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ORGANIC MATTER ENRICHMENT AFFECTS ARCHAEA COMMUNITY IN LIMESTONE CAVE SEDIMENTS

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Abstract

Caves are unique environments filled with complex microbial communities that have adapted to oligotrophy. Communities of fungi and bacteria are commonly studied in touristic caves or are associated with guano or other sources of organic matter, but the archaeal community is often overlooked in these conditions. Based on this gap in the existing literature, the present study aims to evaluate the effect of a unique in vitro contamination event by organic matter in the archaeal community over the course of one year. For that purpose, samples were collected in Gruta Manoel Ioiô, a limestone cave located in Iraquara, Brazil. The collected samples were transported to the laboratory to undergo an enrichment of 0.25% or 0.5% mixture 1:1 (w/w) of yeast and meat extract. Samplings were collected at 0, 1, 6, and 12 months to evaluate the effects on the archaeal community by polymerase chain reaction followed by Denaturing Gel Gradient Electrophoresis (PCR-DGGE). PCR-DGGE profiles show that Operational Taxonomic Units (OTUs) remained in all samples, but variations were observed among the contaminated and control samples, especially at 6 months. Also, an increase in the number of OTUs was observed in samples that received the addition of organic matter in relation to the control. These OTUs were identified as Euryarchaeota and Crenarchaeota. This study showed that the archaeal community could be impacted by organic matter contamination in caves.

Introduction

Caves are unique natural environments, usually characterized by the absence of light and by oligotrophy (Barton and Jurado, 2007; Simon et al., 2007). Despite those characteristics, caves present a high potential for microbial life that is little known, especially archaea (Gonzalez et al., 2006; Tetu et al., 2013; Barton et al., 2014; Ortiz et al., 2014). These micro-organisms are a monophyletic group of phenotypically diverse prokaryotes. Initially, it was believed that they were found only in extreme environments; however, several articles have demonstrated that archaea probably occur in all environments that support life, including caves (Gonzalez et al., 2006; Chen et al., 2009; Jarrel et al., 2011). This group was found to be important for ecological relationships in several environments, particularly because of their role in biogeochemical cycles (Kudo et al., 1997; Chen et al., 2009; Jarrel et al., 2011).

Microbial life in caves has typically adapted to an oligotrophic environment, but several factors can increase the nutritional offerings inside caves, such as runoff, animal feces like bat guano, and tourism activity (Nieves-Rivera et al., 2009; Nováková, 2009; Borda et al., 2014). However, archaea are rarely studied in such conditions. Studies commonly focus on bacteria and fungi communities (Chelius and Moore, 2004; Gonzalez et al., 2006; Ikner et al., 2007; Chelius et al., 2009; Adetutu et al., 2011), with a few studies involving protozoa (Sigala-Regalado et al., 2011; Garcia-Sanchez et al., 2013). The focus on bacteria and fungi is related to organic matter input that can present problems linked to uncontrolled proliferation of fungi and bacteria, causing risks to the cave itself and to humans (Jurado et al., 2010; Saiz-Jimenez, 2012). In general, fungi, protozoa, and bacteria proliferation in caves is related to the input of organic matter and micro-climatic changes (Bastian et al., 2010; Saiz-Jimenez, 2012). However, little knowledge exists about archaea in caves associated with guano or runoff. The present study in microcosms evaluated the effects of the addition of organic matter on the archaeal community in limestone cave sediments.

Materials and Methods

Gruta Manuel Ioiô is a touristic limestone cave (12°20′9.96″S, 41°33′50.04″W) where the major attraction is the numerous stalactites, stalagmites, and columns. It is located in Iraquara, Bahia, Brazil, near to several others touristic caves in Chapada Diamantina. Unlike Manoel Ioiô, most of these caves were opened earlier for tourism and have a large variety of speleothems that attracted many more tourists resulting in the studied cave having lower numbers of visitors. It is important to mention that in this dry cave there aren't any platforms for visitors; they walk on the floor of the cave itself. The sampling was done in an area closed for tourists. Sediment samples were collected (SISBIO authorization number 38453) approximately 500 m from the entrance. Five subsamples were collected in sterile plastic bags approximately 1 m apart and mixed together to form a composite sample (Marques et al., 2016). Organic matter present in the sediment was less than 5000 ppm.

Microcosms were set up with 75 g of cave sediment in 250 mL Enlermayer flasks. Two concentrations of yeast and beef extract (Acumedia) mixture (1:1) were tested, at 0.25 and 0.5%. They were diluted in 10 mL of distillated water,

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and all experiments were made in triplicate. Flasks with beef extract, yeast extract, and water, but without soil, were used as controls for medium contamination, and additional controls were made with only sediment and distillated water (Marques et al., 2016). All flasks were incubated in the dark for one year at 25 °C, a temperature close to the recorded in the cave's chamber (22°C).

At the days 0, 30, 180 and 365, approximately one gram was collected from each triplicate with a water-washed and fire-sterilized collector. 0.25 g was used for DNA extraction utilizing the MoBio PowerSoil DNA kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer protocols. PCR was performed in a thermocycler (Eppendorf, Hamburg, Germany) using the archaea-specific primers 1100F (5′- CAC GGG GGG AGT CAG GTA ACG AGC GAG -3′) and 1400R (5′- GTG CAA GGA GCA GGG AC -3′) (Kudo et al., 1997) in a 25 µL reaction mixture containing 0.2 µM of each primer, 0.2 mM of each dNTP, 1.25 U Taq DNA polymerase (Invitrogen, São Paulo, Brazil), 3 mM MgCl2, 1X PCR buffer, and approximately 60 ng DNA.

For Denaturing Gel Gradient Electrophoresis (DGGE), primer 1100F was attached with a GC-clamp (5′- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG G-3′) (Muyzer et al., 1993). After PCR, the samples were subjected to DGGE in a DCode universal mutation detection system (Bio-rad Laboratories, Hercules, CA). Amplicons were electrophoresed on 8% polyacrylamide gel (w/v) (37.5:1 acrylamide:bisacrylamide) in a denaturing gradient of 20–60% [4 M of urea, with 40% (v/v) formamide representing a 100% denaturing gradient]. Electrophoresis was performed in a TAE 1X buffer for 16 h at 70 V and 60°C. The gel was stained with silver nitrate (Marques et al., 2016). The presence and absence of bands were used for a nonmetric MDS and dendrogram analysis. Both analyses were performed on the PAST 3.0 software platform (www.folk.uio.no/ohammer/past/).

Selected bands were aseptically excised from DGGE and eluted in ultrapure water. After 36 h to 48 h at 4°C, the elution bands were stored at −20°C until a new PCR was performed using 1400R and 1100F primers without a GC-clamp (Kudo et al., 1997). PCR products were visualized in 1% agarose gel, purified using a Purelink PCR purification kit (Invitrogen, Brazil), and cloned with a TA cloning kit (Invitrogen, Brazil); all procedures were performed according to the manufacturer's instructions. PCR reaction tests were performed using transformed colonies with M13F (5′-GTA AA ACG ACG GCC AGT-3′) and M13R (5′-CAG GAA ACA GCT ATG AC-3′) primers at the same concentrations of reagents as described above. The PCR conditions used were 30 cycles of 94°C for 20 s, 55°C for 15 s, and 72°C for 60 s. Amplicons were sequenced in an ABI-Prism 3100 genetic analyzer (Applied Biosystems). Sequences were processed and analyzed with the Phrap/Phred/Cross Match software (Ewing and Green, 1998) and the BLASTN tool (Altschul et al., 1990). The phylogenetic tree was inferred using the Neighbor-Joining and Jukes-Cantor methods in Mega7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown and bootstrap values inferior to 40 were removed.

Results and Discussion

Microbial communities in caves can be seen as geologically isolated from the surface (Barton and Jurado, 2007); however, several factors, such as runoff, guano, and touristic activity, can disturb the microbial community by adding organic matter. Thus, yeast and meat extract were used as a method to evaluate the in vitro effect of organic matter on the archaea community.

The results observed in the community after a simulated contamination event (Fig. 1) show that the archaeal community changed during treatment, but maintained a similar structure of 6 OTUs in all tested conditions, different from what was observed with fungi (Marques et al., 2016). The nonmetric MDS (NMMDS) and Jaccard similarity dendrogram analyses (Fig. 2) better represent these changes, where the controls maintained a similar structure throughout the year. However, the contaminated samples at months 1 and 12 showed a closer proximity to their respective controls than the samples at 6 months. The month 6 sample also presented a more distinct profile with respect to an increase in the OTU number (Fig. 1). Despite small variations in the profiles of each concentration, the contaminated samples group together according to the month (Fig. 1). The similar impacts caused by the two concentrations tested are an indication that these microorganisms are sensitive to an increase in organic matter and that some of them develop better under these conditions. These archaea that were better adapted in contamination experiments must be organotrophic because of the availability of organic matter, but the archaea shared with the controls could be either organotrophic or autotrophic. Curiously, the major changes did not occur immediately in either concentration, as in month 1 the archaeal community showed less change than at 6 months, where major changes were observed (Fig. 1). These changes must have happened because of competition between archaea, bacteria, and fungi for the organic matter. Archaea commonly have a slower metabolism than the other two groups, leading to a slower change, especially in comparison with the observed changes in fungi in such conditions (Marques et al., 2016). It is interesting to note that at 12 months after the single contamination event, the archaeal community appears to return to profiles close to that of the controls (Figs. 1 and 2); however, more time would be required to provide a better comparison of the probable partial recovery of the native archaeal community. These findings are interesting because of several attempts reported in the literature to recover

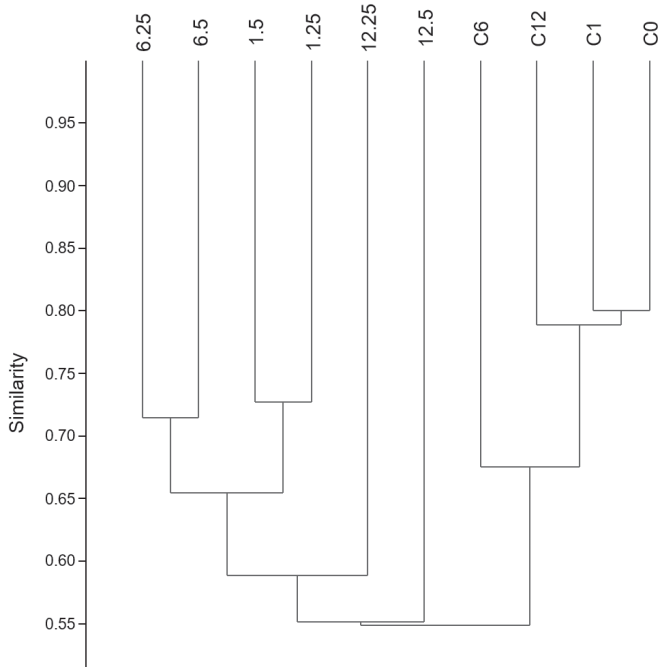

Figure 1. Denaturing Gel Gradient Electrophoresis fingerprint and similarity dendrogram of archaeal 16S rRNA gene from organic-matter-contaminated limestone-cave sediment. "C" indicates control samples, while the number before the decimal point indicates the month of sampling followed by the concentration of organic matter: 25 for 0.25% and 5 for 0.5% of organic matter.

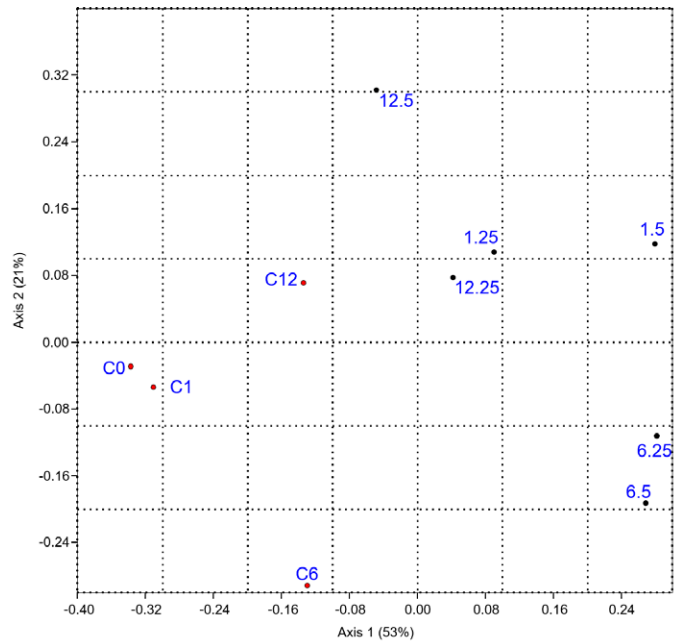

Figure 2. Nonmetric multidimentional scaling (NMDS) ordination of Archaeal 16S rRNA gene Denaturing Gel Gradient Electrophoresis fingerprinting from organic-matter-contaminated cave sediment. The stress value was 0.151 based on Bray-Curtis coefficient distance. Red dots are controls samples, while black dots are experiment samples. Numbers before the decimal point identify the month of sampling, followed by organic matter concentration, 25 for 0.25% and 5 for 0.5% of organic matter.

microbial communities after contamination events (Bastian et al., 2010; Martin-Sanchez et al., 2012) and may indicate that, just as seasonal modifications are found to happen with archaeal communities outside caves based on weather and temperature (Williams et al., 2012; Sher et al., 2013), the same could happen with the archaeal community in caves with respect to organic matter contamination.

Bacteria and fungi have become a major concern in touristic caves because of, among other things, the input of organic matter transported to the cave by touristic activity (Chelius et al., 2009; Martin-Sanchez et al., 2012) and the proliferation of microrganisms, especially pathogenic ones. Because of the lack of known human pathogenic archaea and the general difficulty to culture them, this group is often ignored in such caves. However, recent studies have shown the importance of archaea to a cave's ecosystem (Legatzki et al., 2011; Jones et al., 2014; Ortiz et al., 2014), and it is shown in this study that they suffer major changes over time because of a single event of contamination by organic matter (Figs. 1 and 2). Nevertheless, some considerations need to be noted regarding our experiments. Direct or indirect transport of organic matter is a reality in several caves (Chelius et al., 2009; Jurado et al., 2010; Saiz-Jimenez, 2012) that can drastically change the food web in a cave's ecosystem. These results did not show the real effect in natural conditions, because several others factor also influence contamination events that cannot be easily controlled and mimicked in vitro. One example is that microorganisms will be transported into the caves and among microenvironments, while another is that continuous artificial light will affect the microbial community in touristic caves (Mulec and Kosi, 2009; Cennamo et al., 2012; Saiz-Jimenez, 2012) and could also indirectly affect archaea. In addition to the input of organic matter, animals could start to proliferate in the cavern, prompting major changes in the community (Chelius et al., 2009; Yoder et al., 2009).

Despite these limitations, the use of yeast and beef extract for an in vitro organic-matter study shows that some previously undetected archaea were favored by the contamination event (Fig. 1); these organisms had a low representativeness, less than 1%, which is associated with undetected bands in DGGE (Muyzer et al., 1993), independent of the concentration tested. Considering that there was only one contamination event, these Operational Taxonomic Units could represent potential archaea related with microbial blooms. In caves, such blooms are only associated with bacteria and fungi (Jurado et al., 2010; Saiz-Jimenez, 2012); archaeal blooms have been reported in other environments (Oren and Gurevich, 1995; Fan and Xing, 2016). Phylogenetic analysis of those OTUs identifies them as Crenarchaeota (8 OTUs) and Euryarchaeota (2 OTUs), as shown in Figure 3. Among the eight Crenarchaeota OTUs, three were grouped with soil archaea, one was related to the Nitrosocaldus genus, and four were archaea from a marine environ-

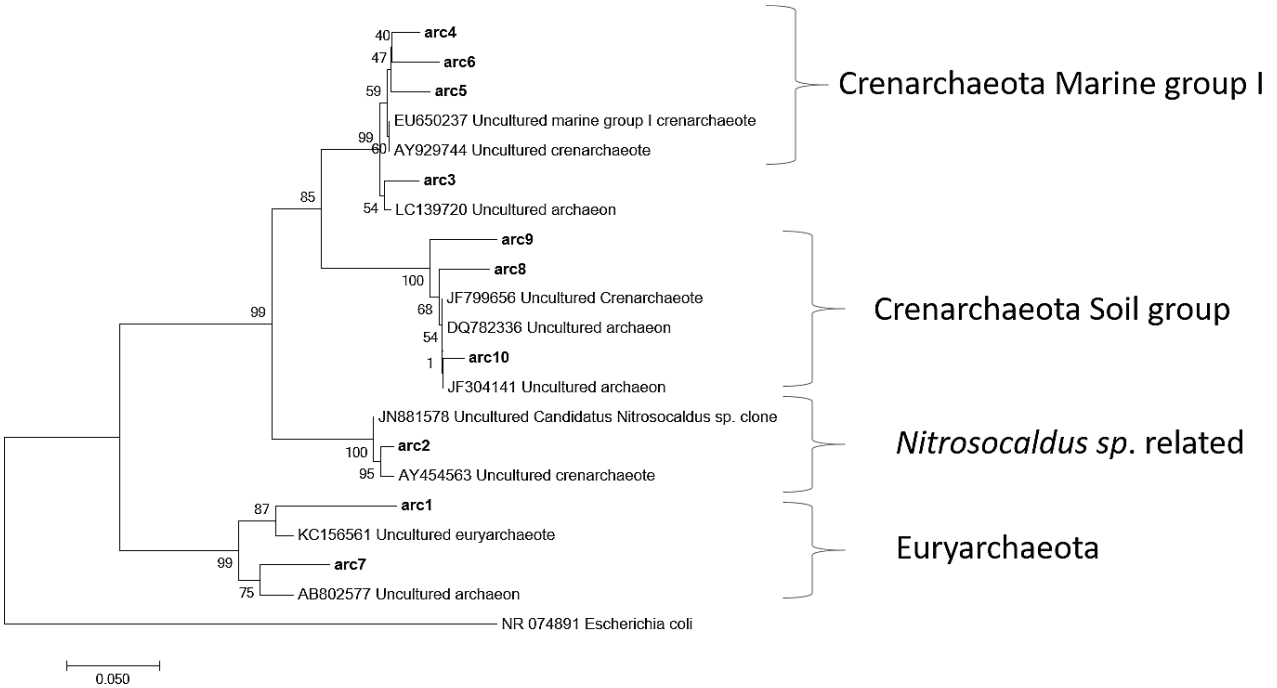

Figure 3. Phylogenetic analysis of archaeal Denaturing Gel Gradient Electrophoresis bands from cave-sediment-contaminated with organic matter.

ment. The Euryarchaeota and Creanarchaeota taxa are dominant in archaea communities in limestone caves (Chelius and Moore, 2004; Chen et al., 2009), including Crenarchaeota from marine environments that have previously been found in caves (Chelius and Moore, 2004).

Conclusions

This work shows the importance of understanding archaeal dynamics in organic matter contamination. Although no human pathogens are known from this group, their ecological importance is unquestionable, and in vitro organic matter input changes the microbial community. The slow change and partial recovery of the archaea community after 12 months could be useful for considering the effect in studies of microbial communities and for recovery of native microorganisms in caves after organic matter contamination events, especially in touristic caves. While we found variation among controls and among times tested, more studies must be done to validate these results, including studies in different caves, different distances from the entrance, and in situ studies in touristic caves.

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