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SHORT COMMUNICATION

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Metabolically active Crenarchaeota in Altamira Cave

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Abstract Altamira Cave contains valuable paleolithic paintings dating back to 15,000 years. The conservation of these unique paintings is attracting increasing interest, and so, understanding microbial proliferation in Altamira Cave represents a prioritary objective. Here, we show for the first time that members of the Crenarchaeota were metabolically active components of developing microbial communities. RNA was extracted directly from the studied environment, and a number of 16S rRNA gene sequences belonging to the low-temperature Crenarchaeota were detected. Although low-temperature Crenarchaeota detected in a variety of ecosystems by using molecular techniques remain uncultured, this RNA-based study confirms an active participation of the Crenarchaeota in cave biogeochemical cycles.

Introduction

The biosphere on Earth is constituted of three domains: the Eukarya (comprising protists, fungi, animals, and plants), the Bacteria, and the Archaea [30]. For a long time, Archaea were considered to occur exclusively in extreme environments. However, recent findings have showed that the occurrence of Archaea, and specifically the Crenarchaeota, is not restricted to extreme environments because they also thrive in moderate habitats such as soils [7]. So far, these low-temperature Crenarchaeota have not been cultivated using classic microbiological tools and have only been detected by using culture-independent methods [11]. As a consequence, we know of their presence and diversity in a variety of ecosystems, but whether they show significant metabolic activity and their potential role in natural environments remains to be discovered. Low-temperature Crenarchaeota might represent not only a critical microbial group in our planet biodiversity but also a unique and untouched biotechnological resource [5].

Microorganisms occupy all cave niches [20]. DNA-based molecular analyses of microbial communities allow for the detection of microorganisms present in a sample without the need for culturing [29]. However, only a portion of the microorganisms in an environment are showing detectable physiological activity. The use of RNA-based molecular analysis of environmental samples allows the detection of metabolically active members because the amount of RNA per cell is proportional to its metabolic activity [17].

A long-term microbiological study is being conducted on Altamira Cave [10, 22]. This report is a contribution to deciphering the complex active microbial communities inhabiting cave niches. The aim of this study was to detect whether Archaea were participant in developing biodeteriorating processes in Altamira Cave and to determine the phylogenetic position of these archaeal communities.

Materials and methods

Small (<1 mg) samples were collected from different colored microbial colonies developing on the walls and ceiling of Altamira Cave (Cantabria, Spain). RNA was immediately preserved with the solution RNAlater (Ambion Inc., Austin, USA). Total RNA was extracted with the RNAqueous-4PCR kit for the isolation of DNA-free RNA (Ambion Inc.). The cDNAs from 16S rRNA genes were synthesized at 55°C using the reverse-transcriptase Thermoscript (Invitrogen, Carlsbad, USA) and the primer 518 R (5′-ATT ACC GCG GCT GCT GG) [18]. Polymerase chain reaction (PCR) amplifications targeting the archaeal 16S rRNA genes were performed with the primer pair 20bF (5′-YTC CSG TTG ATC CYG CSR GA) and 518 R using *Taq* DNA polymerase (Extaq, Takara, Tokyo, Japan). PCR products were cloned into DNA libraries as previously described [8]. Clones were sequenced following standard

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procedures. Sequences were manually edited using Chromas http://www.technelysium.com.au/chromas.html) and analyzed by a homology search using the basic local alignment search tool (BLAST) algorithm [2]. A phylogenetic tree was constructed with the software suite ARB [14] by maximum-likelihood methods and the quartet-puzzling method [27]. The sequences retrieved during this study can be accessed under the numbers AY972855–AY972865.

Results

Yellow-, gray-, and white-colored conspicuous colonies, visible to the naked eye, develop on the walls and ceiling of Altamira Cave. Similar colonies in Tito Bustillo Cave were composed of at least 15 different microorganisms [23]. In Altamira Cave, microbial colonization is actually progressing over time, demonstrating the active growth of microbial communities. This is revealed by the incipient colonization of the far extreme end of the Polychromes Hall by white colonies (Fig. 1).

A number of sequences were retrieved from RNA-based molecular surveys performed from Altamira Cave samples. A total of 34 sequences were classified in 11 different Operational Taxonomic Units (OTUs) [31] corresponding to the GenBank accession numbers AY972855–AY972865.

The phylogenetic position of the Crenarchaeota sequences detected in Altamira Cave and their relationship to previously reported low-temperature, uncultured Crenarchaeota and the high-temperature, cultivated Crenarchaeota are shown in Fig. 2. The Crenarchaeota detected in Altamira Cave belong to novel lineages represented by the so far uncultured low-temperature Crenarchaeota previously detected only during DNA-based molecular surveys.

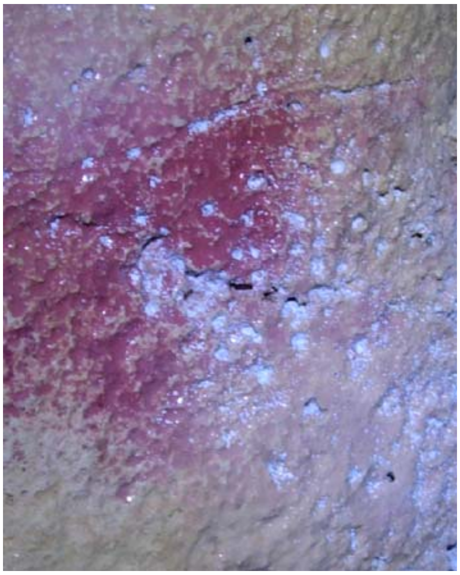

Fig. 1 Microbial colonization of pigmented areas at the Polychromes Hall in Altamira Cave

Discussion

Complex microbial communities have been detected in Altamira Cave. The bacterial components of the communities have been studied in detail somewhere else and were mainly composed of Proteobacteria and Acidobacteria [9, 22, 31].

RNA-based molecular surveys allow the detection of metabolically active microorganisms in naturally occurring communities [16], as opposed to DNA-based surveys which only show their presence. This study was focused on Archaea and RNA-based molecular surveys from samples taken from the walls and ceiling of Altamira Cave. Interestingly, our findings have revealed the presence of a diverse metabolically active archaeal community, mostly constituted by members of the low-temperature Crenarchaeota, corroborating the importance of metabolically active archaeal communities in the cave. The Crenarchaeota remained unreported from previous DNA-based molecular studies in Altamira Cave, which usually have targeted the bacterial domain.

The presence of active low-temperature Crenarchaeota in Altamira Cave suggests a significative role in biogeochemical cycles. Previous studies on the presence of low-temperature Crenarchaeota in a variety of environments confirms a widespread distribution in nature [5, 7, 11], although no previous information on their physiological stage and their in situ activity was available. For instance, the presence of low-temperature Crenarchaeota have been reported in caves [6, 20], soils [15, 19, 26], marine environments [3, 28], and freshwater [21]. Simon et al. [25] showed that low-temperature Crenarchaeota colonized plant roots, suggesting an active role in nature for these microorganisms.

The Crenarchaeota sequences retrieved from Altamira Cave in this study are related to previously reported marine and terrestrial low-temperature Crenarchaeota, suggesting that phylogenetic divergence and environmental origin are not strictly related to the low-temperature Crenarchaeota [7]. As previously suggested [24], the low-temperature Crenarchaeota could be ammonium oxidizers, although this remains to be confirmed, and this will require the cultivation and physiological analysis of these microorganisms.

Our findings indicate that metabolically active low-temperature Crenarchaeota represent significant participants in actively growing microbial communities. If these results are confirmed in other subsurface environments, they would suggest the need for refocusing current initiatives on understanding global environmental issues and biogeochemical element cycling. Besides, the active participation of Crenarchaeota in colonization processes on cave walls with paleolithic paintings indicates that current efforts in understanding microbial colonization and conservation of cultural heritage are to be reoriented. Understanding the diversity and activity of microbial communities developing therein will be critical for the design of conservation strategies. This study emphasizes the interest in understanding the huge, mostly unknown, microbial diversity existing in our planet and presents an example where

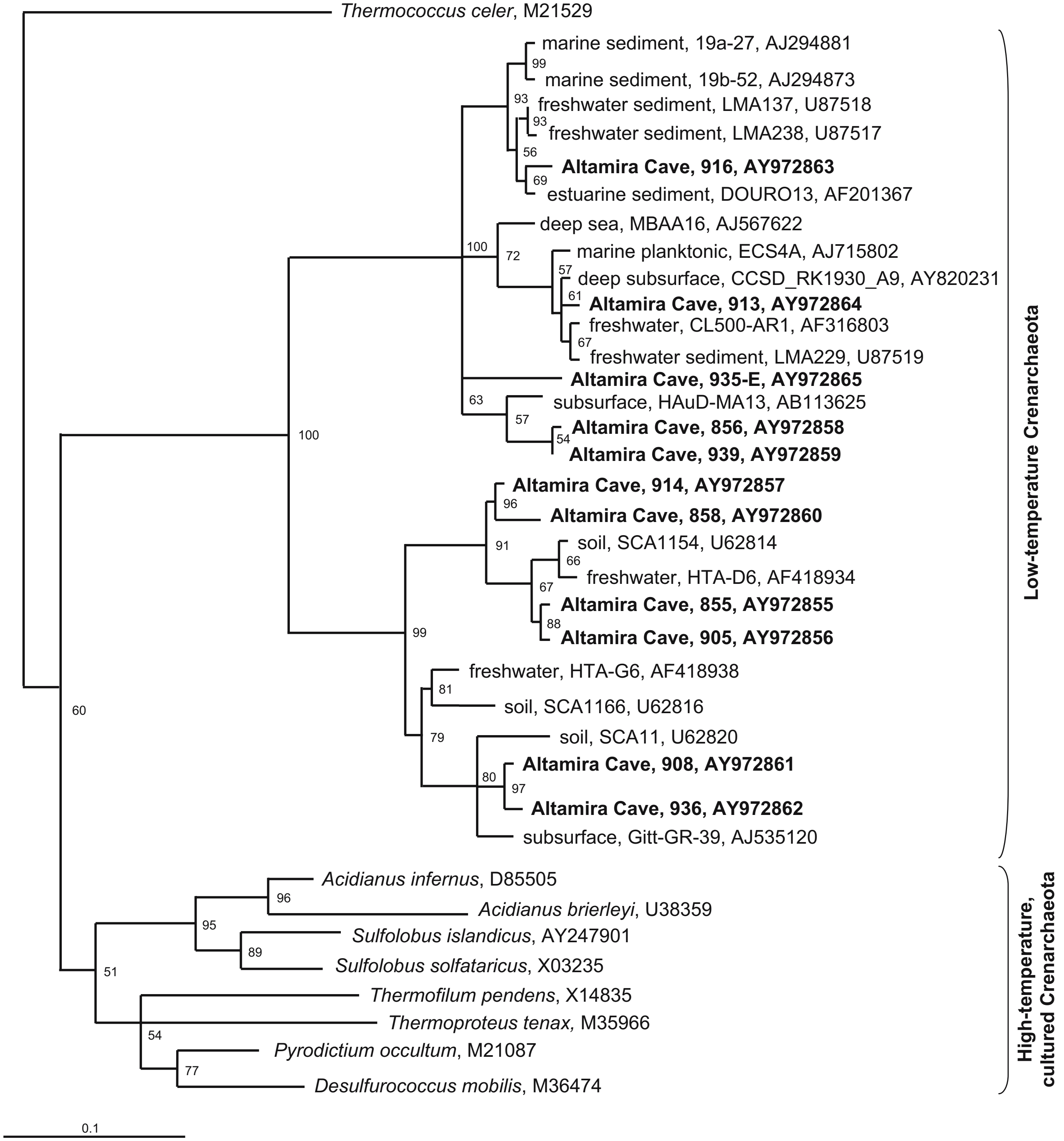

Fig. 2 Phylogenetic analysis based on 16S ribosomal RNA gene sequences showing the relationships among cultured (high temperature) Crenarchaeota, environmental (low temperature) Crenarchaeota, and the metabolically active Crenarchaeota detected in this study (in *bold*). The environmental origin, clone number, and accession numbers for their 16S rRNA gene sequences are shown for uncultured Crenarchaeota. Cultured Archaea are presented by their species name and accession number for their 16S rRNA gene sequence. *Thermococcus celer* (a hyperthermophilic Euryarchaeon) sequence is used as an outgroup. *Bar* indicates the changes per nucleotide. Reliability values are provided at bifurcations as percentages from 10,000 trials

potential negative consequences could be generated if their role is underestimated.

There is great media interest in the conservation of paleolithic paintings and the possible application of new technologies in their preservation [1, 4, 12, 13]. However, the design of novel, scientifically based conservation strategies requires, from the microbiologist point of view, knowledge on the microbial communities developing in an environment (i.e., Altamira Cave). At present, the microorganisms composing these microbial communities and their natural functions are practically unknown. This gap in our knowledge needs to be filled before deciding which technologies are to be applied and which microorganisms should be the targets of conservation initiatives. The recent invasion of the fungus *Fusarium solani* in Lascaux Cave, after other microorganisms were eradicated, is a clear example of how man's attempts to control or manage nature provokes further unpredicted changes in a cave's biodiversity [1, 13].

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