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Bacterial diversity in dry modern freshwater stromatolites from Ruidera Pools Natural Park, Spain

Fernando Santos[a], Arantxa Peña[a], Balbina Nogales[b], Elena Soria-Soria[a,1], Mª Ángeles García del Cura[c], Juan Antonio González-Martín[d], Josefa Antón[a,*]

[a] División de Microbiología, Departamento de Fisiología, Genética y Microbiología, Universidad de Alicante, 03080 Alicante, Spain
[b] Grup de Microbiologia, Departament de Biologia, Universitat de les Illes Balears, 07122 Palma de Mallorca, Spain
[c] Laboratorio de Petrología Aplicada, Unidad Asociada CSIC (Instituto Geología Económica – UA), Universidad de Alicante, 03080 Alicante, Spain
[d] Departamento de Geografía, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

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ABSTRACT

Ruidera Pools Natural Park, Spain, constitutes one of the most representative systems of carbonate precipitation in Europe. The prokaryotic community of a dry modern stromatolite recovered from the park has been analyzed by molecular techniques that included denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone library analysis, together with microscopic observations from the sample and cultures. Ribosomal RNA was directly extracted to study the putatively active part of the microbial community present in the sample. A total of 295 16S rRNA gene sequences were analyzed. Libraries were dominated by sequences related to *Cyanobacteria*, most frequently to the genus *Leptolyngbya*. A diverse and abundant assemblage of non-cyanobacterial sequences was also found, including members of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes* and *Chloroflexi* groups. No amplification was obtained when using archaeal primers. The results showed that at the time of sampling, when the pool was dry, the bacterial community of the stromatolites was dominated by groups of highly related *Cyanobacteria*, including new groups that had not been previously reported, although a high diversity outside this phylogenetic group was also found. The results indicated that part of the *Cyanobacteria* assemblage was metabolically active and could thus play a role in the mineralization processes inside the stromatolites.

Introduction

Ruidera Pools Natural Park in Central Spain (38°58′N 02°52′W) is a fluvio-lacustrine system consisting of a series of successive lagoons stepped along the longitudinal profile of the High Guadiana Valley, connected by streams, waterfalls and springs. The lagoons are formed due to the impermeability of the geological substrate at the bottom of the valley and the presence of a natural tufa barrier, constituted by carbonate precipitation, which create natural dams separating the different pools. Ruidera Pools represents one of the most important sites of current carbonate precipitation in Europe (Ordóñez et al. 1997) and contains well-preserved examples of present day fluvial and lacustrine tufas developed under semi-arid conditions (Ordóñez et al. 2005). Tufas are formed by physico-chemical and biological precipitation, a process mediated by the presence of microbial biofilms generally dominated by diatoms, cyanobacteria and heterotrophic bacteria. These lithified, laminated, calcareous and benthic microbial deposits are frequent in Ruidera Pools and constitute the stromatolites that are organosedimentary layered structures accreted by sediment trapping, binding and *in situ* precipitation due to the growth and metabolic activities of microorganisms (Walter 1976). Although many naturally occurring microbial mat communities are known to trap and bind sediments, only a few ecosystems, such as the one described here, are conducive for the process of lithification and fossilization (Havemann and Foster 2008). In addition, living microbialites, such as the ones studied here, are only found within a few selected locations, as pointed out by Breitarbt and collaborators (Breitbart et al. 2009). Stromatolites present in Ruidera Pools have been studied morphologically, geologically and microscopically (Ordóñez and García del Cura 1983; Ordóñez et al. 1997; Souza-Egipsy et al. 2005), although very little is known about their microbial diversity. Microscopy analysis of submerged stromatolites shows abundant diatoms in the surface of calcite build-ups interspersed with a layer of EPS and unicellular and filamentous cyanobacteria and algae. It seems clear that microorganisms play an important role in the

* Corresponding author at: División de Microbiología, Departamento de Fisiología, Genética y Microbiología, Universidad de Alicante, Apto. 99, 03080 Alicante, Spain. Tel.: +34 965903870; fax: +34 965909569.
E-mail address: anton@ua.es (J. Antón).
[1] Present address: LABAQUA, Dpto. de Microbiología, C/Dracma, 16-18 Polígono Industrial Las Atalayas, 03114 Alicante, Spain.

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building of these organosedimentary structures and that microbial cell walls, sheaths and exopolymeric substances influence the process of calcite biomineralization (Souza-Egipsy et al. 2005). In fact, differences between cell walls and sheaths of cyanobacteria and algae, as well as the presence of exopolymeric substances, lead to differences in the process of biologically induced calcite mineralization between these two microbial groups (Souza-Egipsy et al. 2005).

We chose a pool located in an erosional channel between two larger pools (Tomilla and Tinaja) that was generated in 1947 as a consequence of a flooding-related spillover (García del Cura et al. 1997). The water of Tomilla Pool, which feeds the small pool sampled, is almost saturated with calcite and dolomite. The pool is periodically dried due to drought and/or seasonal changes in the rain regime. The bottom of the pool is covered with stromatolites of different morphology which grow by the formation of mineral-entrapping microbial layers that can be up to 2 mm thick. Below the surface of the stromatolite, a clear green band was observed (Fig. 1A), formed by the mineral-entrapping microbial community. These layers are formed only when the pool is covered by water and, therefore, stromatolite formation depends on the periodical flooding of the pool, as well as the development of an active microbial community that contributes to mineral precipitation. However, microorganisms have also been repeatedly observed by scanning electron microscopy inside dry lithified stromatolites from Ruidera Pools (A. de los Ríos, personal communication), although it is unknown whether these communities remain active when not covered by water, and which microbes compose them. To address these questions, culture-independent methods were chosen for examining the microbial diversity and composition of a dry stromatolite. The bacterial diversity was analyzed by using denaturing gradient gel electrophoresis (DGGE) and clone libraries from 16S rRNA genes amplified by PCR from total environmental DNA, and libraries from 16S rRNA, reversely transcribed and amplified from total environmental rRNA. Since RNA molecules are more labile than DNA, the detection of 16S rRNA in an environmental sample is an indication of the presence of microbial cells which contain ribosomes and, therefore, they are presumably metabolically active (Nogales et al. 2001). In addition, the microbial community in the dry stromatolite was analyzed by culture and microscopic methods.

Materials and methods

Samples

A stromatolite sample (10 cm × 7 cm × 3.5 cm, 150 g, Fig. 1A) was taken from the bottom of the pool on September 15th, 2000 (the pool was dry at the time of sampling), and kept at 4 °C for 3 h, during transportation to the laboratory. The stromatolite chosen for this study had a pinnate dome shape (h = 20 cm) and was located in the subcritical turbulent flow and in the deepest part of the pool. The main component of the pinnacle domes consists of layers of superposed light lamina of fibrous calcite macrocrystals with visible cyanobacterial trichomes in an intracrystalline position (Souza-Egipsy et al. 2005). The sample was stored at −70 °C, and subsamples were taken for nucleic acid extraction by using a sterile spatula to scrape off material from the green ("algal") layer (Fig. 1A), which was collected in a sterile Petri dish. Care was taken to avoid scraping detritus material deposited on the surface of the stromatolite. A piece of the original sample was kept at room temperature and used for culture inoculation.

Microscopy

Scraped material from the green layer of the stromatolite sample was used for phase contrast optical microscopy (DM 4000B, LEICA) and confocal laser scanning microscopy (TCS SP2, LEICA) examinations after rehydrating in sterile water.

Nucleic acid extraction and purification

For nucleic acid extraction, two protocols were assayed. Protocol I, an adaptation of the protocol described previously (Cifuentes et al. 2000), was followed. A total of 5 g from the greenish layer of the stromatolite was mixed in a 15 mL polypropylene tube and washed twice with 7 mL cold AE buffer (20 mM sodium acetate pH 5.5, 1 mM EDTA) by centrifuging at 3900 × g for 5 min. Then, 10 mL of phenol–chloroform–isoamylalcohol 25:24:1 (PCI), pre-warmed at 60 °C, and 300 μL of 10% SDS were added. This mixture was incubated at 60 °C for 5 min with vortexing every minute. After cooling on ice, the tubes were centrifuged at 3900 × g for 5 min at 4 °C. The aqueous phase was transferred to a new tube to which 500 μL of 2 M sodium acetate pH 5.2 and 10 mL of PCI were added, vortexed, and centrifuged as above. The removal of the aqueous phase, addition of sodium acetate and PCI and centrifugation were repeated until a clear interphase between the aqueous and the organic phases was observed (i.e. 2–3 times). Finally, nucleic acids were precipitated with ethanol and resuspended in 100 μL of sterile deionized water.

A second nucleic acid extraction protocol (protocol II) was also carried out for comparison (see Results). For this extraction, 5 g from the greenish layer of the stromatolite were mixed as described above with 7 mL of extraction buffer (100 mM Tris–HCl pH 8.0, 100 mM EDTA pH 8.0). Then, 80 μL lysozyme (final concentration: 3 mg mL^{-1}) were added and the sample was incubated for 15 min at 37 °C (180 rpm). A total of 120 μL proteinase K (final concentration: 150 μg mL^{-1}) and 800 μL 10% SDS were added, followed by incubation for 30 min at 37 °C (180 rpm). The sample was mixed with 1600 μL 5 M NaCl and then incubated at 65 °C for 10 min with 1200 μL CTAB (10% CTAB, 0.7 M NaCl). Three freeze/thaw steps were carried out by using liquid nitrogen and a water bath at 65 °C. One volume of PCI was added and the sample was centrifuged at 3900 × g for 5 min (4 °C). After centrifugation, the aqueous phase was transferred into new tubes and the addition of PCI was repeated until a clear interphase between the aqueous and the organic phases was observed (i.e. 2 or 3 times). Nucleic acids were precipitated with a 0.1 volume of 3 M sodium acetate (pH 4.8), 0.01 volume of 1 M magnesium chloride and 0.6 volume of cold isopropanol and finally resuspended in 100 μL sterile deionized water.

Crude nucleic acid extracts were purified with GENECLEAN Kit II (Bio 101), according to the manufacturer's protocol. To check the quality of the extracted nucleic acids, they were subjected to electrophoresis on 0.8% LE agarose gels (FMC Products, Rockland, ME, USA) in 1× Tris–acetic acid–EDTA (TAE) buffer, and visualized under UV light after ethidium bromide staining. All glassware was washed with 2N NaOH and rinsed with sterile deionized water to minimize RNA degradation.

PCR amplification of 16S rRNA genes

Forward primers 27f and 21F, and universal reverse primer 1492r were used for complete 16S rRNA gene amplification, as previously described (DeLong 1992). Gene fragments for denaturing gradient gel electrophoresis (DGGE) were amplified with forward primer 341f-GC for *Bacteria*, and 344F-GC for *Archaea* and universal reverse primer 907r, as previously described (Muyzer et al. 1993, 1998; Nagy and Johansen 2001), using different conditions for the touch down PCR (from 65 to 55 °C, and from 60 to 50 °C) that did not have an effect on the DGGE patterns. Primers CYA359-GC and CYA781R (equimolar mixture between primers CYA781Ra and CYA781Rb) were used for PCR amplification of cyanobacterial 16S rRNA genes from environmental DNA, as previously described (Nübel et al. 1997).