Viable cyanobacteria in the deep continental subsurface

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Cyanobacteria are ecologically versatile microorganisms inhabiting most environments, ranging from marine systems to arid deserts. Although they possess several pathways for light-independent energy generation, until now their ecological range appeared to be restricted to environments with at least occasional exposure to sunlight. Here we present molecular, microscopic, and metagenomic evidence that cyanobacteria predominate in deep subsurface rock samples from the Iberian Pyrite Belt Mars analog (southwestern Spain). Metagenomics showed the potential for a hydrogen-based lithoautotrophic cyanobacterial metabolism. Collectively, our results suggest that they may play an important role as primary producers within the deep-Earth biosphere. Our description of this previously unknown ecological niche for cyanobacteria paves the way for models on their origin and evolution, as well as on their potential presence in current or primitive biospheres in other planetary bodies, and on the extant, primitive, and putative extraterrestrial biospheres.

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The deep terrestrial biosphere is receiving increasing interest as it harbors a significant fraction of the total microbial biomass of the planet (1–5), yet also imposes severe energy and nutrient limitations on its inhabitants (4). Subsurface life is dependent on buried organic matter and lithogenically sourced compounds such as molecular hydrogen (5). Despite its inhospitality, the deep subsurface is a stable and sheltered environment, which makes it a good candidate habitat for the development of early life on Earth, as well as on potential extra-terrestrial scenarios (6). Despite the increasing interest in deep subsurface ecosystems, obtaining reliable data on their microbiology is severely hampered by the high cost and technical difficulty of retrieving pristine samples, its heterogeneity and extent, and the vanishingly small microbial loads (7). The majority of deep subsurface habitats thus remain significantly underexplored (6).

The Iberian Pyrite Belt (IPB) in southwestern Spain hosts one of the largest sulphide deposits in the world, as well as the Río Tinto Mars analog (8–10). In previous work, we characterized pyrite-rich drill core samples down to 166-m depth with several molecular ecology techniques, and revealed a subsurface ecosystem with active iron and sulfur cycles (9). To investigate the microbiota and their activities in a deeper, more pristine location, we carried out a second drilling at a site ~500 m from the previous one, where geophysical studies and tritium measurements showed the presence of an aquifer formed by ~60-y-old groundwater at a depth of around 400 m (10). A 613-m-deep borehole was drilled, implementing procedures for aseptic sampling and for tracing potential contamination events during drilling and subsequent processing of the retrieved rock cores. These procedures minimized contamination of samples with extraneous microbes, enabled us to identify any samples that had been contaminated, and if so, to quantify any such contamination.

Results and Discussion

Biogeochemical Characterization of the Deep Subsurface of the Iberian Pyrite Belt. Rock cores from a 613-m-deep borehole were retrieved and processed following protocols for aseptic sampling and the tracing of potential contaminations (SI Appendix, Fig. S1). The geochemistry of the borehole was characterized with several complementary techniques (SI Appendix, Fig. S2), and microbial profiles were obtained via MiSeq 16S rRNA gene amplicon.

Cyanobacteria were responsible for the origin of oxygenic photosynthesis, and have since come to colonize almost every environment on Earth. Here we show that their ecological range is not limited by the presence of sunlight, but also extends down to the deep terrestrial biosphere. We report the presence of microbial communities dominated by cyanobacteria in the continental subsurface using microscopy, metagenomics, and antibody microarrays. These cyanobacteria were related to surface rock-dwelling lineages known for their high tolerance to environmental and nutritional stress. We discuss how these adaptations allow cyanobacteria to thrive in the dark underground, a lifestyle that might trace back to their nonphotosynthetic ancestors.

Significance

The authors declare no conflict of interest.

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Data deposition: Raw sequence data and assemblies that support the findings of this study have been deposited in NCBI under the BioProject ID PRJNA4766489. MG-RAST results are deposited under project ID mgp83581.

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sequencing (SI Appendix, Fig. S3) and an in situ antibody microarray immunoassay (11) (SI Appendix, Fig. S4).

**Detection of Deep Subsurface Cyanobacteria.** Strikingly, the on-site immunoassay detected, inter alia, cyanobacterial markers in samples from several depths, and 16S rRNA gene sequencing showed a predominance of cyanobacteria, whose exact sequence variants (ESVs) were related to endolithic and hypolithic representatives of the genera *Calothrix*, *Chroococcidiopsis*, and *Microcoleus* (SI Appendix, Fig. S4 and Dataset S1). The presence of cyanobacteria was associated with local decreases in hydrogen concentrations (Fig. L4). The cyanobacterial ESVs present in the subsurface samples were absent from the drilling fluid and the internal laboratory controls (SI Appendix, Fig. S3), which confirms that their detection is not a consequence of contamination during sample retrieval and processing, and that they are indigenous to the retrieved cores.

We further focused on the samples from 420 and 607 m of depth (from now on referred to as samples 420 and 607, respectively), as they showed higher amounts of fatty acids (SI Appendix, Fig. S2). The rocks from 420 m below the surface floor (mbsf) are dominantly made up of quartz, with minor proportions of pyrite, carbonates (ankerite), and white mica, and show a conspicuous layering between some millimeters and 1 cm. Permeability is mainly controlled by widespread unoriented fracturing, with very variable openings between 0.01 and 0.1 mm. The rocks from 607 mbsf, on the other hand, consist of alternating dark shale (with abundant centimeter-sized nodules of pyrite) and sandstone. Fractures appear in the abundant contact zones between both minerals (SI Appendix, Fig. S2). Overall, the rocks at both depths have low porosity, but the presence of fractures provides space for microbial colonization and allows for a limited input of water and nutrients.

Cyanobacteria were the most abundant organisms in the metagenomes of both samples, followed by the Ascomycota, Alphaproteobacteria, and Bacteroidetes groups (Fig. 1B and Dataset S2). These organisms may form a microbial consortium similar to those found in cyanobacterial crusts (SI Appendix, Supplementary Text). In previous work, we described the presence of biofilms in the same borehole using fluorescence microscopy (12), but only universal probes were used. In this work, we confirmed the presence of viable cyanobacteria by catalyzed reported deposition fluorescent in situ hybridization (CARD-FISH) with specific probes against the cyanobacterial 16S ribosomal RNA. CARD-FISH is the best-practice method to search for viable cells—as defined by the presence of ribosomes—in deep subsurface settings (7, 13, 14). Ribosomal RNA has a half-life of days (15) and readily degrades upon cell starvation (16).

Further, our samples contain pyrite, which is known to mediate the degradation of RNA via hydroxyl radicals under oxic and anoxic conditions (17). Sorption on certain mineral surfaces, such as clays, can increase the extracellular stability of ribosomal RNA, but complete degradation still occurs after a short time (18). Therefore, under the conditions of this study, positive CARD-FISH signals are a strong proof of extant viability.

CARD-FISH with specific probes revealed clusters of cyanobacterial cells tightly attached to the mineral matrix and associated with other microorganisms (Fig. 2). These cells did not show photosystem II-related autofluorescence, indicating that they lacked active photosynthetic pigments. The inactivation of the photosynthetic apparatus when under environmental stress is a known trait of desert-dwelling cyanobacteria, such as *Microcoleus* sp (19), which helps them cope with both desiccation and photoinhibition.

Cyanobacteria have long been known to be ecologically versatile microorganisms (20) capable of light-independent energy generation (21), but until now, their ecological range appeared to be restricted to environments with at least occasional or prior exposure to sunlight (22). A few studies have reported the presence of cyanobacteria in deep subsurface environments (23–25), but to the best of our knowledge, only in ref. 25 have the authors attempted to discuss their origin. They proposed that a bloom of aquatic cyanobacteria had been trapped thousands of years ago into a groundwater aquifer with no further connection with the surface. That scenario strongly differs from the one described in this study: we analyze rock samples instead of groundwater, the IBP subsurface aquifer has recent connection to the surface (10), and the cyanobacterial lineages detected in this work are endolithic rather than aquatic. We thus believe that our results correspond to modern cyanobacteria with the ability to colonize deep subsurface environments.

**Hydrogen as an Electron Donor for Cyanobacteria.** We found an apparent inverse correlation between the cyanobacteria predominance and hydrogen concentration in our samples (Fig. L4). Hydrogen can be produced in the subsurface by several abiotic mechanisms, and its concentration in deep continental settings has recently been found to be controlled by biological sinks (26). To identify putative hydrogen consumers, we tested whether hydrogen concentration was dependent on taxa abundances using multiple linear regression. We considered the phylum, class, order, and family levels and tested models including all possible combinations of one to six taxa. Cyanobacteria was the only taxon that significantly explained hydrogen abundances when considered alone (negative correlation, $P = 0.03, R^2 = 0.33$). The addition of more taxa to the model helped explain residual variance. The best model included the cyanobacterial families...
In several cyanobacterial genera, the overreduction of plastoquinone triggers the transfer of electrons from hydrogen to oxygen via the electron transport chain (29). Cyanobacteria also have a bidirectional hydrogenase (Hox), which is hypothesized to function as an electron valve, providing a rapid way to balance the redox state of the cell. Hox can transfer electrons from/to either NAD(P) or plastoquinone via the NDH-I complex, contributing to both photosynthetic and respiratory electron transport chains are overreduced (32).

**Cyanobacterial Electron Transport Chains in Deep Subsurface Environments.** In several cyanobacterial genera, the overreduction of plastoquinone triggers the transfer of electrons to extracellular acceptors, via a cytochrome bd quinol oxidase (33). This has a protective effect in light-intense conditions, where cytochrome bd is unable to accept electrons from plastoquinone at a sufficient rate. We note that growth under the dark, anoxic conditions of the deep subsurface would also lead to an overreduction of the plastoquinone pool, potentially triggering electron transfer from plastoquinone to cytochrome bd quinol oxidase. We thus propose that this protection mechanism would also provide the means for the anaerobic oxidation of hydrogen or other compounds using extracellular electron acceptors such as iron and manganese oxides, or phenolic compounds derived from the degradation of recalcitrant organic matter by other members of the microbial community (SI Appendix, Supplementary Text).

An additional potential electron acceptor could be nitric oxide, as we found a quinol-dependent nitric oxide reductase in the cyanobacterial pangenome from sample 420 (Dataset S2). Cyanobacterial nitric oxide reductases connected to the electron transport chain have been proposed to participate in nitric oxide detoxification and energy conversion (34). Interestingly, incomplete denitrification by noncyanobacterial partners is predominant in cyanobacteria-dominated biological crusts, leading to the emission of nitric and nitrous oxides (35, 36). Thus, cyanobacteria might profit from nitric oxide reductases by using them to exploit the nitric oxide produced by other members of the consortium as an alternative electron acceptor (34).
Evolution of Light-Independent Electron Transport Chains in Cyanobacteria. Höchler and Jørgensen (4), and more recently Starnawski et al. (37), have argued that the slow rates of biomass turnover in deep subsurface environments provide minimal opportunities for the introduction and propagation of beneficial mutations. Survivability will thus be determined by traits gained in other ecosystems showing similar (to some extent) restrictions, but with higher energy fluxes. In this context, endolithic cyanobacteria are perfect candidates for inhabiting the deep subsurface, as they are already adapted to living inside rocks and are able to withstand severe nutritional and environmental stresses and experience periodic anoxia during the diel cycle (38). Further, some cave-dwelling cyanobacteria survive for long periods in the near-total absence of light, where photosynthesis is no longer possible (39). For these reasons, they have developed mechanisms that, having evolved to cope with light stress and desiccation in their original habitats (40), could also be triggered under the reducing conditions found in the deep subsurface and result in functional electron transport chains. This proposed mechanism relies on traits that are conserved across cyanobacterial lineages, and might reflect the lifestyle of the nonphotosynthetic ancestor of cyanobacteria (27). Under this second hypothesis, part of the energy transduction machinery of such an ancestor would have been coopted to serve as stress defense mechanisms in cyanobacteria, while still retaining its original capabilities in the absence of light.

Conclusions

We report the existence of cyanobacteria-dominated microbial communities in the deep continental subsurface, and discuss their potential metabolism based on geochemical and metagenomic data. Our proposal of cyanobacterial hydrogenotrophy is consistent with a large body of literature, as well as several parallel lines of evidence presented in this work. While the dark metabolism of cyanobacteria is still under investigation, this putative hydrogenotrophic approach could allow samples from this and other studies calls for a reevaluation of their potential roles in deep subsurface ecosystems and increases their relevance in early life and astrobiological scenarios.

Materials and Methods

Drilling and Sampling. Boreholes were continuously cored by rotary diamond-bit drilling using a Boart Longyear HQ wireline system producing 3 m of 60-mm-diameter cores. Well water was used as a drilling fluid to lubricate the bit and displace the mud to the surface. Fluids were recirculated. To detect potential contamination of the samples, sodium bromide (200 ppm) was added to the drilling fluid as a marker. Upon retrieval from the drilling rig, cores were divided into 60-cm-length pieces, inspected for signs of alteration, and stored in boxes for permanent storage and curation in the Instituto Geológico Minero de España lithoteque in Peñarroya. Selected cores were deposited in the Museo Minero de España lithoteque in Peñarroya. Selected cores were deposited in the Museo Minero de España lithoteque in Peñarroya. Selected cores were deposited in the Museo Minero de España lithoteque in Peñarroya.

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Lipid Extraction and Characterization. Rock powders were extracted using a modified Bligh–Dyer method (42). Samples were placed in a 250-mL Teflon bottle, submerged in a monophasic solution of 4:1:0.5 water:methanol: dichloromethane, and disrupted with a sonicator wand (Branson Ultrasonics) for 1 h while maintained on ice. Following sonication, the bottles were shaken at 200 rpm for 1 h and centrifuged at 1,500 rpm for 15 min. The supernatant was removed and the extraction repeated twice. A total of 10 mL of dichloromethane and 10 mL of water were added to the pooled supernatant to induce phase separation, and the organic phase was collected. The aqueous phase was extracted with 10 mL of dichloromethane two additional times, and the pooled organic phases were dried under N2 and weighed. A total of 2.5 μg of pregnane diol was added to each sample as an internal standard. The extracts were acetylated by dissolving them in 50 μL of acetic anhydride and 50 μL of pyridine and heating them at 60 °C for an hour, after which they were dried and redissolved in 100 μL of dichloromethane. Samples were analyzed on a Trace 1310 GC coupled to an ISQ LT single quad mass spectrometer (Thermo Scientific). The programmable temperature vaporizing inlet was operated in constant temperature splitless mode at 300 °C. Separation was achieved on a Rxi-5HT fused silica column (Restek; 30 μm; 0.25 mm inner diameter; 0.25 μm film) with a He flow rate of 1.5 mL/min using the following temperature program: 2 min hold at 40 °C; 25 °C/min to 120 °C; 6 °C/min to 320 °C; 30 min hold at 320 °C. The ISQ LT was operated in electron ionization mode with a 230 °C source temperature, scanning a mass range of 43–800 Da with a 0.2-s dwell time. Pregnan-5α-ether, (23α,24α,27α),and pregnane-3α,24α,27α-triol were quantified by comparing analyte peak areas to the area of the internal standard pregnane diacetate, assuming a 1:1 response factor. Finally, the calculated amount of 5α-pregnan-3α,24α,27α-triol in each sample was normalized to sample weight.

Sandwich Microarray Immunoassays with LDChip. Sandwich-type microarray immunoassays (SMIs) were performed as described previously (11). Briefly, printed microarray slides with LDChip300 antibody microarrays were blocked with 0.5% (vol/vol, final concentration) BSA in 5% NaCl; 2.7 mM KCl; 10 mM Na2HPO4; 1.8 mM KH2PO4 at 4 °C for 2 h. The samples were washed twice with 1× PBS and stored at −20 °C in 1:1 ethanol:1× PBS. Approximately 150 mg of small samples were subsequently subjected to analysis. Samples were embedded in 0.2% (wt/vol) agarose. Endogenous peroxidases were inactivated in 0.1% H2O2 in methanol for 30 min at room temperature. Hybridization was performed following the method described in ref. 43 with some modifications to facilitate the handling of small fragments of rock. Alexa Fluor 594-labeled tyramide was used as a fluorochrome. Samples were decontaminated with 4% 2,6-diaminodino-2-phenyllindole (DAP). The oligonucleotide probe used in this study for targeting RNA genes was CY3A361 (5′-CCCCATTCGGAAGATTCC-3′) (44) at 35% (vol/vol) formamide concentration. HRP-labeled probes

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were synthesized by Biomers.net GmbH. Negative controls were performed with the control probe NON338 (5′-ACTCTCTAGGGAGGCACG-3′) (45). Additionally, negative controls of amplicon DNA isolation were conducted by subjecting the sample to the whole workflow without adding the probe, to evaluate whether the complex fluorophore-tyramide could interact with some minimal signal giving rise to a false positive. No signal was obtained from these controls. Further controls were conducted to evaluate whether the CARD-FISH signal could have been overlapping with autofluorescence from the cyanobacterial cells. CYA361 probe with both Alexa 488-labeled tyramide and Alexa 594-labeled tyramide were tested separately, and fluorescence was measured at 488 nm (green), 594 nm (red), and 633 nm (far red). In none of these controls could we achieve any autofluorescence signal from the cyanobacterial cells. Samples were mounted onto ibidi µ-slides 8 Well (ibidi GmbH) embedded in Citifluor/Vectashield (4:1) and examined with a Nikon A1R+ Resonant Scanning Confocal System (Nikon) at the Confocal Microscopy service at the Centro de Biología Molecular Severo Ochoa, CSIC-UAM.

DNA Extraction, Amplification, and Sequencing. DNA extraction was performed in a UV- and ethanol-sterilized flow chamber according to ref. 46. Briefly, 0.5 g of powdered core sample was introduced into an Ultra-Clean Bead Tube (MoBio Laboratories), whose original buffer had been previously removed and substituted with 1 mL of phosphate buffer (1 M sodium phosphate, 15% ethanol). After adding 60 μL of MoBio Ultra-Clean Soil DNA solution S1, the tubes were vortexed to remove DNA (30 s, power setting of 5.5 m/s) separated by 1 min of ice cooling. Subsequently, the tubes were incubated in a thermostimulator at 80 °C for 40 min, while shaking at 300 rpm. The MoBio Ultra-Clean Soil DNA extraction protocol was then followed from the addition of solution S2, according to the manufacturer’s instructions. All materials and stock solutions were UV sterilized for 5 min in either a G2 Linker UV Chamber (Bio-Rad Laboratories) or a Stratalinker 1800 UV crosslinker (Stratagene) to eliminate trace DNA contaminations. The isolated DNA was later subjected to multiple displacement amplification (MDA) using either the MagnifiPhi Phii29 polymerase (Genetix, formerly X-Pol Biotech) or the REPLi-g Single Cell Kit (Qiagen). The nonenzymatic MDA reagents and the random hexamers were decontaminated following ref. 47. Briefly, they were aliquoted into 0.2-mL PCR tubes, which were laid down horizontally on the UV crosslinker chambers for the total UV dosage of 15 mJ·cm⁻2. The remaining MDA amplification products were finally purified using a MicroSpin G-50 column (GE Healthcare). Successful amplification was confirmed by PCR of the 16s rRNA gene using primers 16SF (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SR (5′-CAGCACGCTTACGAGGCGG-3′). Negative controls were also run from MDA reagents without template DNA. Furthermore, in the cases where the MDA-amplified DNA gave no 16S PCR product, another nine individual rounds of DNA isolation were performed using the same extraction protocol (for a total of -5 g of powdered core sample). The 10 DNA isolations from the samerock core sample were immediately pooled, cleaned with phenol:chloroform:isoamyl alcohol (25:24:1), and precipitated with 70% ethanol (also UV sterilized) as described elsewhere. The DNA pellet was finally eluted in 50 μL of 10 mM Tris pH 8.0, amplified by MDA, and purified using the MicroSpin G-50 columns.

Additionally, an up-scaled modification of the previous protocol was applied using the PowerMax Soil DNA Isolation Kit (MoBio Laboratories). A total of 15 mL of phosphate buffer was mixed with up to 10 g of powdered core sample into the provided PowerMax Bead Tube. After the addition of 5 mL of PowerMax Soil DNA solution C1, the tubes were vortexed vigorously for 30 s and subjected to two FastPrep cycles (40 s, power setting of 6.0 mJ/s). Samples were then incubated at 80 °C in a water bath during 40 min and centrifuged at 2,500 × g for 3 min at room temperature. The supernatant was recovered in a new collection tube and the protocol was followed from the addition of solution C2. Once again, the solutions employed were UV sterilized as described above, and the resulting DNA was also subjected to MDA amplification, purification, and PCR of the 16s rRNA gene.

The extraction of DNA from different depths with either one or several of the above-described methods is summarized in SI Appendix, Table S1. DNA was additionally isolated from a drilling water sample, to trace contamination events occurring during the core retrieval process. Two 250-mL drilling water samples, collected with a 15-d difference, were pooled and filtered through a 0.22-μm pore size filter (Millipore). The filter was introduced into an empty Ultra-Clean Bead Tube and the DNA was isolated using the first protocol described above. Additionally, during the preparation of the aliquots used for MiSeq sequencing (see below) DNA was also extracted from an empty PowerMax Bead Tube to account also for laboratory contamination during extraction of the nucleic acids (DNA isolation control). DNA was finally eluted in 50 μL of 10 mM Tris HCl buffer and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The VS-V6 hypervariable regions of the bacterial 16s rRNA gene were PCR amplified using primers 807F and 1050R (48). The barcoding of the DNA amplicons, as well as the addition of Illumina adaptors, was carried out as described previously (49). The PCR reaction was carried out with 2 μL of NEBNext dsDNA fragmentase (New England Biolabs, Inc.) and carried out for 25 min at 37 °C. After incubation, the fragmentation was halted by the addition of 5 μL of 0.5 M EDTA. The ensuing DNA was purified with the QIAquick PCR Purification Kit (Qiagen) and eluted in a final volume of 35 μL before quantification with a Nanodrop (Thermo Scientific). Metagenomic libraries were prepared with the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Inc.) using ~200 ng of fragmented DNA as initial input. Size selection of 400–500 bp DNA library fragments was carried out using the Agencourt Ampure XP magnetic beads (Beckman Coulter, Inc.) according to the NEBNext Ultra DNA Library Prep Kit instructions. Each metagenomic DNA library was sequenced (100-nt paired-end sequencing) with the Illumina HiSeq. 2500 platform using the TruSeq SR Cluster Kit, v3-cBot-HS (Illumina).

16S Community Profiling. Raw 16S MiSeq paired reads were assembled and quality filtered with moira (50) (v 1.3.2) with the -q posterior flag, and then PCR-generated amplicon libraries, were subjected to the following: the non-PCR amplicon libraries were subjected to the whole workflow without adding the probe, to evaluate whether the complex fluorophore-tyramide could interact with some minimal signal giving rise to a false positive. No signal was obtained from these controls. Further controls were conducted to evaluate whether the CARD-FISH signal could have been overlapping with autofluorescence from the cyanobacterial cells. CYA361 probe with both Alexa 488-labeled tyramide and Alexa 594-labeled tyramide were tested separately, and fluorescence was measured at 488 nm (green), 594 nm (red), and 633 nm (far red). In none of these controls could we achieve any autofluorescence signal from the cyanobacterial cells. Samples were mounted onto ibidi µ-slides 8 Well (ibidi GmbH) embedded in Citifluor/Vectashield (4:1) and examined with a Nikon A1R+ Resonant Scanning Confocal System (Nikon) at the Confocal Microscopy service at the Centro de Biología Molecular Severo Ochoa, CSIC-UAM.

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