lavoie, 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 one of these mechanisms operates within the biofilm, covering the pisoids in Perlová Cave. In such a case, it could have contributed to the crystallization of calcium carbonate and, hence, the formation of the pisoids studied.

Bearing in mind these reservations, the postulated influence of hydrogen-oxidizing bacteria on the precipitation of calcium carbonate should be tested, using other methods. The precipitation of calcium carbonate in cultures of such bacteria, conducted in monitored laboratory conditions, could test the hypothesis, formulated in this paper.

CONCLUSIONS

Chemolithoautotrophic, hydrogen-oxidizing bacteria can cause biologically induced calcification. The essence of the process is a shift in calcite supersaturation, due to biogenic CO_2 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.

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