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Biological production of H_2, CH_4 and CO_2 in the deep subsurface of the Iberian Pyrite Belt

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Summary

Most of the terrestrial deep subsurfaces are oligotrophic environments in which some gases, mainly H_2, CH_4 and CO_2, play an important role as energy and/or carbon sources. In this work, we assessed their biotic and abiotic origin in samples from subsurface hard-rock cores of the Iberian Pyrite Belt (IPB) at three different depths (414, 497 and 520 m). One set of samples was sterilized (abiotic control) and all samples were incubated under anaerobic conditions. Our results showed that H_2, CH_4 and CO_2 remained low and constant in the sterilized controls while their levels were 4, 4.1 and 2.5 times higher respectively, in the unsterilized samples compared to the abiotic controls. The $\delta^{13}C_{CH4}$-values measured in the samples (range −31.2 to −43.0 ‰) reveals carbon isotopic signatures that are within the range for biological methane production. Possible microorganisms responsible for the biotic production of the gases were assessed by CARD-FISH. The analysis of sequenced genomes of detected microorganisms within the subsurface of the IPB allowed to identify possible metabolic activities involved in H_2 (*Rhodoplanes*, *Shewanella* and *Desulfosporosinus*), CH_4 (Methanobacteriales) and CO_2 production. The obtained results suggest that part of the H_2, CH_4 and CO_2 detected in the deep subsurface has a biological origin.

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Introduction

The deep subsurface, the dark biosphere, is one of the largest ecosystems on Earth and, by far, the least studied until now. Microbial life is known to reach depths of several kilometres within the continental crust (Zhang *et al*., 2005; Magnabosco *et al*., 2016; Stepniewska *et al*., 2018; Purkamo *et al*., 2020) and diverse microbial communities, mainly bacteria and archaea, are known to inhabit fractures and pores in the deep subsurface of Earth's crust (Itävaara *et al*., 2016; Escudero *et al*., 2018a). In 2018, Magnabosco and colleagues estimated that almost 90% of the Earth's prokaryotic biomass is within the dark, anoxic and oligotrophic environment of the deep subsurface (Magnabosco et al., 2018). Thus, addressing the question of how all this biomass is sustained over time is vital to understanding the operation of the system. Although groundwater samples provide information on the physicochemical characteristics and diversity of the system, this data lacks precise information on the relationship between the microorganisms and the mineral substrate in which they develop.

There is a considerable consensus that some gas molecules such as hydrogen, methane and carbon dioxide are important components of the deep subsurface ecosystems, because they have been detected in most drilling operations (Stevens and McKinley, 1995; Pedersen, 1997; Chapelle *et al*., 2002; Itävaara *et al*., 2011; Brazelton *et al*., 2012; Nyyssönen *et al*., 2012; Ino *et al*., 2016; Lau *et al*., 2016; Rempfert *et al*., 2017), but their source is not always clear due to the fast diffusion of gases. Molecular H_2, CH_4 and CO_2 can be generated by both biotic and abiotic processes. H_2 can be produced by radiolysis of water or as a result of water interaction with different minerals, among other systems (Apps and van de Kamp, 1993; Stevens and McKinley, 1995). CO_2 can be produced by dissolution of carbonates and methane by volcanic activity and gas–water–rock interactions (Etiope and Sherwood Lollar, 2013). All of them, however, are also produced biologically. Heterotrophic bacteria release H_2 through fermentation, nitrogen fixation or anaerobic oxidation of CO (Diender *et al*., 2015; Madigan *et al*., 2020). Methanogenic archaea obtain their energy by producing CH_4 through CO_2 reduction with H_2 or by acetate or

methyl-compound reduction (Kietäväinen and Purkamo, 2015). CO_2 is released in diverse metabolic processes, mainly anaerobic respiration or fermentation (Madigan *et al*., 2020).

Generally, in subsurface environments, while CH_4 is considered a biological product, it is assumed that H_2 and CO_2 are of abiotic origin. Actually, studies have shown that deep subsurface life is supported by chemolithoautotrophic microorganisms, mostly methanogenic archaea, which take advantage of the H_2 and CO_2 generated abiotically in the geosphere to produce methane (Stevens and McKinley, 1995; Pedersen, 1997). This scenario has been demonstrated in the South Africa Gold mines, where concentrations of up to 7.41 mM of dissolved H_2 were detected and isotope analysis corroborated their geological origin, likely by water radiolysis (Lin *et al*., 2005; Lin *et al*., 2006). Compositional and isotopic signatures from hydrogen exsolving from Precambrian Shield rocks in Canada and South Africa showed its abiogenic nature. The detection of methanogens and isotopic analysis of ^{13}C-enriched CH_4 suggests the possibility that abiogenic gases may support H_2 autotrophy linked to methanogenesis in the deep subsurface (Sherwood Lollar *et al*., 2006). Methane cycling was confirmed by detection of functional genes, i.e. methyl coenzyme M reductase (*mcr*A) and methane monooxygenase (*pmo*A) genes, in a 600 m deep borehole at the Fennoscandian Shield (Purkamo *et al*., 2018).

In the deep continental subsurface, alternative energy sources, such as organic matter can be found buried in sedimentary rock systems. Organic matter can be used by heterotrophic microorganisms as an energy source, and its utilization may lead to the generation of H_2 or CO_2, which can sustain chemolithoautotrophic microorganisms (Fredrickson and Balkwill, 2006; Purkamo *et al*., 2015). Excreted metabolic products together with decomposition compounds released after cell death could be the main substrates for heterotrophic microorganisms. Thus, the question of how important the biological production of these gases actually is within the deep subsurface systems, remains, to the best of our knowledge, unanswered.

The IPB stretching for some 250 km across SW Iberia hosts the largest concentration of volcanogenic massive sulfides on Earth (Tornos, 2006). Its formation through hydrothermalism took place during the Hercynian orogenesis. The IPB gives rise to Río Tinto, a 92 km long extreme acidic river with a high concentration of heavy metals (Fe, Cu, Zn, Ni) and an exceptional level of microbial diversity (González-Toril *et al*., 2002; Amils, 2016).

Two drilling projects, Mars Astrobiology Research and Technology Experiment (MARTE project, 2003–2006) and Iberian Pyrite Belt Subsurface Life Detection (IPBSL project 2011–2015) revealed a subsurface ecosystem with active iron and sulfur cycles (Amils *et al*., 2014; Puente-Sánchez *et al*., 2014, 2018) in which variable concentrations of H_2, CH_4 and CO_2 were detected along the length of the boreholes. In this work we employed stimulation through the addition of water and comparison with sterilized samples to assess the biotic and abiotic origin of these gases in three IPB deep subsurface hard-rock core samples from different depths.

Results

Samples for the analysis were selected due to their high methane production in enrichment cultures. The mineral composition of the analysed samples (Table S1) shows quartz dominance at all depths. However, while at 497 m below surface (mbs) iron containing minerals such as pyrite and illite stand out, the presence of carbonates (dolomite, Mg-calcite, siderite and ankerite) at 414 mbs is also noticeable. At 520 mbs, the minerals dolomite and illite are present in equal concentrations. Elemental composition analysis shows significant concentration of heavy metals, mainly iron, in all samples (Table S2). Carbohydrates were detected in the analysed samples: 2, 40 and 15 $\mu g\ g^{-1}$ in the 414, 497 and 520 mbs samples respectively. In addition, four samples from different depths of the borehole BH10 were analysed for stable isotope composition of methane. The carbon ($\delta^{13}C_{CH4}$) isotopic values were −30.9 (519 m), −31.2 (538.4 m), −37.6 (357.7 m) and − 43.1 (544 m) ‰.

Gases released under different experimental conditions

In natural samples with no or low humidity, possible abiotic and/or biological production of H_2, CH_4 and CO_2 was, in general, low. However, release of occluded gases in the mineral matrix was observed in some samples when subjected to the heat treatment for sterilization (Fig. 1). Consumption of H_2, as well as the other gases studied, cannot be ruled out. Therefore, the amount generated could likely be greater than the gases measured.

Hydrogen. During the first phase of the experiment (stage 1), there was some release of occluded H_2 (Fig. 1A). The level remained constant over time, although the sterilized samples had a higher concentration of released H_2 than the non-sterilized ones, probably because the heat applied during the sterilization process promoted the release of potential occluded gases. Small fluctuations observed in this period can be merely attributed to technical variations, although some reactivity after addition of water cannot be ruled out.

The addition of water (stage 2) promoted the release of H_2 in the non-sterilized samples. This effect was

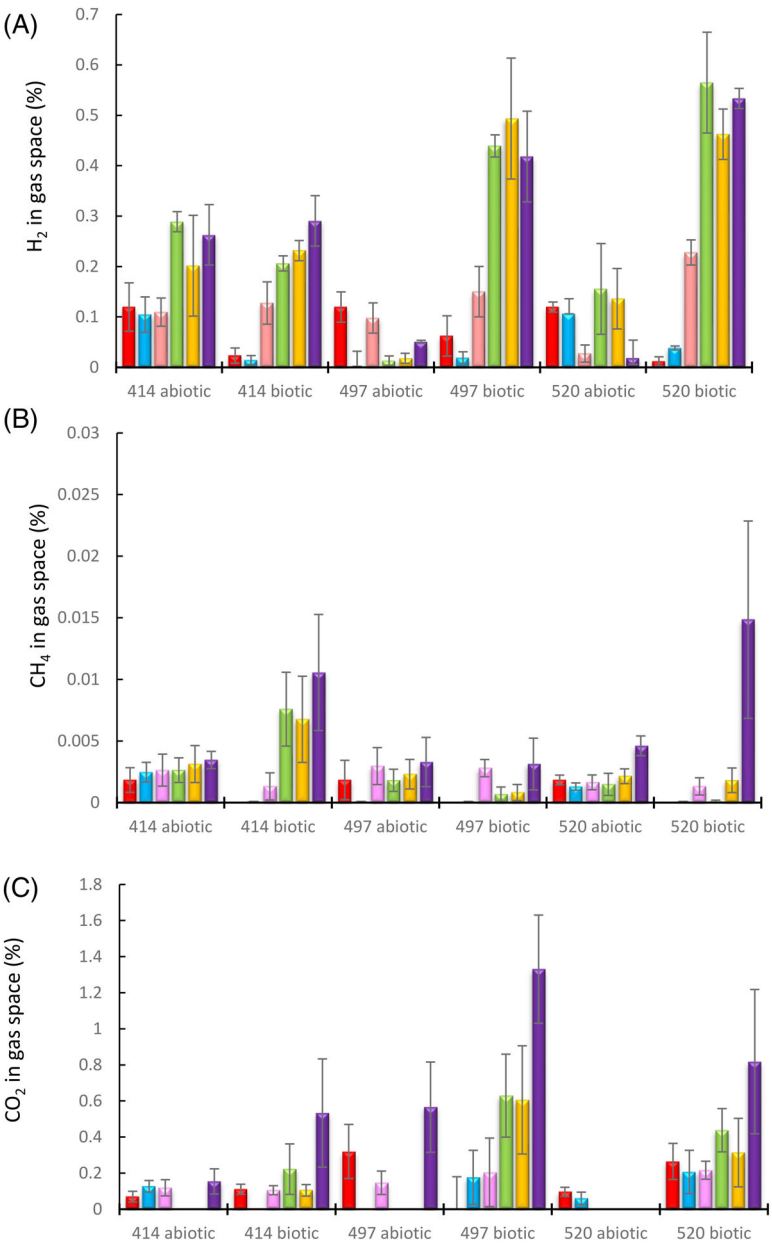

Fig. 1. Hydrogen (A), methane (B) and carbon dioxide (C) released throughout the experiment. The first three measurements were made at 50, 75 and 100 days since start point. The last three measurements were made after 3, 7 and 15 days of adding water. [Color figure can be viewed at wileyonlinelibrary.com]

especially remarkable in samples obtained at 497 and 520 mbs, which showed a maximum H_2 production of 0.64% and 0.51% of the gas space (corresponding to 4.3 and 3.4 μmols in our experimental conditions) respectively. Since the level of H_2 remained constant in the sterilized controls from these two depths, these results strongly suggest that the H_2 released by the non-sterilized samples are the metabolic product of microorganisms present in the sample. Most of the detected H_2 was released in the first 3 days, with no noticeable increase after 2 weeks of stimulation. In the 414 mbs sample, H_2 accumulation was also observed after the addition of water, but its concentration was similar in the sterilized and unsterilized samples. In samples from 497 and 520 mbs the produced biotic H_2 after stimulation by addition of sterile water was between 3.1 and 5.0 times the release observed in the sterilized samples.

Methane. The levels of CH_4 measured in stage 1 were very low in all samples. The small fluctuations do not appear to be significant or show a definite trend, since CH_4 levels were close to the limit of reliably detection (10 ppm) by Gas Chromatography (Fig. 1B). After the addition of sterile water, the production of methane in the non-sterilized 414 and 520 mbs samples showed a remarkable stimulation (between 2.7 and 5.5 times). The

comparison with the corresponding sterilized samples strongly suggests that this methane was produced by methanogenic microorganisms. The 520 mbs sample showed a steady increase of methane production over time after stimulation (maximum value of 0.015% (0.1 μmols) after 14 days incubation). This effect was not observed in the 414 mbs sample in which most of the detected methane had been already observed during the first 3 days of stimulation. There was no difference between biotic and abiotic methane release in the sample obtained at 497 mbs. In this case no important differences between sterilized and unsterilized samples in methane production prior to stimulation were observed.

Carbon dioxide. Although the obtained results are somewhat variable, it is clear that the addition of water stimulates the release of CO_2, particularly in the non-sterilized samples (Fig. 1C). In this case, the CO_2 concentration in the stimulated samples increased over time in all analysed samples. The highest values were observed after 14 days of incubation and were, on average, 2.5 times higher than those released in sterilized samples. The abiotic release was very low, with the exception of the 497 mbs sample.

Microbial diversity responsible for the biotic release of gases

To identify the possible microorganisms responsible for the biotic production of the different detected gases, CARD-FISH hybridizations were performed in the selected samples. General probes were used to detect the presence of microorganisms in both the sterilized and the unsterilized samples. In the sterilized samples, no hybridizations were detected, while in the unsterilized samples the presence of both Bacteria and Archaea was observed (Fig. 2A–F, Fig. S1).

In addition, we used specific probes to detect the presence of microorganisms that have been isolated or identified in the subsurface of the IPB (Leandro *et al*., 2018) and for which sequenced genomes are available (Leandro *et al*., 2017; García *et al*., 2018; Mariñán *et al*., 2019; Rodríguez-Robles *et al*., 2019; de Polanco *et al*., 2020; Martínez *et al*., 2021). This allowed us to identify possible metabolisms that could generate the detected gases. Members of the *Brevundimonas*, *Pseudomonas*, *Rhizobium*, *Rhodoplanes* and *Tessaracoccus* genera were identified in the analysed samples (Fig. 2, Table 1 and Fig. S1). We also incorporated *Desulfosporosinus* sp., detected at these depths in enrichment cultures, in the analysis. Of the detected microorganisms, *Desulfosporosinus, Rhodoplanes* and *Shewanella* showed the presence of genes related with H_2 production (Table 1).

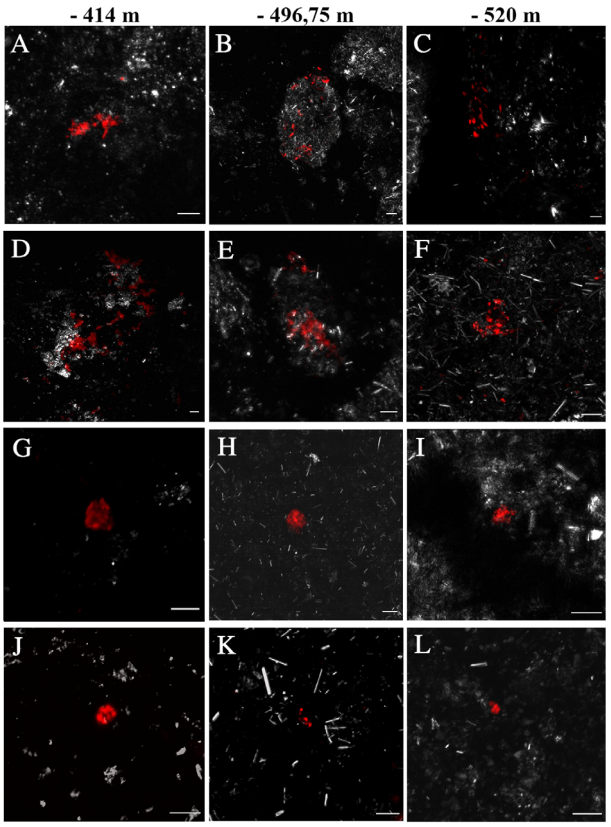

Fig. 2. Detection of microorganisms in subsurface rock samples by CARD-FISH. (a-c) Bacteria detected at: 414 mbs (A), 497 mbs (B) and 520 mbs (C). (D–F) Archaea detected at: 414 mbs (D), 497 mbs (E) and 520 mbs (F). (G) *Tessaracoccus* spp. detected at 414 mbs, (H) *Rhizobium* spp. detected at 497 mbs, (I) Methanobacteriales detected at 520 mbs, (J) *Brevundimonas* spp. detected at 414 mbs, (K) *Rhodoplanes* spp. detected at 497 mbs, (L) *Shewanella* spp. detected at 520 mbs. In red, CARD-FISH signal. In grey, reflection. Scale bars, 5 μm. [Color figure can be viewed at wileyonlinelibrary.com]

As mentioned, the increase in the release of methane in the unsterilized 414 and 520 mbs samples after water was added indicates methanogenic activity in these samples. Archaea were detected in all samples with the general archaeal probe (Fig. 2) but only a positive hybridization signal was obtained with the specific probe for Methanobacteriales in sample 520 mbs (Fig. 2I). Unfortunately, no methanogenic archaea could be isolated from enrichment cultures, thus genus specific hybridization probes for methanogenic archaea could not be developed.

In the case of CO_2 production, different microorganisms were identified in all analysed samples that have the genes for anaerobic respiration or fermentation (Table 1, Fig. 2).

Discussion

In natural, untreated samples the production of H_2, CH_4 and CO_2 was very low. The addition of sterile water

Table 1. Detected microorganisms in the analysed samples and metabolic activities producing H_2, CH_4 and CO_2 identified in their genome sequences.

Identified microorganisms	414	497	520	CO_2res	CO_2fer	COoxi	H_2fer	$H_2$for	N_2fix	CH_4
Bacteria	H	H	H	+	+	+	+	+	+	−
Archaea	H	H	H	+	+	−	−	−	−	+
Methanobacteriales	−	−	H	−	−	−	−	−	−	+
Brevundimonas	H	−	−	+	−	−	−	−	−	−
Desulfosporosinus	E	−	E	+	+	+	−	−	+	−
Pseudomonas	H/I	−	H	+	−	−	−		−	−
Rhizobium	H	H	H	+	+	−	−	−	−	−
Rhodoplanes	I	H	H	+	+	−	?	−	+	−
Shewanella	H	H	H	+	+	−	−	+	−	−
Tessaracoccus	H/E	−	H/E	+	+	−	−	−	−	−

H: CARD-FISH; I: isolation[1]; E: enrichment culture[1]; CO_2res: CO_2 produced by anaerobic respiration; CO_2fer: CO_2 produced by fermentation; COoxi: CO_2 produced by oxidation of CO; H_2fer: H_2 produced by fermentation; $H_2$for: H_2 produced from formate; N_2fix: H_2 produced by nitrogen fixation reaction, CH_4: methane produced by methanogenic archaea.

[1] For enrichment cultures, splitting rock samples were used as inoculum in a mineral medium. After 1 year of incubation in anaerobic conditions, the cultures were microbiologically characterized. Isolation of microorganisms was performed by the Hungate roll-tube method. Detailed protocols for enrichment, isolation and characterization can been found in Leandro and colleagues (2018).

Table S3 (annotated genes): Nitrogen, hydrogen and carbon metabolism related genes present in the genomes of the microorganisms isolated from the IPBSL subsurface.

stimulated the release of these gases in the non-sterilized samples that strongly suggests that a significant part of these gases has a biological origin. Carbon isotopic data ($\delta^{13}C_{CH4}$ −43.0 to −31.2 ‰) measured in samples from the borehole, confirm the biological production of methane into the subsurface hard-rock of the IPB, possibly associated with acetoclastic (carbon isotopic signature between −15 and −30 ‰), and hydrogenotrophic methanogenesis (between −40 and −60 ‰; Whiticar, 1999). These results are within the range of other similar deep subsurface studies where microorganisms contributing to the methane cycle have been reported, e.g., in Finland, $\delta^{13}C_{CH4}$ from −63 to −22 ‰ (Nyyssönen *et al*., 2012; Bomberg *et al*., 2015); or in South Africa, $\delta^{13}C_{CH4}$ from −33 to −28‰ (Lin *et al*., 2005; Sherwood Lollar *et al*., 2006). H_2 was detected in the samples obtained at 497 and 520 mbs, CH_4 in samples obtained at 414 and 520 mbs, and CO_2 in all tested samples. At these depths microorganisms with genes responsible for diverse metabolic activities that produce the detected biogases had been previously identified. Genes related to hydrogen production, e.g., nitrogen fixation in *Desulfosporosinus* and *Rhodoplanes*; CO-oxidation in *Desulfosporosinus*; periplasmic [Ni—Fe] hydrogenase in *Shewanella*, are present in bacteria identified in subsurface environments. Interestingly enough, although *Rhodoplanes* lacks the gene coding for the subunit FdhF of the formate hydrogenlyase complex, which catalyses formate oxidation for the generation of H_2 through fermentation in model systems, it does have the gene that encodes the catalytic [NiFe] hydrogenase subunit (HycE) of the complex, which catalyses the formation of H_2 (McDowall *et al*., 2014).

Although we could not identify the methanogenic archaea responsible for CH_4-generation, given the incubation conditions, it is difficult to find an alternative explanation for the generation of methane. By extension, methane can be associated to the production of H_2 and CO_2. The generation of both H_2 and CO_2 in the ecosystem suggests that hydrogenotrophic methanogenesis can be potentially active in the deep subsurface of the IPB, although other methanogenic activities cannot be ruled out. Many different microorganisms that are able to generate CO_2 metabolically through anaerobic respiration or fermentation have been detected in the analysed samples (Table 1, Fig. 2).

Parkes and colleagues (2011) reported in their work with subseafloor sediments that CH_4, CO_2 and, a to a lesser extent, H_2 were released in the presence of microorganisms, while none were generated in the sterile controls after the temperature was increased gradually from 0 to 100°C, over the course of 83 days. Gas production improved when minerals (e.g., quartz, basalt, pyrite, hornblende, olivine) were added to the incubations. The authors concluded that the effect of microorganisms on the minerals generated reactive surfaces that were able to interact with water generating the detected gases, such as H_2, in the subsurface. Although we cannot rule out this possibility due to the iron-containing minerals present in our samples, the much shorter incubation times and the temperature used in our stimulations fit better with the product of metabolic activities than with a secondary effect of microbial growth.

In the 414 mbs sample, the addition of water stimulated H_2 production, but the effect was similar in both the sterile and non-sterile samples. The result observed for CH_4 production in the 497 mbs sample followed the same pattern. We conclude that in these cases the generation of gases was abiotic, produced in water-rock reactions. Detailed information on abiotic generation of these gases can be found in Kietäväinen and Purkamo (2015). Therefore, the mineral matrix plays an important role in the abiotic generation of these gases at these depths.

The carbohydrates detected in the analysed samples (between 2 and 40 $\mu g\ g^{-1}$ sample) could be used as substrates for heterotrophic metabolic reactions. Actually, those depths which showed higher production of H_2 and CO_2, contained a higher concentration of reduced organic matter favouring the metabolic reactions that lead to H_2 and CO_2 production. The detection and identification of fermenting microorganisms by CARD-FISH at these depths support this assumption because it allows the generation of these gases to be correlated with the metabolic abilities detected in the genomes of native isolated microorganisms from the same borehole (Fig. 2).

The described results demonstrate that the biological production of H_2, CH_4 and CO_2 in the deep subsurface of the IPB is important, much more than has been predicted. We strongly believe that this is not a peculiarity of the studied ecosystem, but that the biological production of gases in the deep subsurface should be considered and incorporated into any model system describing the dark biosphere.

Experimental procedures

Drilling and sampling subsurface materials. Borehole BH10 drilled, in Peña de Hierro area of the IPB (37°43′45.74″ N/ 6°33′22.37″ W) was continuously cored by rotary diamond-bit drilling on lexan liners using a Boart Longyear HQ wireline system producing 3 m of 60 mm-diameter cores. Drinking water was used as drilling fluid to lubricate the bit and return cuttings to the surface. To detect potential contamination of samples, sodium bromide (10 $mg\ L^{-1}$) was added to the drilling fluid as a marker. Upon retrieval from the driling 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 y Minero de España lithoteque in Peñarroya (Córdoba, Spain). Selected cores were deposited in plastic bags, oxygen was displaced with N_2, which were then sealed and transported to a field laboratory located at the Museo Minero in Riotinto village, close to the drilling site for their processing.

Sample processing. Upon arrival at the field laboratory, cores were placed in an anaerobic chamber (5% H_2, 95% N_2, < 50 ppm O_2), logged and photographed. The anaerobic chamber and the airlock were decontaminated daily with Virkon S (Antec International Limited), a mixture of surfactants, organic acids and strong oxidizers that disrupt bacterial membranes and degrade their nucleic acids. Furthermore, the chamber and the airlock were cleaned with a 50:50 bleach: water solution along with ethanol before the introduction of a new core sample. Aseptic subsamples were obtained by splitting cores with a hydraulic press and drilling out the central untouched face with a rotary hammer drill mill cutter to produce powdered samples using sterile bits. Temperature of the hand drilling was strictly controlled with an infrared thermometer (maximum allowed drilling temperature 40°C). The presence of bromide was analysed by Ion Chromatography (Metrohm 861 Advanced Compact Ion Chromatographer IC). Samples in which bromide was detected, were discarded from further studies.

Serum bottles (40 ml) were filled with small fragments and rock splinters. Forty gram of rock samples per bottle were weighed using a scale with 0.5 g of precision. The size of the splinters was smaller than 22 mm (the diameter of the bottles neck). The bottles were sealed while inside the anaerobic chamber, with gas-tight butyl stoppers. Samples used in this study were stored at 4°C in anaerobic closed bottles in the dark for approximately 2 years before their use. Mineralogical analysis was performed on pieces adjacent to the aseptic subsample.

Sample characterization

The mineral components of the material were determined by Powder X-Ray Diffraction using a Seifert 3003 TT instrument with Cu Kα radiation ($\lambda = 1.542$ Å). The X-ray generator was set to an acceleration voltage of 40 kV and a filament emission of 40 mA. Samples were scanned between 5° (2θ) and 60° (2θ) using a step size of 0.05° (2θ) and a count time of 1 s.

For ICP-MS analysis of powdered samples, a total acidic extraction was carried out according to the EPA 3052 Microwave Assisted Acid Digestion of Siliceous and Organical based matrixes method, using ultrapure HNO_3, HF, HCl, H_2O_2 mixture (9:4:1:2) from Merk. The digestion was done in an Ethos Touch Control Screen from Milestone in high pressure and temperature resistant containers: 15 min from room temperature to 180°C, plus 10 min at 180°C. A NexION 2000 (PerkinElmer, Shelton, Conneticut, USA) instrument was used. A multielemental semiquantitative analysis was performed and ARD-01, was used as matrix reference. All the analyses were performed in mode KED to avoid polyatomic interferences.

The carbohydrate content of the samples was determined in the field laboratory. Crushed samples (1 g) were subjected to 3 × 1 min ultrasonication cycles in 2 ml of

distilled water with 1–2 min stops by using a handheld sonicator. Samples were centrifuged at 2000 g to sediment the mineral particles and the supernatants were directly assayed for sugar content as described (Dubois *et al*., 1956). A NanoDrop instrument was used for spectrophotometric measurements.

Experimental design

Samples obtained from drilling cores at 414, 497 and 520 mbs, were selected for this analysis due to the high methanogenic activity detected in enrichment cultures (Leandro *et al*., 2018). The following experiments were performed in duplicate:

Stage 1. For each duplicate sample two replicas were made: one was sterilized according to the protocol described by Zhang and colleagues (2015) for chalcopyrite samples (abiotic production). Splinters from the first replica were washed with a 0.1 M EDTA and 0.4 M NaOH solution, rinsed extensively with deionized water and heated at 120°C for 10 h (sterilized control). The other replica was left untreated (biotic production) in anaerobic conditions in the dark. All bottles were bubbled with N_2 for 3 min and then incubated in the dark in a 30 $\pm$ 2°C thermostatic room. The H_2, CH_4 and CO_2 content in the headspace was determined periodically by gas chromatography after 50, 75 and 100 days.

Stage 2. After maintaining the bottles in the above mentioned conditions for 100 days (after which the potential release of occluded gases can be considered to have ceased), 0.5 ml of sterile deionized MilliQ water (boiled, gassed with N_2 during cooling and sterilized under N_2 atmosphere at 121°C for 20 m) was added to the rock containing bottles using sterile syringes. Subsequently, gas production was followed sampling at 3, 7 and 14 days.

Analytical methods

Gases were analysed with a Bruker Series Bypass 450GC chromatograph. The chromatograph was equipped with a column CP2056 0.6 m $\times$ 1/8'Ultimetal Cromsorb GHP 100-120 mesh, and a column CP81073 0.5 m $\times$ 1/8' Ultimetal Hayesep Q 80–100 mesh, a detector TCD at 200°C for the detection of H_2 and CO_2 and a detector FID at 250° C for the detection of CH_4. N_2 was used as carrier gas.

After the gas production experiment was finalized, bottles were opened in sterile conditions. CARD-FISH analysis and their respective controls were carried out on the rock splinters as described previously in detail (Escudero, 2018; Escudero *et al*., 2018b). Briefly, rock samples were fixed with 4% formaldehyde in Mackintosh minimal media [KH_2PO_4 27 mg L^{-1}, $(NH_4)_2SO_4$ 132 mg L^{-1}, $MgCl_2$*6 H_2O 53 mg L^{-1}, $CaCl_2$* 2 H_2O 147 mg L^{-1}, pH 1.8] at 4°C for 2 h, washed with Mackintosh minimal media and PBS (KCl_2 0.2 g L^{-1}, NaCl 8 g L^{-1}, Na_2HPO_4 1.44 g L^{-1}, KH_2PO_4 0.24 g L^{-1}) and then stored in a solution PBS:ethanol (1:1) at −20°C.

Rock splinters were gently ground to the size of grains of sand in a sterile mortar and pestle under sterile conditions. Powdered rock samples were immobilized with agarose at 0.2%, dried at 37°C, dehydrated with absolute ethanol and stored at −20°C. CARD-FISH hybridization was performed as described (Pernthaler *et al*., 2004) with minor modifications. Endogenous peroxidases were inactivated as described (Ishii *et al*., 2004). Samples were permeabilized with lysozyme and achromopeptidase. Hybridizations were carried out with 5′-HRP-labelled probes (Table S4) (Biomers) for 2 h at 46°C after which samples were washed at 48°C for 10 min. Formamide (FA) and NaCl concentration in hybridization and washing buffer respectively were regulated for each probe (Table S4). Tyramide Signal Amplification was performed for 45 min at 46°C.

Subsequently, rock samples were counterstained with Syto9 (Thermo Fisher Scientific) according to manufacturer recommendation, covered with a mix of 1:4 Vectashield (Vector Laboratories): Citifluor (Citifluor) and mounted onto eight-well glass bottom slides (Ibidi).

Hybridized samples were visualized using a confocal laser scanning microscope LSM710 coupled with an inverted microscope AxioObserver (Carl Zeiss, Jena, Germany) and equipped with diode (405 nm), argon (458/488/514 nm) and helium and neon (543 and 633 nm) lasers. Images were collected with a 63 $\times$ 1.4 oil immersion lens.

FISH probes design and determination of probes hybridization conditions

Two probes were designed for fluorescence in situ hybridization as described in (Hugenholtz *et al*., 2002) with the PROBE DESIGN tool from ARB software (Ludwig *et al*., 2004). S-G-Rdop-1255-a-A-20 (Rho1255) probe was designed to detect members of *Rhodoplanes* genus and S-G-Brevu-1242-a-A-22 (Bre1242) to detect members of *Brevundimonas* genus. Designed probes were synthetized and labelled with CY3 fluorophore (Biomers, Germany). Specificity and optimal astringency conditions of the probes were determined by FISH as described in (Hugenholtz *et al*., 2002) in triplicate experiments using EUB338-I probe (see Table S1) labelled with FITC fluorophore as positive control.

Brevundimonas sp. T2.26MG-97 and *Rhodoplanes* sp. T2.26MG-98, isolated from enrichment cultures carried out in the IPBSL project, were generously provided by Tania Leandro (University of Coimbra, Portugal) and

used as positive hybridization control of the designed probes. *Methylobacterium hispanicum* and *Brevundimonas halotolerans*, which had one base pair mismatch with the designed probes, were selected as negative control of Rho1255 and Bre1242 respectively and were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). All microorganisms were grown in liquid media at 28°C. *Brevundimonas* and *Rhodoplanes* strains were grown in R2A medium and *M. hispanicum* in Plate Count Broth. Optima formamide concentration was determined at 20% for Rho1255 probe and 40% for Bre1242 probe (Table S1).

The rest of the used probes are shown in Table S4, which includes hybridization conditions and the corresponding references.

Gene prediction analysis of sequenced microorganisms

Gene prediction analysis and functional annotation of *Desulfosporosinus meridiei* DEEP (IMG genome ID 2721755100), *Tessaracoccus* sp. T2.5-30 (IMG genome ID 2751185744), *Rhizobium* sp. T2.30D1-1 (NCBI accession number NZ_UEYP01000001.1), *Brevundimonas* sp. T2.26MG-97 (NCBI accession number NZ_UXHF01000001.1), *Rhodoplanes* sp. T2.26MG-98 (NCBI accession number NZ_UWOC01000001.1), *Shewanella* sp. T2.3DD-1.1 (ENA accession number CACVBT0200000010) and *Pseudomonas* sp. T2.31D-1 (ENA accession number CAJFAG010000000.1) was performed with the Integrated Microbial Genomes annotation pipeline (Chen *et al*., 2019), NCBI annotation pipeline (Tatusova *et al*., 2016) and PROKKA v1.12 software (Seemann, 2014). Enzymes of interest were defined based on the metabolic pathways described in the KEGG and MetaCyc databases (Kanehisa and Susumu, 2000; Caspi *et al*., 2014).

Stable isotope composition

Stable isotope composition of methane ($\delta^{13}C_{CH4}$) was measured on the head space gas of selected samples using a PreCon-GasBench preconcentration unit-gas chromatograph interfaced with a MAT 253 isotope-ratio mass spectrometer (Thermo Scientific, Bremen, Germany). Gas samples were flushed into a chemical trap and a nitrogen trap (to trap water, CO_2 and other minor hydrocarbons). Then, the CH_4 flowed into a NiO furnace (heated to 1000°C), the carbon dioxide product was transferred to a second liquid nitrogen trap (to pre-concentrate), and then, a third trap to cryo-focus the CO_2. The $\delta^{13}C$ of the carbon dioxide was determined with a MAT 253 IRMS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and reported in the standard per mil notation (‰). A certified standard from Indiana University (Methane#2) was used. The analytical precision of the $\delta^{13}C$ values were within $\pm$0.5 ‰. Detailed description of the analytical method can be found in Yarnes (2013).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Counterstaining of hybridized samples shown in Fig. 2 with Syto9. (a-c) Bacteria detected at: 414 mbs (a), 497 mbs (b) and 520 mbs (c). (d-f) Archaea detected at: 414 mbs (d), 497 mbs (e), and 520 mbs (f). (g) *Tessaracoccus* spp. detected at 414 mbs, (h) *Rhizobium* spp. detected at 497 mbs, (i) Methanobacteriales detected at 520 mbs, (j) *Brevundimonas* spp. detected at 414 mbs, (k) *Rhodoplanes* spp. detected at 497 mbs, (l) *Shewanella* spp. detected at 520 mbs. In green, Syto9 signal. In grey, reflection. Scale bars, 5 μm.

Table S1. Minerals detected by XRD.

Table S2. ICP-MS elemental analysis of core samples (ppm).

Table S3. Nitrogen, hydrogen and carbon metabolism related genes present in the genomes of the microorganisms isolated from the IPBSL subsurface.

Table S4. Fluorescence in situ hybridization probes.