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Characterization of bacterial diversity associated with calcareous deposits and drip-waters, and isolation of calcifying bacteria from two Colombian mines

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ABSTRACT

Bacterial carbonate precipitation has implications in geological processes and important biotechnological applications. Bacteria capable of precipitating carbonates have been isolated from different calcium carbonate deposits (speleothems) in caves, soil, freshwater and seawater around the world. However, the diversity of bacteria from calcareous deposits in Colombia, and their ability to precipitate carbonates, remains unknown. In this study, conventional microbiological methods and molecular tools, such as temporal temperature gradient electrophoresis (TTGE), were used to assess the composition of bacterial communities associated with carbonate deposits and drip-waters from two Colombian mines. A genetic analysis of these bacterial communities revealed a similar level of diversity, based on the number of bands detected using TTGE. The dominant phylogenetic affiliations of the bacteria, determined using 16S rRNA gene sequencing, were grouped into two phyla: *Proteobacteria* and *Firmicutes*. Within these phyla, seven genera were capable of precipitating calcium carbonates: *Lysinibacillus, Bacillus, Strenotophomonas, Brevibacillus, Methylobacterium, Aeromicrobium* and *Acinetobacter*. FTIR and SEM/EDX were used to analyze calcium carbonate crystals produced by isolated *Acinetobacter gyllenbergii.* The results showed that rhombohedral and angular calcite crystals with sizes of 90 μm were precipitated. This research provides information regarding the presence of complex bacterial communities in secondary carbonate deposits from mines and their ability to precipitate calcium carbonate from calcareous deposits of Colombian mines.

1. Introduction

Biomineralization is a common process by which organisms move different metals to form minerals. In recent years, there has been growing interest in biomineralization by microbes with the ability to fix or mobilize metals, sequester CO^2 and precipitate different compounds of interest in various applications, such as calcium carbonates (Boquet et al., 1973; Weiner and Dove, 2003; Perito and Mastromei, 2011; Dhami et al., 2013a,b).

At a biotechnological level, studies related to calcium carbonate precipitating bacteria have focused on the conservation of monuments, (Tiano et al., 2006; Zamarreño et al., 2009; Dhami et al., 2014), soil improvement (Gurbuz et al., 2011; Hata et al., 2011; González et al., 2012; Cheng and Cord-Ruwisch, 2014; Sarmast et al., 2014) the removal of heavy metals, (Achal and Pan, 2014; Kang et al., 2014), self-healing concretes (De Muynck et al., 2010; Qiu et al., 2014), and, more recently, carbon dioxide fixation (Han et al., 2013; Lee et al., 2014; Yasumoto et al., 2014).

Despite the technological interest in these microorganisms, the ecological and physiological roles of biomineralization and bacterial diversity in secondary calcium carbonate deposits are still unknown (Banks et al., 2010; Perito and Mastromei, 2011; Dhami et al., 2013a,b). However, it has been demonstrated that bacteria from cave environments are capable of precipitating calcium carbonate *in vitro*. Different species and genera have been isolated from secondary deposits (speleothems) in caves, including *Bacillus pasteurii*, *Bacillus subtilis*, *Myxococcus xanthus*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Pseudomonas flurescens*, *Micrococcus* sp.,

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Rhodococus sp., *Arthrobacter* sp. and many others (Chekroun et al., 2004; Rivadeneyra et al., 2006; Achal et al., 2010; Rusznyák et al., 2012). In addition, it has been suggested that these bacteria have a direct relationship with carbonate deposition and speleothem development (Cacchio et al., 2004, 2012; Rusznyák et al., 2012).

Most research concerning the profiles of microbial communities in speleothems has taken a culture-dependent approach, which has revealed that the common phyla in these environments are *Firmicutes, Proteobacteria* and *Actinobacteria* (Laiz et al., 1999; Cacchio et al., 2003, 2004, 2012; Ikner et al., 2007; Banks et al., 2010; Rusznyák et al., 2012; Dhami et al., 2014). Culture-independent studies based on partial analysis of the 16S rRNA gene using clone library methods (Rooney et al., 2010; Rusznyák et al., 2012; Ortiz et al., 2013, 2014) and genetic fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) (Schabereiter-Gurtner et al., 2004; Legatzki et al., 2011), have suggested that *Proteobacteria*, *Actinobacteria* and *Acidobacteria* are the dominant phyla. Metagenomic approaches in a karstic cave showed functional bacterial genes associated with low nutrient, high calcium adaptations and nitrogen-based metabolism (Ortiz et al., 2014).

Two hypotheses have been proposed regarding what is responsible for the precipitation of calcium carbonate in bacterial processes. The first focuses on passive or influenced biomineralization, where the metabolic activity of a heterotroph (ureolytic bacteria and sulfur-reducing bacteria) or an autotroph (cyanobacteria) favors an increase in pH followed by the generation of carbonate ions (CO_3^{2-}), which could precipitate as $CaCO_3$ in the presence of calcium ions (Ca^{+2}) (Almahamedh, 2013). The second hypothesis is activated or induced biomineralization. In this mechanism, bacteria regulate calcium input and output through channel proteins, non-proteins and calcium antiporter ATP-dependent pumps. Ca^{2+} ions expelled by bacteria in the presence of CO_3^{2-} lead to the formation of calcium carbonate. Both hypotheses suggest that the cell wall of the bacteria or an extracellular matrix may act as nucleation sites for the crystallization of calcium carbonate (Bosak and Newman, 2003; Aloisi et al., 2006; Perito and Mastromei, 2011).

Until now, calcifying bacteria have been isolated mainly from seas (Rivadeneyra et al., 2006; Rivadeneyra Torres et al., 2013), secondary deposits such as stalactites and stalagmites in caves without human intervention, and soils (Banks et al., 2010; Rusznyák et al., 2012; Dhami et al., 2014). In most previous studies, bacterial isolation was performed using artificial media and natural media (especially seawater) (Rivadeneyra Torres et al., 2013). However, there are few related diversity studies of microorganisms from Colombian calcareous deposits located in areas with human intervention, and of their ability to precipitate calcium carbonate (Montoya et al., 2005).

To obtain additional information regarding the bacterial composition of calcareous deposits from Colombian mines, both conventional microbiological methods and culture-independent molecular tools were used. These included temporal temperature gradient electrophoresis (TTGE), which is based on the fingerprints of the amplified 16S rDNA gene. This was the first study to use the TTGE molecular approach to examine the bacterial diversity of speleothems and drip-water in the "El Zancudo" and "El Toro" mines. This approach enables the detection of dominant bacterial communities that could be of importance in calcareous deposits in Colombian mines. Further, scanning electron microscopy (SEM) and Fourier transformation infrared spectrometry (FTIR) were used to describe the structure of crystals produced by biomineralization.

2. Materials and methods

2.1. Description of sampling sites

The samples were collected in March 2013 and June 2013. The El Toro and El Zancudo mines are located in the Cordillera Central, northeast of Colombia (5°47′26″N 75°25′37″W, 6°3′45″N 75°47′37″W). El Toro is a limestone mine located near Abejorral (Antioquia, Colombia). The rocks in the mine are mainly marbles; these are interspersed with schist and quartzite, consisting mainly of calcite, graphite, some white mica and, occasionally and at low rates, pyrite (Castro et al., 2007). The climate is warm, with an average temperature of 20 °C throughout the year. The mine has four levels, or blocks, above the Buey River. Two sites that contained secondary calcium carbonate deposits (stalactites and curtains) were sampled (Fig. 1): Zone I in Level 1, located at 1032 m.a.s.l, and Zone II in Level 4, located at 920 m.a.s.l. El Zancudo, meanwhile, is a gold mine located near the town of Titiribí (Antioquia, Colombia). The mine was operational in the late nineteenth century and was for decades the center of gold mining in the region, but was closed due to the depletion of this resource. The average temperature in the region is 20 °C throughout the year, with annual rainfall between 1500 mm and 2000 mm. Three zones with calcium carbonate speleothems were sampled in the first 140 m from the mine entrance in the Independence tunnel.

2.2. Collection of samples

In both mines, speleothem fragments (dripstones and flowstones) were carefully removed with a chisel and a geological hammer. Approximately 30 g of each speleothem, and 250 ml of drip-water, were collected under aseptic conditions.

In Zone I of El Toro mine, samples of different types of speleothems, such as curtains (flowstones), which displayed white and reddish colors, were taken from a slope that was about 7 m long and 6 m high (Fig. 1A–C). In Zone II, located in Level 4, samples were taken from a slope that was 5 m long and 6 m high; the deposits there were also curtains, showing similar white and reddish colors (Fig. 1D). A total of 17 samples were taken from the two zones (Table 1).

Three areas were sampled in El Zancudo gold mine; these were located in the entrance of the Independence tunnel, 140 m from the pithead. Twenty samples were taken, including curtains with different colors (white, red and black), stalactites and cave pearls (Fig. 1). Table 1 describes the samples that were collected.

The material collected was put in sterile wide-mouth bottles of 500 ml and stored at ~4 °C in Styrofoam coolers for transportation to the laboratory. In the case of the liquid samples (drip-water), pH, redox potential (Eh), temperature and dissolved oxygen were measured *in situ,* using HACH HQ30d Multi-Parameter Meter equipment, with an Ag/AgCl electrode and a KCl electrolyte.

2.3. Isolation of precipitating bacteria

Precipitating bacteria were isolated in a B4 medium (Boquet et al., 1973) (4 g yeast extract, 2.5 g of calcium acetate, 10 g of glucose and 18 g agar per liter of distilled water, final pH: 6.63 ± 0.01) and in a B4 medium supplemented with drip-water from each mine (B4DT = B4 with drip-water from El Toro and B4DZ = B4 with drip-water from El Zancudo) (4 g yeast extract, 2.5 g of calcium acetate, 10 g of glucose and 18 g agar per liter of drip water). Final pH was 6.73 ± 0.006 for the B4DT medium and 6.91 ± 0.004 for the B4DZ medium. All media were autoclaved at 121 °C for 20 min.

Drip-water samples were plated directly on the Petri dish with the media. Speleothems were crushed with a sterilized hammer, and 10 g of each sample was re-suspended in 40 ml of sterile dis-