Microbially induced calcite precipitation in culture experiments: Possible origin for stalactites in Sahastradhara caves, Dehradun, India

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Laboratory experiments involving calcium carbonate precipitation by bacteria isolated from stalactites sampled from three caves in Sahastradhara, Dehradun, India were conducted to determine whether geomicrobiological processes might be involved in stalactite formation. Dominant bacteria inhabiting the Sahastradhara caves, confirmed by PCR amplification of 16S rRNA genes (16S rDNA), were *Bacillus thuringiensis* and *Bacillus pumilis*. Culture experiments confirmed their role in mineral precipitation. The ability of these bacteria to form $CaCO_3$ crystals at different incubation temperatures (5, 15, 25, 32°C) indicated that 25°C was optimum for calcite precipitation. The microbial community detected by DAPI staining showed a significant number of cells (9×10^5 cells, g sed^{-1}). Application of fluorescence *in situ* hybridization techniques, based on the presence of rRNA, shows a large number of active microbial cells (around 55% of the total cell number). The microbial community is dominated by Eubacteria, mainly sulphate-reducing bacteria (representing 10% of the total microbial community), but Archaea were also present. Thin section petrography reveals that the stalactites consist of microcrystalline calcite, which occurs in chains probably attributed to bacterial precipitation. Thus, microbial activity and optimum temperature appear to be key factors promoting calcite precipitation and ultimately stalactite formation.

Keywords: Bacteria, biomineralization, calcite, caves, geomicrobiology.

MICROORGANISMS have been agents for geochemical change for over 85% of the earth's history, and linkages between the geochemical and biological evolution of the earth are profound. It is widely accepted that microorganisms were largely responsible for production of oxygen in the earth's atmosphere and that through their metabolism they can dramatically alter elemental distributions. Interactions between the biosphere and geosphere are complex because organisms are able to transform the chemistry of their environment. Calcium carbonate precipitation is a general phenomenon in the bacterial world under appropriate conditions[1]. Indeed, some bacteria and fungi can induce precipitation of calcium carbonate extracellularly through a number of processes that include photosynthesis, ammonification, denitrification, sulphate reduction and anaerobic sulphide oxidation[2–4]. Additionally, the activity of sulphate-reducing bacteria has been shown to mediate precipitation of dolomite[5,6]. The primary role of bacteria in the precipitation process has been ascribed to their ability to create an alkaline environment through various physiological activities[3,7]. In addition to field observations, calcium carbonate has been formed in the laboratory in association with different bacterial cultures, such as marine bacteria[8], soil bacteria[9], *Pseudomonas flurescens*, *Myxococcus xanthus*[10], and various other autotrophic and heterotrophic bacteria[3].

Caves host diverse microbial populations and are sites of active mineral precipitation. Chemical processes mediated by microbial activity are fundamentally related to the distribution of microbes throughout the cave system. Mineral precipitation is, however, commonly considered to be abiogenic despite the fact that microbes are present in caves. Analysis of cave substrates from a geological perspective shows that microbes can mediate constructive and destructive processes. Microorganisms have been shown to be important active and passive promoters of redox reactions influencing geological processes. Potentially these processes can significantly influence the formation and preservation of any cave deposit. Although there is extensive documentation of microbial precipitation of calcium carbonate in the non-cave carbonate literature[11], studies of microorganisms in caves have been predominantly descriptive, with only a few experimental studies reported. The past decade has produced extensive research into microbial interactions with minerals within cave environments. Fungi, algae and bacteria are implicated in the precipitation of carbonate dripstone in caves[12,13]. A large variety of heterotrophic microbial communities in stalactites are well documented in cave ecosystems[14]. Monitoring modern sites of active precipitation can provide valuable insights

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into the factors that control precipitation and evolution of various cave deposits[15]. Various microbiological techniques have also been used to show that microbes are present in most cave environments and modify the composition of fluids and/or influence precipitation of various minerals, including calcite[16].

Microbial diversity in selected habitats cannot be comprehensively studied by traditional approaches. Because DNA analysis of bacteria provides no information on metabolism, physiology, ecology, biochemistry or geomicrobiology of a strain[16], only by growing microorganisms in the laboratory under controlled conditions is it possible to delineate their ability to alter the chemistry of their microenvironment and produce biominerals. Hence laboratory-based culture experiments, as well as geochemical and molecular biological techniques were used in this study to understand the possible extent of microbial involvement in stalactite formations in the Sahastradhara caves.

Geological setting

The study area is situated in the Dehradun Valley, a crescent-shaped intermontane valley formed within the Siwalik Formations in Garhwal Himalaya (Figure 1). The valley is separated from the Lesser Himalayan Formations in the north by a major intraplate thrust, the Main Boundary Thrust (MBT). The Siwalik group underlying the valley and the Southern Siwalik Range are thrust over the Indo-Gangetic Plain sediments along the Himalayan Frontal Fault, forming the Dehradun Valley as a piggyback basin[17]. The study area (lat. 30°17′ to 30°24′N; long. 78°00′ to 78°06′S) is between the Lesser Himalayan hills in the north and the topographic relief of the Siwalik ranges in the south, enclosed by the rivers Ganga in the east and Yamuna in the west. The thick alluvium deposit that fills the synclinal depression consists of eastern, central and western fans descending from the Lesser Himalayan hills, occupying a major portion of the area as surficial deposits. Coalescence of these fans has resulted in a piedmont plain[18]. Caves and circulating groundwater are an essential part of this terrain.

Sahastradhara, meaning place of the 'thousand-fold spring', is situated at a distance of 14 km from Dehradun. Of the 1545 caves throughout India[19], the Sahastradhara springs (Figure 2) are pH neutral as well as being exposed to light, allowing photosynthetic activity to take place. These caves are rather small in size (10 m long, 2 m wide), but they are important because of their exceptional beauty and the associated springs, which are of high medicinal value. The spring hosts a complex microbial community. The cold spring water flows downward about 9 m, leaving an incrustation of carbonate on the surface. Accumulating over the centuries, the incrustations form projecting ledges with caves, from the roofs of which falls a perpetual shower.

Methodology

Sample collection and preservation

Samples were taken from small stalactites, which were approximately 15–20 cm in length and 3–4 cm in diameter. For the geomicrobiological study, samples were brought to the laboratory (Geomicrobiology Laboratory, ETH, Zurich, Switzerland) and preserved at 4°C before aseptic cutting to collect uncontaminated sub-samples. Classical cultivation methods select specific organisms and are inadequate to quantify active cells. To overcome this 'plate count anomaly', the molecular approach with rRNA analysis was employed. Samples collected from different caves were fixed for 6 h in 4% para formaldehyde in phosphate buffer saline (PBS; 0.13 M NaCl, 7 mM Na_2HPO_4,

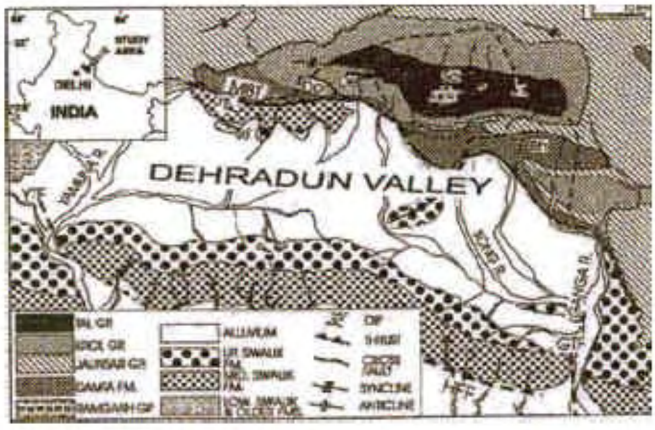

Figure 1. Regional geological map of the study area. YTF, Yamuna Tear Fault; GTF, Ganga Tear Fault; MBT, Main Boundary Thrust; HFF, Himalayan Frontal Fault. Drainage areas of the Donga fan (Do), Dehradun fan (De), Song River (So) and Bhogpur fan (Bh) (after Singh *et al.*[18]).

Figure 2. Stalactites from Sahastradhara caves, Dehradun.

3 mM NaH_2PO_4, pH 7.2 in water). Samples were washed in PBS and stored at –20°C in ethanol : PBS (1 : 1) before subsequent analysis.

Isolation of calcifying bacteria

Stalactite samples (1 g) were powdered in a mortar and pestle, suspended in 9 ml sterile saline solution and vortexed. Triplicate B-4 medium (2.5 g calcium acetate, 4 g yeast extract, 10 g glucose and 18 g agar per litre of distilled water[1]) spread plates were inoculated with sample dilutions ranging from 10^1 to 10^6 and incubated at 32°C for two weeks. Individual colonies were selected and purified by repeated streaking on B-4 agar. For short-term preservation, the isolates were streaked on B-4 agar slants and stored at 4°C. Bacteria were identified by DNA sequencing using identification methods – PCR amplification of 16S rRNA genes (16S rDNA) and Gapped BLAST and PSI–BLAST protein database search programs[20] at IMD (Institut für medizinische Diagnostik), Zurich.

Calcite precipitation by bacteria

Bacterial isolates cultured on B-4 agar were incubated aerobically at different temperatures (5, 15, 25, 32°C) for two weeks. Petri plates were examined for the presence of crystals by optical microscopy periodically for up to 25 days. Controls consisted of uninoculated culture medium along with experimental samples. The morphology and characteristics of the bacteria and crystals were studied by SEM–EDAX (Geophysics Laboratory, ETH) gold-shadowed and analysed.

Analysis of microbial communities

Total number of microorganisms was investigated using DAPI staining (4′,6-diamidino-2-phenylindole)[21]. Microbial community structure was studied by fluorescence *in situ* hybridization (FISH) using three general oligonucleotide probes targeting the domain level of Eubacteria (Eub338) and Archaea (Arch915), and most of the members of the δ-subclass of Proteobacteria, which are sulphate reducers (SRB385)[22]. Next 0.5 g of fixed samples was unfrozen and diluted with 20–40 ml of 0.1% pyrophosphate. The calcite present was dissolved using 0.1 M EDTA (pH 4.5) followed by centrifugation. Bacteria were detached using an ultrasonic homogenizer (2 min, power 7, Sonifer B-12 Branson sonic power). Then 20 µl aliquots were spotted onto gelatin-coated slides and air-dried. Next 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS, 30% formamide, pH 7.2), DAPI solution (final concentration of 20 ng ml^{-1}) and one of the oligonucleotide probes labelled with Cy3 fluorochrome (25 ng μl^{-1}) were placed onto the slides and incubated for 2 h at 40°C in the dark. Excess stain was washed in buffer (10 mM Tris, 5 mM EDTA, 0.01% SDS and 0.9 M NaCl) for 20 min at 48°C. Slides were air-dried and mounted with citifluor. The preparations were examined at 1000 x magnification (Zeiss Plan-Neofluar) with a Zeiss Axiophot microscope fitted for epifluorescence with a 50 W high-pressure mercury bulb and filter set for Cy3 (G 546, FT 560, BP 575-640) and filter set for DAPI (G365, FT395, LP420). Four hundred bacteria (10×40 fields) were counted per slide triplicate.

Mineralogy and geochemistry

Using a Scintag X-ray diffractometer (XRD) (Mineralogy/Petrology Laboratory, ETH), powdered stalactite samples were scanned at a rate of 1° per minute in the scanning range of 2θ values between 4° and 50° and the spectra of minerals produced were identified using the ICDD database (JCPDS)[23]. Electron microscope studies were conducted using a SEM CamScan CS44LB equipped with an energy dispersive X-ray system (SEM–EDAX). Representative chip samples were mounted on stubs and coated with gold of thickness 100–150 Å and then transferred to the sample chamber of the instrument prior to imaging. Major element geochemistry was analysed using XRF (Geochemistry Laboratory, ETH).

Results and discussion

Direct microscopy revealed the presence of a variety of indigenous heterotrophic bacteria, actinomycetes, cyanobacteria and mosses. *Bacillus thuringiensis* and *Bacillus pumilis* strains were the dominant bacteria identified. Colonies that formed minerals were isolated by repeated streaking. These pure cultures precipitated calcite *in vitro* (Figure 3 *a–f*). Actinomycetes cultures were also observed to precipitate calcite (Figure 4). Predominance of bacilli is in agreement with the established hypothesis that *Bacillus* species play a major role in carbonate deposition in natural habitats[1,24,25].

The temperature, number of strains and time required for calcite precipitation were studied by cultivating the strains at different incubation temperatures (5, 15, 25, 32°C). The number of bacterial strains increased from 5 to 25°C and the maximum number of strains was observed at 25°C. There was a decrease in the number of strains at 32°C (Figure 5). The optimum temperature for calcite precipitation was 25°C. Crystal precipitation at 25°C started after a week of inoculation (Figure 6). Petri plates were examined periodically up to 25 days by optical microscopy for the presence of crystals. At lower temperatures (5–15°C), crystal precipitation started after 15 days of inoculation (Table 1). In contrast, control experiments without bacteria precipitated no carbonate minerals.

Microscopic observations showed that this precipitation takes place in a microenvironment provided by bacterial

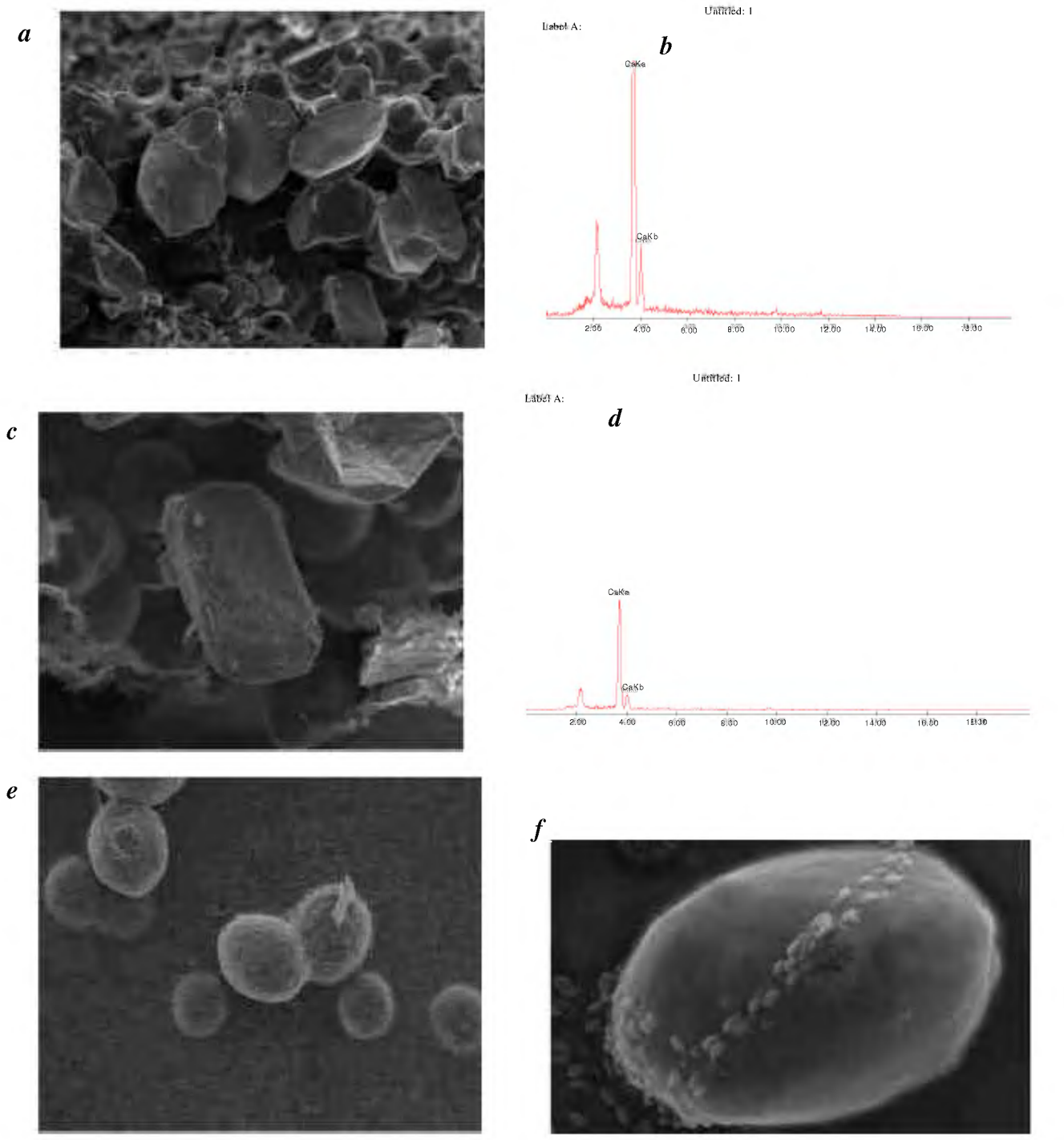

Figure 3. ***a***, ***c***, Calcite precipitated by *Bacilli* sp. ***b***, ***d***, SEM–EDAX of calcite crystals in (***a***) and (***c***) respectively. ***e***, Bacterial calcite seen as oval structures. ***f***, Bacteria across the crystal.

growth and metabolism. The size and quantity of crystals increased with time (Figure 7 *a*, *b*). These changes show the influence of bacteria in the geochemical alteration of their closed environment and undoubtedly, in the mediation of calcite precipitation. The nucleation and growth of calcite crystals occurred in an organic matrix and bacteria were found to be associated with mineral surfaces (Figure 3*f*), indicating that microbial mediation could be an active process. The organic mucus encapsulating the bacteria might be promoting diffusion gradients, whereby ions can diffuse through it and enable the development of physico-chemical conditions enhancing mineral precipitation. Culture

Table 1. Calcite precipitation at different temperatures

	Incubation temperatures (°C)				Calcite precipitated at various temperatures (°C)			
Microorganism	5	15	25	32	5	15	25	32
Bacillus sp.	+/–	++	+++	++	–	–	+++	++
Actinomycetes	–	+	++	++	–	–	+	+

–, No growth; +/–, Poor growth; +, Moderate growth; ++, Good growth; +++, Optimal growth.

Figure 4. Calcite precipitated by actinomycetes.

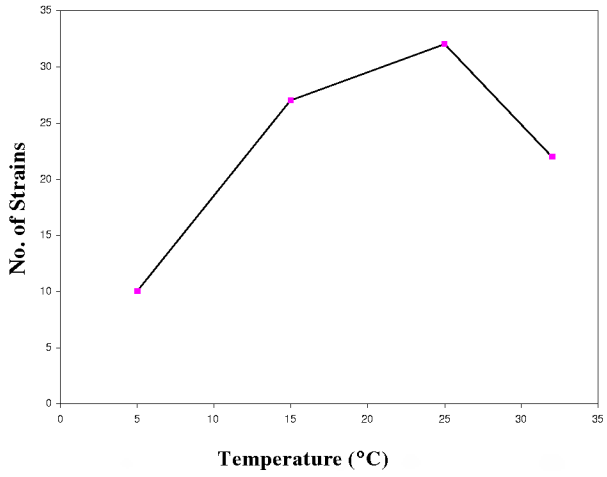

Figure 5. Temperature vs bacterial strains.

Figure 6. Mineral precipitation by bacilli.

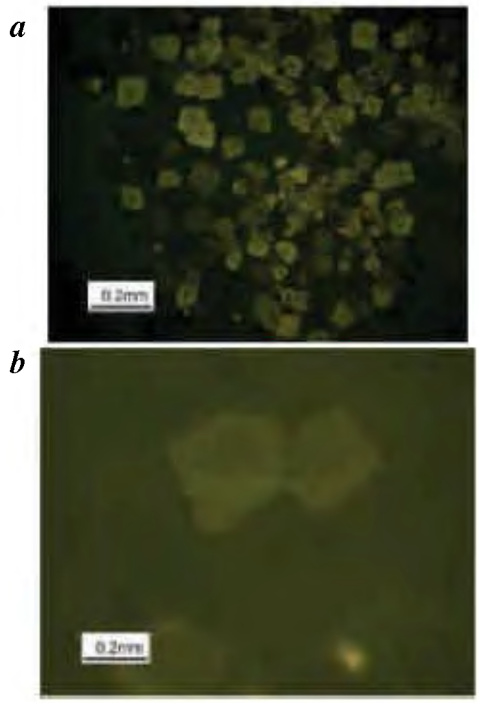

Figure 7. Bacterial calcite: ***a***, after a week and ***b***, after 20 days.

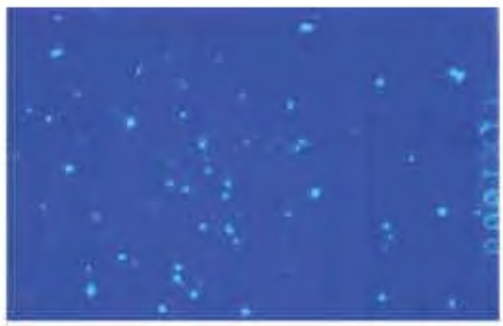

Figure 8. Microbes detected by DAPI staining.

experiments using sulphate-reducing bacteria in the mediation of dolomite formation in a synthetic anoxic hyper saline medium demonstrated the same[6,26].

An abundant microbial community (9×10^5 cells, g sed^{-1}) was detected by direct microscopic observation after DAPI staining (Figure 8). FISH demonstrates the presence of a large number of active microbial cells representing 55% of the total cell number. The percentage of hybridization obtained with the two domain probes (Eub and

Arch) depends on the content of ribosomes; FISH detects reliably only the physiologically active cells. The microbial community is dominated by Eubacteria, mainly sulphate-reducing bacteria (Figure 9; representing 10% of the total microbial community), but Archaea were also present (Figure 10). A significant fraction of these cells are active, indicating the high probability of their participation in biomineralization processes involved in stalactite formation[27]. A large number (about 26%) of detected Eubacteria belong to the δ-subgroup of Proteobacteria, which is the largest group of organisms capable of dissimilatory sulphate reduction[28].

XRD indicated that calcite is the dominant mineral, with aragonite and dolomite being present in trace amounts. EDAX analysis indicated that the Ca peak is strong, and rhombic morphology of calcite is observed. Major element geochemistry showed the presence of 42.86% CaO. Thin-section petrography reveals the presence of microcrystalline calcite. Microcrystalline calcite deposition observed in chains may be related to bacterial involvement in the precipitation process (Figure 11).

Calcium carbonate precipitation is governed by the calcium concentration, concentration of dissolved inorganic carbon, pH and availability of nucleation sites[3,29–32]. Microbial mineral precipitation resulting from metabolic activities of microorganisms can occur inside or outside the cells. Often, bacterial activity simply triggers a change in solution chemistry that leads to over-saturation and mineral precipitation.

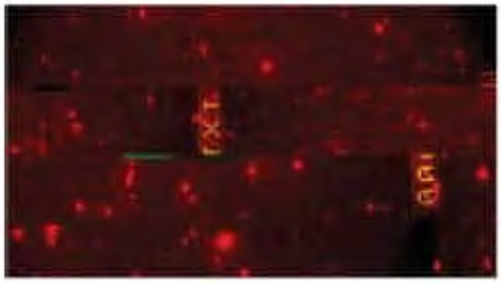

Figure 9. SRB detected after hybridization with Cy3-labelled sulphate-reducing bacteria probes.

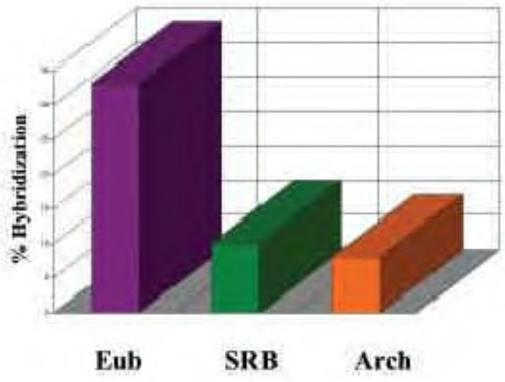

Figure 10. DAPI-stained cells (%) detectable after hybridization with Cy3-labelled sulphate-reducing bacteria (SRB), Eubacteria (Eub) and Archaea (Arch) probes.

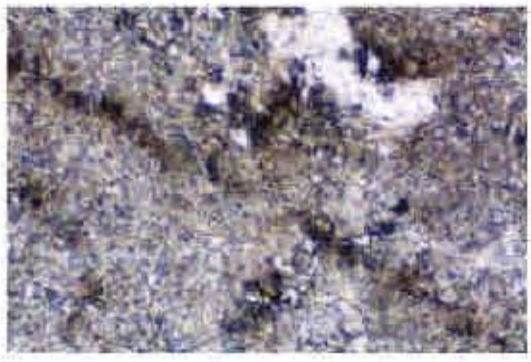

Figure 11. Micrite deposition observed in chains.

The role of heterotrophic bacteria (for example *Bacillus* sp.) in carbonate formation may be twofold. First, actively growing cells (or cell aggregates) alter their local environment by establishing a concentration gradient of calcium, magnesium, carbonate ions and pH, all of which are necessary to promote ideal conditions for carbonate formation. This is comparable to the role of the metabolic pathway of heterotrophic bacteria proposed for calcium carbonate formation, which consists of a passive process involving change in pH and increase in metabolic end products, such as carbonate ions[3,33], Secondly, the cell surfaces of metabolically active bacteria (SRB) are involved in the concentration of Ca^{2+} and Mg^{2+} ions around the cell[26].

In the present study, the metabolic activity of bacterial strains and temperature are key factors in carbonate deposition. The optimum temperatures have a positive effect on bacterial precipitation of calcite, increasing the ability of the strain to form crystals. Calcium carbonate precipitation chemically favours bacterial survival and proliferation[33]. Seepage of water saturated with carbonates has a partial pressure of CO_2 greater than that of the cave atmosphere. Degassing of CO_2 takes place from water to air leading to a decrease in the solubility of calcium carbonate, and hence saturation with respect to calcium carbonate and ultimately its deposition. The classical model is based on inorganic processes associated with carbonate solubility, shown by the following equation:

$$Ca^{2+} + 2HCO_3^- \Leftrightarrow CaCO_3\downarrow + CO_2\uparrow + H_2O.$$

The equilibrium constant expression for $CaCO_3$ dissolution/precipitation is: $K = [Ca^{++}]\ [CO_3^{--}]$. When K = Ksp* (solubility product constant in water at any given temperature, pressure and salinity), the solution is considered to be exactly saturated. Carbonate precipitation occurs when the product of the concentration of calcium and carbonate exceeds Ksp*. The metabolic activity of *in situ* microbes facilitates in exceeding the solubility product of carbonate minerals and also by providing nucleation sites that promote stability of mineral nuclei.

Conclusions

The culture experiments demonstrate that *B. thuringiensis* and *B. pumilis* mediate the precipitation of calcite under well-defined conditions. The optimum temperature for calcite precipitation was 25°C. The presence of an active microbial community with high biodiversity colonizing the Sahastradhara caves, suggests that carbonate precipitation could be mediated or catalysed by microbes, since microbial metabolism can influence the bicarbonate–carbonate equilibrium. Thus we propose that microbial activity and optimum temperature appear to be key factors promoting calcite precipitation and ultimately stalactite formation.

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