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Speleothems from Sahastradhara caves in Siwalik Himalaya, India: Possible biogenic inputs

SUSHMITHA BASKAR

Environmental Studies
School of Agriculture
Indira Gandhi National Open University
New Delhi - 110068, India

RAMANATHAN BASKAR

Department of Environmental Science and Engineering
Guru Jambheshwar University of Science and Technology
Hisar - 125001, Haryana, India
Corresponding author: rbaskargjuhisar@yahoo.com

JOYANTO ROUTH

Department of Water and Environmental Studies
Linköping University
581 83 Linköping, Sweden

Stalactites and moonmilk from Sahastradhara caves in Siwalik Himalayas were studied to understand the role of microbes in their genesis. Fourier spectroscopy in the moonmilk indicates a complex milieu of organic compounds that is unusual for inorganic formations. Stable C and O isotopes show trends in the moonmilk and stalactite, which suggest biogenic input; the geochemical inference is consistent with evidence from microscopy and laboratory-based microbial cultures. Light microscopy of moonmilk samples show the presence of a number of microbial forms similar to Cyanobacteria, and scanning electron microscope (SEM) images show microbial structures similar to Spirulina. The total number of microbial cells using SYBR Gold is 6.5×10^5 cells, g sed⁻¹ in moonmilk and 3.2×10^5 cells, g sed⁻¹ in stalactites. FISH indicates approximately 3.5×10^5 cells, g sed⁻¹ in moonmilk and 2×10^5 cells, g sed⁻¹ in stalactites. SEM images of the moonmilk indicate a large network of microbial fila-

ments along with minerals, which are identified as calcite based on their x-ray diffraction pattern. In vitro laboratory cultures with pure monogenic strains isolated from the moonmilk and stalactites raise pH in the medium, which facilitate calcite precipitation. The mineral precipitating isolates were identified as: Bacillus pumilis, B. cereus, B. anthracis, B. lentus, B. sphaericus, B. circulans and Actinomycetes. The Sahastradhara moonmilk and stalactites are colonized by a diverse microbial community and the isolated bacterial strains induce biomineralization on different nutrient media, supporting their biogenic origin.

Keywords: Bacteria, Biogenic deposits, Calcite, Speleothem

Introduction

Cave ecosystems are extreme environments as they are characterized by very low nutrient levels (Pedersen, 2000). Within a cave ecosystem, there are different micro-environments inhabited by various groups of microorganisms, which in turn are affected by specific conditions like: humidity, stable temperature, nutrients and pH (Mulec, 2002). A large number of these heterotrophic and chemolithotrophic microorganisms in caves are involved in lithogenic processes, e.g. speleothem deposition and cave enlargement (Engel et al. 2004; Cañaveras et al. 2006; Mulec et al. 2007). These microbial communities often display a strong association with mineral assemblages, particularly in carbonate formations (Krumbein, 1979; Danielli and Edington, 1983; Chafetz and Buczynski, 1992; Rivadeneyra et al. 1997; Castanier et al. 1999; Warthmann et al. 2000; Northup and Lavoie, 2001; Engel et al. 2004; Barton and Northup, 2007). Microbes actively influence physicochemical precipitation, resulting in the formation of calcite fibers of different morphologies and sizes (Cañaveras et al.

2006). Boquet et al. (1973) reported that CaCO_3 precipitation by bacteria is well-established and common. Microbial activity can bring about changes in the culture media and result in precipitation of carbonate minerals in laboratory experiments (Cañaveras et al. 2001). Carbonate mineralization by bacteria occurs through either active (photosynthesis, urea hydrolysis, sulfate reduction, and iron reduction) or passive pathways (nucleation sites). Microbial metabolic activities induce changes in the surrounding environment i.e. pH and the extent of these processes depends on the metabolized nutrients (Braissant et al. 2002; Portillo et al. 2009). Laboratory cultures have often reported precipitation of calcium carbonate by bacterial isolates in cave systems (Baskar et al. 2006, 2011; Cañaveras et al. 2001; Contos et al. 2001; Cacchio et al. 2001, 2003, 2004, 2012). However, not much is known about the molecular and biochemical processes of these microbial communities involved in carbonate precipitation (Hammes and Verstraete, 2002; Barabesi et al. 2007).

In this study, we investigate the role of microbes in speleogenesis in Sahastradhara caves, situated in Siwalik Himalaya, India. A combination of geochemical analyses, microscopic studies, biological assays, and laboratory based microbial experiments conducted in aseptically collected speleothem samples were studied to establish the biogenic nature of these deposits.

Study area and geology

The Sahastradhara caves (700 m.a.s.l; 30°17'to 30°24'N; 78°00'to 78°06'S) are situated at a distance of about 14 km from Dehradun city in Uttarakhand, north-west India. The caves are located in a crescent shaped intermontane valley in the Neoproterozoic dolomitic rocks belonging to the Krol formation (Tewari, 2007). There are seven small caves, which are ca. 10 m long and 2 m wide, and they are easily ac-

cessible. Geologically, the valley is divided into three regions: the Lesser Himalaya, Siwalik Himalaya, and the synclinal basin. The Dehradun Valley lies between the Ganges River in the east and the Yamuna River in the west. Caves, springs, and circulating groundwater are an essential part of the terrain (Figure 1a-b).

The caves are well-known for their karst formations and have beautiful stalactites and stalagmites. In the native language ‘Sahastra’ means ‘thousand’ and ‘dhara’ means spring hence, Sahastradhara literally is the place of a thousand springs. The springs have near neutral pH, and they are closely associated with development of the karst landscape. The cold sulfur-rich springs are believed to cure skin infections and have medicinal value. The springs flow into the nearby Baldi River in the valley.

The region experiences a humid tropical monsoon climate and the annual rainfall is 2000 mm. The minimum and maximum mean annual temperature varies from 2.7°C during December-January and 26°C during May-June. The area has a rich vegetation cover, and the natural forests of Sahastradhara have tropical mixed deciduous scrubby vegetation (tropical moist and dry deciduous communities). Sal trees (*Shorea robusta*) occupy a major part of the thick forests in addition to *Deodar* and *Chir* (conifers). Some of the floral biodiversity observed around the Sahastradhara caves include - shrubs (*Cassia spp.* and *Mimosa spp.*), herbs (*Lantana spp.*) and trees (*Acacia spp.*, *Bauhinia spp.*, *Bombax spp.*, *Dalbergia spp.*, *Melia spp.*, *Ziziphus spp.*, *Asparagus spp.*).

The sampled cave at Sahastradhara has two entrances and structural weakness in the ceilings/floors is absent. The caves are exposed to light, and water continuously drips from the ceiling. This sampled cave is rarely visited by tourists being inaccessible, as it requires crawling and climbing the hill. There is a permanent water line (ca. 0.15 m high) inside the caves. The cave flora at the entrance in the outer photosyn-

thetic regions consists of autotrophic organisms like algae (*Cyanobacteria*), lichen, mosses, ferns and liverworts. During sampling, we encountered various surface forms of fauna inside the cave e.g. spiders, beetles, millipedes, and crayfish. The cave has a total length of 11m, height 1.5 m and width varying between 1-3 m at different places (Figure 1c, inset). The main cave entrance is 2.5 m in height and 1.25 m in width. These caves host a number of spectacular stalactites ranging from 1 cm to 1.5 m in length (Figure 1d), cave wall deposits and stalagmites. The speleothems and cave wall deposits near the entrances have a greenish/grey appearance on the outermost zone, and are creamy white in the inner zones (Figure 1e).

Two moonmilk samples and five stalactites were collected for the study. The studied moonmilk samples were sub-aerial deposits, MM1 was located near the cave entrance and MM2 was located at a distance of 1 m from the second entrance on the inner cave wall in a twilight zone (Figure 1e-f). The deposits lie in a narrow crawl way devoid of human/anthropogenic activity. Physically the deposit appeared moist, smooth, soft, and cream colored with specks of green matter (Figure 1e-f). The moonmilk sampled for the study occurred in a patch of (1) MM1: 10 cm x 5 cm and (2) MM2: 13.5 cm x 16 cm (Figure 1e-f).

Sampling and analytical procedures

Sample collection

Drip waters, moonmilk and stalactite samples were aseptically collected from the cave. Ten aliquots of drip waters (about 2 l each), two moonmilk and five stalactites (ca. 50 g each) were collected with sterile disposable gloves and forceps in autoclaved sterilized bottles, and 250 ml sterile polypropylene tubes, and stored at 4°C. Geochemical analyses were done immediately (within 48 hrs) and microbiological anal-

yses were started within a week of sample collection. Conductivity, pH, humidity and temperature were determined *in situ* during field sampling using portable instruments (HI98130 pH/conductivity/TDS tester, HC520 Thermo-hygrometer). 20 g of fresh moonmilk and each stalactite sample were separately placed in 50 ml sterile polypropylene tubes with 25 ml of 96% cold ethanol for FISH and SYBR Gold assays. All the samples were stored at -20°C until further analyses.

Geochemistry

The drip waters were analyzed using an inductively coupled plasma atomic emission spectrometer (ICP-AES, JOBIN YVON JY-70 Plus), (WIHG, Dehradun) for different elements (Ba, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Si and Sr). The detection limit for most of these elements is <1 µg/l, except for Si and K for which the limits are 10 ppb. Relative standard deviation ranged from 5-25%. Merck certiPUR multi-element standards were used to calibrate and standardize the instrument. The total organic carbon content in the stalactite and moonmilk samples was analyzed using the method by Walkley and Black (1934).

Stable isotope geochemistry

The carbonates in the samples were analyzed for $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ isotopes. H_3PO_4 acid was added in vials containing the sample and flushed with Ar for 10 minutes. After contact between the acid and sample, CO_2 gas released in the sealed container was left to equilibrate for 24 h. The gas was transferred via a Thermo Gasbench II unit into a Finnigan MAT 252 isotope ratio mass-spectrometer for analyses (Debajyoti and Skrzypek, 2006). Various international standards like NBS18, NBS19, IAEA-CO-1, and IAEA-CO-8 were used for standardization. The results

were reported versus the Vienna Pee Dee Belmenite (VPDB) for both ^{13}C and ^{18}O . The standard deviation was better than 0.1‰ for ^{13}C and 0.15‰ for ^{18}O . For stable O isotope analyses in drip water, we used a Thermo Conversion Elemental Analyser connected to a Finnigan MAT Delta V mass spectrometer. The results were reported versus VSMOW. The standard deviation was better than 0.2‰ for ^{18}O .

Mineralogy

A portion of the moonmilk samples were air-dried, and powdered using a mortar and pestle, and the stalactite samples were machine powdered for various mineralogical analyses. Using a PAN Analytical X'pert PRO X-ray diffraction system (XRD) equipped with a Cu anode X-ray tube, the powdered samples were scanned at a rate of 1° per minute in the scanning range of 2θ values between 4° and 50° to identify the different mineral phases. The mineral spectra were identified using the ICDD database (JCPDS, 2000). Fourier Transform Infra Red Spectroscopy (FTIR, Shimadzu) and a Laser Raman Micro Probe - (Horiba JobinYvan) were used to complement the XRD studies, and confirm the phase profiles.

Electron microscopy (SEM and ESEM)

The moonmilk sample was fixed by adding glutaraldehyde to a final concentration of 2.5% and left for 1 hour. Aliquots of 50-100 μl of the suspended material was then vacuum filtered onto 0.2 μm polycarbonate filters, and subsequently dehydrated using a graded series of ethanol solutions (50-75-96 (3) x 100% v/v) for 10 minutes each. The filters were mounted on Al stubs and coated with 5 nm iridium by using a Precision etching coating system (Model 682, Gatan). For x-ray microanalyses by energy dispersive spectroscopy (EDS), moonmilk samples were coated with 5 nm of

carbon using an Agar turbo carbon coater. Samples were examined using a Zeiss Supra 55 VP field emission microscope (FE-SEM) attached to a Thermo Electron Corporation EDS system at the University of Bergen, Norway.

Moonmilk samples were also fixed on Al stubs with two-way adherent tabs and analyzed with an Environmental Scanning Electron Microscope (XL30 ESEM-FEG) at Stockholm University, Sweden. The moonmilk samples were coated in dry mode with a layer of *ca.* 15 nm gold using a Baltic SCD005 sputter gold coater (at $\sim 10^{-1}$ millibar). In addition, moonmilk samples were also observed in wet mode without coating. The ESEM was equipped with an energy dispersive spectrometer (EDS), back-scatter electron detector (BSE) and a gaseous secondary electron detector (GSE). EDS analyses were performed using a Philips EDAX (energy dispersive analysis of x-ray) instrument calibrated with a cobalt standard. A back-scatter electron detector was used, and the penetration depth was 0.5-3 μm . The samples were subjected to a pressure of 0.4 Torr under high vacuum for examination of moonmilk samples with gold coating. Under low vacuum (3.7 Torr) moonmilk samples without coating were observed in wet mode with an accelerating voltage of 20 kV. The point detection of the EDS instrument is in the range of 3 μm . Semi-quantitative analysis was done using EDS and the total was normalized to 100%.

Crystals precipitated by bacteria isolated from moonmilk and stalactite on B4 agar were analyzed using XRD and SEM (ZEISS EVO 40 EP, resolution, 3.0 nm SE and HV; magnification 7–1,000,000x; accelerating voltage 0.2–30 kV) equipped with an energy dispersive X-ray analyzer (EDX microanalyzer Bruker LN2 Free X Flash 4010SDD detector, resolution 129 eV) at Wadia Institute of Himalayan Geology, India.

Microbiological Analysis

Abundance and visualization of microbial cells and DNA extraction

The total microbial cell counts were determined on an epifluorescence microscope by counting cells stained with SYBR Gold, a fluorescent dye, which intercalates to the DNA. For cell enumeration, the moonmilk and stalactite samples were fixed with 2% paraformaldehyde (PFA) and stored in the dark at 4°C overnight. Then, 500 µL of the supernatant was removed, diluted in phosphate-buffered saline (PBS) and filtered onto 0.2 µm Anodisc filters (Whatman) and stained with the DNA binding fluorochromes SYBR® Gold and DAPI (molecular probes; Einen et al. 2008). The filters were then mounted in PBS/glycerol 1:1 with 0.1% *p*-phenylenediamine as an antifading reagent. The cells were counted using a Zeiss Axioplan fluorescence microscope at 1000 x magnification (Zeiss Plan-Neofluar). The microbial community was studied by fluorescence *in situ* hybridization (FISH) using a general oligonucleotide probe targeting the domain level Bacteria (Eub338). DNA was extracted from the moonmilk and stalactite samples with a FASTDNA SPIN Kit for isolation from soil (Catalog #6560-200, MP Biomedicals, USA), following the manufacturer's instruction. The amount and purity of DNA extracted was determined with a spectrophotometer.

Enumeration of bacterial colonies on various media

Bacteria enumerations in different media were performed on both the moonmilk and stalactites to understand the microbial diversity, metabolism, and growth requirements in terms of nutrients present in the media. The pour-plate technique was used to determine the number of microbes in each sample. The petri plates were inoculated with aliquots of sample dilutions (in both moonmilk and stalactites) ranging from 10^1 to 10^6 . Then 15 ml of the autoclaved media (45°C) was poured and mixed with the sam-

ple by gently swirling the petri plate. The petri plates were allowed to completely gel for 10 minutes and then inverted and incubated at 25°C for 24-48 hours. Enumerations were performed separately on twelve different media: Nutrient agar (NA, Hi-Media); B4 agar (Boquet *et al.*, 1973); Kliger iron agar (KIA, Hi-Media); sulphite agar (SA, Hi-media); thiosulphate agar (Thio-SA, Hi-media); starch-casein agar (SCA, Hi-media); Mn agar (MNA, Hi-media); Actinomycete isolation agar (AIA, Difco); Pseudomonas King's medium A (KM, Atlas and Parks, 1993); Bg-11 (Atlas and Parks, 1993); Iron agar (Hi-media) and a self-designed cave media (MCM) simulating our geochemical results (glucose 1g l⁻¹, tryptone 2 g l⁻¹, CaCO₃ 0.25 g l⁻¹, MgCO₃ 0.005 mg l⁻¹, ZnSO₄ 0.007 mg l⁻¹, BaCl₂ 0.005 mg l⁻¹, CuCl₂ 0.004 mg l⁻¹).

Isolation of bacteria capable of *in vitro* calcite precipitation

Bacteria capable of precipitating calcite *in vitro* were evaluated. The moon-milk and each stalactite sample in triplicate (1 g) were aseptically dried, and powdered in a sterilized mortar and pestle, suspended in sterile 0.9% saline solution and vortexed. 1 ml of this solution was diluted with 9 ml autoclaved distilled water and followed by successive dilutions. Spread plates containing B4 agar, NA, and the MCM cave media were inoculated with sample dilutions ranging from 10¹ to 10⁶. The pH of the media was checked and adjusted to pH 7 (neutral pH) using 5M NaOH at the time of preparing the media and autoclaved. The samples were incubated under aerobic, aphotic conditions, ranging from 18-32°C (18°C, 20°C, 22°C, 25°C, 28°C, 32°C separately), and were designed to simulate cave temperature and light conditions. A broad temperature range was chosen to study if the bacteria isolated are able to survive and precipitate calcite at temperatures above the range; and also, if temperatures had an effect on the quantity of calcite precipitated. Individual colonies were

selected and purified by repeated streaking on the respective agar. The petri plates were examined periodically by optical microscopy for presence of carbonate minerals. Individual colonies that formed a visible concentric mineral corona were selected based on differences in color and colony morphotypes, and further purified by repeated streaking for identification and mineral precipitation experiments. For short-term preservation, the isolates were streaked on B4 agar slants and stored at 4°C for 30 days before renewed inoculation. The calcifying strains were deposited for identification by conventional phenotypical and biochemical methods in the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, IMTECH, Chandigarh, India. In addition, B4, NA, and MCM broth (without agar) were used to monitor the change in pH. Isolated strains were inoculated at standard concentration, and reconstituted to 10^{-1} , 10^{-2} , 10^{-3} dilutions of the standard concentration. The inoculations were incubated at $25\pm 5^{\circ}\text{C}$ for 7 to 30 days. Aliquots were collected once a week and pH was measured. In addition, bacterial growth was also measured using a spectrophotometer. Three different controls consisting of autoclaved distilled water and 0.9% saline solution (both uninoculated) and uninoculated B4 media were also incubated.

Bacterially precipitated minerals

The bacterial isolates were examined periodically by optical microscopy for up to 30 days for presence of precipitated carbonate minerals. Media plugs of the crystal precipitating cultures were aseptically excised and mounted on SEM stubs with carbon tape. The morphology of bacteria, microbial association with minerals, microbial cells, and precipitated crystal morphologies were examined by SEM–EDX. For analysis of crystals by FTIR and XRD, the carbonate crystals were aseptically extracted

from the solid media and placed in boiling water to dissolve the agar. The supernatant and the sample were re-suspended and washed in distilled water to free them of impurities. The washed carbonate crystals were air-dried at 37°C (Rivadeneira et al. 1994; Sanchez-Roman et al. 2007a). The precipitates on the agar block were cut out and weighed.

Results

Geochemistry and mineralogy

Geochemical analyses of the drip waters indicated concentrations of: Ca 85mg/l, Fe 1.02mg/l, K 2.74mg/l, Mg 55mg/l, Mn 6 µg/l, Si 2.70mg/l, Sr 540 µg/l and minor concentrations of Ba 22 µg/l, Cd <1 µg/l, Cr 3 µg/l and Cu 7 µg/l. The *in situ* pH ranged from 7.4 - 7.6, and conductivity was around 1700 µS. The water temperature was 17 °C, whereas the air temperature inside the cave was 18±2°C. The humidity in the cave was ca. 60%. The sampled moonmilk deposits had high water content (72-74%), and the total organic carbon content of the moonmilk ranged from 3.0 to 3.5 wt % and the stalactite 1.25 wt % (Table 1). X-ray diffraction analysis and laser Raman bands at 1088.118 cm⁻¹, 284.185 cm⁻¹ indicated that the moonmilk deposits were mainly composed of low-Mg-calcite.

FTIR analyses: Several distinct bands of specific wavelengths were evident in the moonmilk. The bands indicate the presence of organic compounds (Sharma, 1989), 500-800 cm⁻¹ (halogenated compounds), 873 cm⁻¹ (acyclic ethers), 1070-1150 cm⁻¹ (ethers), 1449 cm⁻¹ (aromatic compounds and tertiary alcohols), 1634 cm⁻¹ (alkenes), 1794 cm⁻¹ (amino acids, thiols and carboxylic acid dimers), 2516 cm⁻¹ (carbonyl compounds), 2969 cm⁻¹ and 2876cm⁻¹ (aldehyde, methoxyl or *n*-methyl groups) and 3345cm⁻¹ (alcohols).

Isotopic analysis performed on the moonmilk indicated $\delta^{13}\text{C}$ values from -4.45‰ to -4.44‰, and $\delta^{18}\text{O}$ values from -6.64‰ to -6.63‰. The stalactite indicated $\delta^{13}\text{C}$ value from -2.20‰ to -2.50‰ and $\delta^{18}\text{O}$ value from -4.84‰ to -4.90‰ (values relative to VPDB; Table 1). The drip waters had an average $\delta^{18}\text{O}$ value of -6.73‰ to -6.84‰ (relative to VSMOW; Table 1).

Microscopy (SEM and ESEM)

SEM images of moonmilk showed large numbers of: i) spiral filaments (diameter $\sim 1\ \mu\text{m}$); ii) long stalk like spiral and tubular filaments (Figure 2a); iii) calcite minerals in close association with filamentous structures (Figure 2b, c); iv) bacterial cells (Figure 2d); v) empty filamentous organic structures (Figure 2e); vi) calcite formed in the vicinity of extracellular polymeric substances (EPS; Figure 3a), and vii) fibre calcites (Figure 3b). The minerals were closely associated and/entwined with microbial filaments. Some of these filaments were coated with thick mineral deposits, and it appeared these filaments penetrated the mineral grains (Figure 2e). Some minerals exhibited circular cavities lined with organic matter. The size of the cavities conformed to the size of the filaments (Figure 2f). ESEM-EDAX analyses performed on a dense mesh of these calcite filaments indicated presence of Ca.

ESEM (in the wet mode without any coating on the sample) of moonmilk showed the crystalline nature of the deposit and numerous calcite fibers (length $0.5\ \mu\text{m}$ to $1.5\ \mu\text{m}$; diameter $0.1\ \mu\text{m}$ to $0.25\ \mu\text{m}$ Figure 3b). The length of the crystals did not vary much but there is a variation in the thickness and diameter of the crystals (Figure 3b). These nano-scale fibers appeared as dendritic crystals (Figure 3b). X-ray microanalyses by ESEM-EDAX and XRD indicated that the fibers primarily consisted of Ca, O, C and traces of Mg and S (Figures 3c, d).

Microbiology

Direct observation of the moonmilk under a light microscope revealed a large number of diverse indigenous bacteria which appeared similar to *Cyanobacteria* spp. and some that resembled *Spirulina*. The total numbers of microbial cells in the moonmilk were $> 5.8 \times 10^5$ cells g sed⁻¹ and in the stalactites were $> 3.2 \times 10^5$ cells g sed⁻¹ using SYBR gold staining. Using specific probes (Eub338), FISH revealed that approximately 3.5×10^5 cells g sed⁻¹ belonged to the domain Bacteria in moonmilk samples and 2×10^5 cells g sed⁻¹ in stalactites (Table 1; Figures 3e, f). Both circular and rod shaped cells were observed. The bacteria were 1-2.5µm long. Enumeration in nutrient media indicated different cellular abundances for moonmilk: 6×10^5 (NA); 5×10^6 (B4); 2×10^1 (KIA); 3×10^2 (Thio-SA); 1×10^1 (SA); 3×10^2 (SCA); 4×10^1 (MNA); 4×10^3 (AIA); 3×10^1 (KM); 6×10^3 (Bg-11); 2×10^1 (Iron agar); 6×10^2 (MCM) and for the stalactites (Table 2). The DNA content in the moonmilk was 14 - 18 ng/µl and in the stalactites this was 10 – 10.5 ng/µl (Table 1).

Calcite production by isolates

In vitro culture experiments on the moonmilk and stalactite samples showed that many isolates (nearly 47%) formed CaCO₃ crystals in B4 medium. While 31 strains formed calcite in different nutrient media, we selected 25 strains with the best output for further screening and isolation (Table 2). Based on morphological and phenotypic characteristics, and biochemical assays, these mineral precipitating bacterial strains were identified at IMTECH as: *Bacillus pumilis* (3 strains - SM12, SM15, SM23), *B. cereus* (3 strains - SM04, SM06, SM10; MTCC 8776), *B. anthracis* (2 strains - SM08, SM53), *B. lentus* (4 strains - SM05, SM07, SM17, SM22), *B. sphaericus* (4 strains - SM28, SM44, SM51, SM55), *B. circulans* (4 strains - SM26, SM35,

SM38, SM47) and *Actinomycetes* (5 strains - SM03, SM19, SM40, SM41, SM50) (Table 3). Only cultures of interest were given an ascension number by IMTECH. The biomineral precipitated at 20, 22, 25 and 28°C; precipitation was absent in the controls. The time required by these microbial strains for initiation/formation of crystals was 10 days (minimum) to 15 days (maximum). The size of the zone of the precipitated biominerals on B4 agar ranged between 0.8 to 1.5 cm (diameter), and the precipitate per plate (collected and dried after separation from agar) weighed from 0.04 – 0.90g over a period of 3 to 4 weeks (Table 4). In some cases, crystals formed inside the bacterial colonies (Figure 4a). The crystals became completely encrusted with white, opaque, calcified carbonate, which increased in size with time (Figures 4b-f).

SEM imaging indicated tiny discrete microcrystals attached to the bacterial cells. The strains produced oval or spherical crystals (Figures 5a-d). Internal structure of the precipitates indicated the presence of radial arrangement of microcrystals (Figure 5c). EDS confirmed the chemical composition of these crystals and indicated presence of C, O, and Ca (Figure 5e). The crystals were further identified as calcite using XRD (Figure 5f), FTIR, and Laser Raman techniques.

The crystals increased in their dimension which was noted by measuring the size of the corona. The bacterial colonies were eventually totally replaced by the white mineral corona. Crystal precipitation induced a change in pH in the liquid media. *B. circulans* strain SM26 was most effective and increased pH from 7.6 to 8.6 in B4, 7.6 to 8.2 in NA and 7.6 to 8.3 in MCM over a period of 6 weeks (Figure 6). *B. pumilis* strain SM15 increased the pH from 7 to 7.85 in B4 medium during the fourth week, to 8.5 during the sixth week and finally declined to 7.8 in the seventh week. *B. pumilis* brought about an increase in pH during the entire 7 weeks period from 7.1 to 8.8 in MCM (Figures 6a, b).

Discussion

The Sahastradhara speleothems (moonmilk and stalactites) are colonized by diverse microbial communities that influence *in situ* carbonate precipitation and mineralization. The sampled cave wall deposits MM1 and MM2 have the characteristics of moonmilk in terms of their geochemical and biological characteristics. Moonmilk formation according to Cañaveras et al. (2006) follows a progressive accumulation of bacterially-induced calcite fibers. Consistent with this, the biogenic origin of Sahastradhara moonmilk and stalactites are based on the following lines of support: (1) geochemical evidence based on (FTIR and stable isotope records); (2) SEM images show bacteria/cyanobacteria-like filaments, dendritic fibers, microbial cells and exopolysaccharides (EPS); (3) microbiological evidence, which indicate high number of bacterial cells; (4) experimental geomicrobiology demonstrates the ability of these isolated strains to change pH and precipitate carbonate crystals. Similar lines of evidences are also advocated by other researchers to support the biogenic origin of speleothem deposits and their active formation in cave systems (Cañaveras et al. 2006; Sanchez-Moral et al. 2012).

Geochemical characteristics

The sampled moonmilk deposits form under neutral to alkaline conditions on cave walls which have a continuous supply from the drip water of Ca and minor concentrations of other trace elements. The Sahastradhara moonmilk have high (72-74 wt%) water content and are comparable to other moonmilk deposits (63.8 wt%: Curry et al. 2009; 71-75 wt%: Gradzinski et al. 1997; 70 wt%: Bernasconi, 1976). In addition, FTIR spectroscopy of the moonmilk reveals the presence of distinct bands, with

a complex milieu of organic compounds (e.g., hydroxyl compounds, amino groups, aldehydes, n-methyl groups, amino salts, thiols, carboxylic acid dimers, aromatic compounds and ethers) in these deposits. The presence of diverse organic molecules found in these formations is only observed in entities with distinct biological source/origin (Ramseyer et al. 1997; Frisia and Borsato, 2010).

In speleothems, $\delta^{13}\text{C}$ values lower than -4‰ imply that in addition to the supply of enriched C derived from dissolution of carbonates, depleted biogenic C, which is plant-derived CO_2 dissolved in the soil, can contribute towards precipitation of calcite (Turi, 1986; Cacchio et al. 2012). There are however very few studies on the stable C and O isotope records in moonmilk deposits. These studies relate seasonal changes in the stable isotope records to infer climatic changes (Lacelle et al. 2004; Frisia and Borsato, 2010), biogenic processes involved in speleogenesis (Sanchez-Moral et al. 2012), and food-web association in cave systems (Paoletti et al. 2011; Engel et al. 2013). The narrow range in ^{13}C (-4.44‰ to -4.55‰) and ^{18}O (-6.64‰ to -6.63‰) records in the Sahastradhara moonmilk deposits are more enriched compared to the ones in Canada (Lacelle et al. 2004), Italy (Frisia and Borsato, 2010), and Spain (Sanchez-Moral et al. 2012). The enriched value in our study is probably due to infiltration of surface and/ groundwater derived from the Indian summer monsoon depleted in ^{16}O , which has been removed due to kinetic fractionation during rainfall (Sanyal et al. 2005). These deposits are however depleted by $> -2\text{‰}$ compared to those recorded in stalactites in this study and by Tewari (2007) from Sahastradhara caves (Table 1). Microbial respiration removes CO_2 from drip water and by increasing the supersaturation, it promotes precipitation of calcite. The observed negative shift in isotopic values is therefore consistent with microbial activity. It is likely there is significant input of organically derived CO_2 and biological influence on calcite precipitation

that results in additional fractionation (Frisia and Borsato, 2010; Sanchez-Moral et al. 2012).

The drip water indicates similar values for ^{18}O as the moonmilk deposits (Table 1); however, it is possible that the dissolved inorganic carbonate fraction is compatible with precipitation under equilibrium conditions from meteoric water at cave temperature (O'Neil et al. 1969; Lacelle et al. 2004). Lacelle et al. (2004) suggest a mechanism of bedrock dissolution and re-precipitation, which form speleothems, and this process is controlled by seasonal changes inside the cave. Thus, during winter, condensation of water vapor inside the cave results in less humidity and corrosion of cave walls. In contrast, during summer growth is enhanced through slow evaporation of calcite-saturated water (degassing process). While such an abiogenic mode of formation is certainly possible, detailed isotopic studies are required not only from other speleothem deposits in these caves, but also seasonal sampling of interstitial and drip waters to support near-equilibrium evaporation of water and seasonally driven precipitation. The present evidence suggests it is more than likely that both physicochemical and biological processes appear to be co-occurring and even overprinting each other as reported in many cave systems (e.g., Northup and Lavoie, 2001; Blyth and Frisia, 2008; Curry et al. 2009; Sanchez-Moral et al. 2012). Thus, from our observations, it is noted that temperature, $\delta^{18}\text{O}$ in the water, C which may be derived from inorganic and/ organic sources, and microbial metabolism are essential in defining the isotopic composition of calcite in cave systems.

Notably, the Sahastradhara moonmilk records are similar to another cave in northeast India (Krem Mawmluh, Meghalaya). The Krem Mawmluh moonmilk is from the aphotic dark zone inside the cave, and we reported on a biogenic origin of these deposits based on SEM studies and laboratory cultures (Baskar et al. 2011). In

addition, C and O isotope values and fatty acid profiles indicate organic matter characteristic of bacterial cell-wall in these deposits (Singamshetty et al. 2013; J. Routh, unpublished data), and conforms with the biogenic nature of the sampled deposits.

Biogenic features

There are abundant aggregations of fine microcrystalline dendritic fiber crystals in the Sahastradhara moonmilk deposits (Figure 3b). The fibers have dimensions similar to other reported biogenic minerals in caves that are precipitated due to microbial processes (e.g., Hill and Forti, 1997; Borsato et al. 2000, Northup et al. 2000; Melim et al. 2001, Cañaveras et al. 2006; Curry et al. 2009). The fibers resemble microbial/organic matter and filamentous EPS like structures within the interspaces of calcite crystals, and inside the crystals itself (Figures 3a, b). EPS degradation promotes precipitation of carbonate minerals with an ovoid structure, and consist of sub-micron polyhedron crystals of Ca-dolomite and/or high-Mg calcite (Spadafora et al. 2010). This process further continues to form large ovoid crystals, which subsequently merge to form peloids, and represent the first stage of stromatolitic laminae formation (Spadafora et al. 2010). The authors indicate that degradation and calcification of EPS is driven by heterotrophic bacteria in the deposits. Microbes are reported to actively influence physicochemical conditions during precipitation, resulting in formation of calcite fibers of different morphologies and sizes (Cañaveras et al. 2006). Notably, the morphology and size of calcite crystals observed in this study closely resembles that of biogenically precipitated crystals in laboratories (Cacchio et al. 2003, 2004; Cañaveras et al. 2001; Rivadeneyra et al. 2004; Figures 2d and 3b). Thus, co-existence of EPS, filamentous structures and bacterial cells associated with the

mineral aggregates points to a distinct biogenic origin in the Sahastradhara moonmilk deposits.

Microbial enumeration and communities

The total estimates of microbial cells using DNA intercalating dyes (SYBR Gold) staining show high microbial density. The fluorescent dyes however bind to both living and dead cells, and therefore bacterial density estimated by this technique is on the higher side ($>5.8 \times 10^5$ cells, g sed⁻¹ in moonmilk and $>3.2 \times 10^5$ cells, g sed⁻¹ in stalactites). In contrast, the FISH assays using specific probes (Eub338), target the ribosomes, and only intact living cells are tagged. Hence, the probes yield lower number of bacteria (3.5×10^5 cells, g sed⁻¹ in moonmilk and 1.8×10^5 cells, g sed⁻¹ in stalactites).

Calcium carbonate precipitation by bacteria

Boquet et al. (1973) reported that CaCO₃ precipitation by bacteria is well established, and under appropriate conditions, most common bacteria are capable of precipitating CaCO₃. Microbial activity modifies the culture media and results in precipitation of carbonate minerals in laboratory experiments (Cañaveras et al. 2001). Consistent with this observation, similar mineral assemblages commonly occur in karst terrains (Castanier et al. 2000). Carbonate mineralization by bacteria occurs through either active or passive pathways. Active precipitation of minerals occurs as a by-product of microbial processes such as photosynthesis, urea hydrolysis, sulfate reduction, and iron reduction (Knorre and Krumbein, 2000). These processes can increase pH in the micro-environment surrounding the bacterial cell, and change the saturation state of carbonate and other ions. These processes facilitate precipitation of

various carbonate minerals (e.g. calcite, aragonite, vaterite, magnesite, siderite, and dolomite). In contrast, during passive carbonate precipitation bacterial cells act as nucleation sites, and adsorb Ca^{2+} and Mg^{2+} ions on their cell walls or EPS (Beveridge and Fyfe, 1985; Ferris et al. 1991; Frankel and Bazylynski, 2003). Notably, biochemical factors (i.e. pH, alkalinity) affected by bacterial metabolism influence the shape and morphology of the carbonate-rich precipitate, and extracellular organic matter attached to the mineral surface (Chafetz, 1986; Pedley, 1992; Folk, 1993).

We observe that 47% of the cultivable heterotrophic microorganisms isolated from the moonmilk and stalactite samples in Sahastradhara caves mediate calcite precipitation under oxic conditions in laboratory experiments (Table 4). Two strains (*B. anthracis* SM08, SM53) can grow under anaerobic conditions, and induce an increase in pH, which probably occurs through urea hydrolysis. In addition, three strains (*B. pumilis*; SM12, SM15 and SM23) are aerobic urea hydrolyzers. The possible sources of nitrogenous substrates in caves include organic-rich ammonia or ammonium ions carried in from surface soils, bat guano, bacterial nitrogen fixation, fertilizers, and forest litter (Barton and Northup, 2007). Likewise, the possible sources for nitrogenous compounds in Sahastradhara include bacterial nitrogen fixation, guano and ammonium ions transported into caves from surface soils. *Bacillus* spp. can readily metabolize various nitrogen-rich organic compounds (e.g., proteins, amino acids, nucleic acids), and release CO_2 and NH_4^+ . It is likely this process leads to the observed increase of pH (Sánchez-Roman et al. 2007a, b), which affects precipitation of carbonate minerals as reported in other studies (Barton and Northup, 2007; Portillo et al. 2009; Portillo and Gonzalez, 2010). Four strains (*B. circulans*; SM26, SM35, SM38 and SM47) are capable of growing under anaerobic conditions, and follow the nitrate reduction pathway. Eleven strains are aerobic and follow the nitrate reduction path-

way (SM04, SM05, SM06, SM07, SM10, SM17, SM22, SM28, SM44, SM47, SM51, SM55; Tables 2, 3). The isolated consortium represents a small component of the total microbial community in Sahastradhara caves, although we have increased success in cultivation with the use of low-nutrient media. The isolated consortium represents a subset of the genetic diversity. Nonetheless, the microbial strains isolated in this study indicate different possible metabolic pathways that can be undertaken by these microorganisms to survive in cave ecosystems.

The isolated *Bacillus* spp. both from the moonmilk and stalactites increased the pH from 7.6 to 8.8 in liquid media and aided in the precipitation of calcite. Precipitation is initiated at pH 7.8 and continues to increase as alkalinity increases in the medium. However, in those strains, where pH remains below 7.5 in the medium, precipitation is absent. The microbial cells and filamentous hyphae observed in these deposits under SEM most likely provide the necessary sites for crystal nucleation and growth because they display high affinity for Ca^{2+} ions (Cañaveras et al. 2001; Caccchio et al. 2004). We also observed in the laboratory cultures that a large proportion of aragonite compared to calcite is produced in the liquid media. In contrast, in the solid media more calcite is precipitated. The obtained results are similar to the results obtained by Rivedeneyra et al. (1998, 2006) in laboratory cultures. It is possible that solid medium helps the bacteria to capture Ca^{2+} ions more selectively, and create microenvironments with less Mg:Ca ratio than the liquid media (Rivedeneyra et al. 2004). Our results clearly demonstrate that some of the isolated bacterial strains are capable of altering the solution chemistry, and induce *in vitro* precipitation of carbonate minerals through various metabolic processes. Microbial cells and filamentous hyphae, as evidenced by SEM in this study, provide sites for crystal nucleation and

growth as they display high affinity for calcium ions (Cañaveras et al. 2001; Cacchio et al. 2004).

Several processes (e.g., urea hydrolysis, denitrification, production of sulfate, and iron reduction) have been identified, where by bacteria are able to induce precipitation of CaCO_3 (Hammes and Verstraete, 2002). Bacteria and fungi can precipitate carbonate minerals extracellularly through different metabolic processes (Ehrlich 1996; Castanier et al. 1999; Melim et al. 2001). Some of the processes include:

Photosynthesis: During this process CO_2 is removed and it leads to an increase of carbonate ions in a bicarbonate-rich solution. If Ca^{2+} ions are present, it will precipitate as CaCO_3 .

Oxidation: When organic compounds are oxidized in a well-buffered environment with Ca^{2+} ions, some of the CO_2 generated precipitates as CaCO_3 . When organic nitrogenous compounds are oxidized, NH_3 and CO_2 are released resulting in increase of pH and precipitation of carbonate minerals (Melim et al. 2001).

Hydrolysis: Urea hydrolysis forms ammonium carbonate, which causes precipitation of Ca^{2+} and Mg^{2+} carbonates. Microbial urease catalyzes urea hydrolysis (DeJong et al. 2006), which spontaneously forms ammonia and carbonic acid (Monty et al. 1995).

Ammonium and carbonic acid form bicarbonate, ammonium and hydroxide ions in water.

The hydroxide ions increase pH and shift the bicarbonate equilibrium to form carbonate ions.

The carbonate ions precipitate as CaCO_3 crystals.

We hypothesize that similar microbial processes are most likely ongoing in the Sahastradhara caves. These metabolic pathways are reported to actively result in speleogenesis in other cave systems (e.g., Castanier et al. 1999). *In vitro* studies using inoculated lab cultures produced the same carbonate mineral precipitates as observed in microbial mats, supporting the metabolic pathway (Buczynski and Chafetz, 1993; Chafetz and Buczynski, 1992). Thus, *in vitro* studies help in understanding and demonstrating the metabolic pathways that lead to mineral precipitation in caves. Such studies also have practical applications due to their role in the conservation of ornamental stone (*Bacillus cereus*; Rodriguez-Navarro et al. 2012); and their use in the bio-deposition of carbonates on degraded limestones (*Bacillus lentus* and *Bacillus sphaericus*; Dick et al. 2006).

Conclusions

Besides stalactites, the secondary deposits isolated from the cave walls in Sahastradhara have characteristics of moonmilk. Various geochemical, microscopic observations and laboratory culture add support to the biogenic nature of these deposits. A variety of organic compounds and depleted stable C and O isotope signatures suggest a biogenic origin for these deposits. Likewise, the strong association of carbonate minerals with microbial colonies supports the hypothesis that formation of moonmilk

and stalactite deposits in Sahastradhara is influenced by microbial processes. *In vitro* experiments confirm that subtle changes, such as increase in pH induced by microbes catalyze carbonate precipitation. The nature and morphology of the carbonate minerals observed *in situ* is similar to other reported biominerals. Our observations show a direct relationship between pH, *in situ* microbial strains, and precipitation of carbonate minerals. Further detailed investigations will only help us appreciate the nature of active and passive roles played by bacteria in the formation of these speleothem deposits.

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Figure captions:

FIGURE 1a-b Field details of study area, Sahastradhara caves, Dehradun, India

(a) Map showing Shiwalik region (Map after Sanyal et al. 2005); (b) Regional geological map of study area YTF, Yamuna Tear Fault; GTF, Ganges 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) (Map after Singh et al., 2001)

FIGURE 1c-f Field details of study area, Sahastradhara caves, Dehradun, India

(c) Dehradun road map showing the exact location of Sahastradhara caves; Inset shows the cave; (d) Stalactites in the cave; (e) Whitish cream moonmilk (MM1) sampled for the study (1) with specks of green matter (2); (f) Creamish Moonmilk sample (MM2) (arrows); pool of water seen below stalactites in the cave

FIGURE 2 SEM micrographs of critical-point dried and Ir-coated moonmilk samples, MM1 and MM

(a) MM1: Microbial structures similar to Cyanobacteria; arrow shows spiral (1) and tubular (2) filaments (b) MM2: calcite minerals formed in close association with microbial filaments; (c) MM2: calcite (d) MM2: bacterial cell (e) MM1: calcite formed around a microbial filament (1) empty filamentous organic structure (2) (f) MM1: A hole formed in calcite, showing remains of a formerly occupied microbial filament

FIGURE 3 ESEM micrographs of the moonmilk MM1 without coating (wet mode) and DAPI stain images

(a) Cave wall deposit showing calcite (1) formed in close association with EPS (2, 3) (b) Fibre calcite crystals (1) EPS (2); (c, d) EDX and XRD of the deposits showing calcite, calcium minerals (e) Bacteria identified by DAPI stain and FISH showing hybridized Eubacteria (f) SYBR gold stain showing hybridized bacterial cells in moonmilk

Figure 4 Optical Microscopy images showing crystal precipitation *in vitro*

(a) Crystal precipitation inside a bacterial colony (b) Bacilli strain SM15 completely covered by calcite (c) Crystals seen after ten days (d-f) Crystals increase in size after 18 days and 25 days

FIGURE 5 SEM micrographs showing calcite precipitation by Bacilli sp. in laboratory experiments

(a) Ovoid calcite crystals precipitated by *Bacillus pumilis* on B4 agar after 20 days of incubation (b) 1. Bacilli cells 2. Calcite crystal with a different morphology and rugged surface showing interior of the biolith (c) Inside the biolith 1, 2 showing radial arrangement of crystals (d) *Bacillus circulans* forming crystals on B4 agar (e) EDX showing semi-quantitative analysis of precipitated crystals with calcite peaks (f) XRD of the precipitated crystal with Ca peak

FIGURE 6 pH changes induced by two different strains of Bacillus sp *in vitro*

(a) *Bacillus circulans* strain SM26 inducing an increase in pH to alkaline conditions over a six week period

(b) *Bacillus pumilis* strain SM15 inducing an increase in pH to alkaline conditions over a seven week period